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STUDIES ON THE DISTRIBUTION AND METABOLISM OF STEROLS AND OTHER ISOPRENOIDS IN THE BOVINE RETINA

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Studies on the Distribution and Metabolism of Sterols and Other Isoprenoids in the Bovine Retina

by

Steven Jay Fliesler

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

George J. Schroepfer, Jr., Professor of Biochemistry and Chemistry, Chairman

Graham Palmer, Professor of Biochemistry

James B. Walker, Professor of Biochemistry

Florante Quiocio, Associate Professor of Biochemistry

Ronald L. Sass, Professor of Biology and Chemistry

HOUSTON, TEXAS

August, 1979
ABSTRACT

Studies on the Distribution and Metabolism of Sterols and Other Isoprenoids in the Bovine Retina

by

Steven Jay Fliesler

The sterol composition of bovine retinas and rod outer segment (ROS) membranes was examined. Cholesterol accounted for >98% of the total sterol and ~2% of the dry weight of both the retina and ROS membranes. Minor amounts of components having the chromatographic properties of 5α-cholestan-3β-ol and 5α-cholest-7-en-3β-ol were detected in whole retinas, and a "cholestanol-like" component was also detected, in minor amounts, in ROS membranes. Calculations indicated that the molar ratio of cholesterol:rhodopsin in ROS membranes was ~5. Using literature values for the phospholipid content of bovine ROS membranes, the calculated cholesterol:phospholipid molar ratio was ~0.06 and cholesterol only represented ~5-7 mole % of the total ROS membrane lipid.

The metabolism of $^3$H-labelled mevalonic acid was studied in vitro with "intact" retinas and with the 10,000 x g supernatant ($S_{10}$) fraction. The major nonsaponifiable products obtained from incubations of "intact" retinas had the chromatographic properties of squalene and lanosterol; only minor amounts of label were incorporated into material having the chromatographic properties of $C_{27}$ monohydroxy sterols. The retinas also converted mevalonic acid to isoprenoid acids; no label was incorporated into retina n-fatty acids. In the incubations with the $S_{10}$ fraction, <1% of the label incorporated into nonsaponifiable lipids was precipitable with digitonin. The major nonsaponifiable lipids had the chromatographic properties of squalene and various open-chain isoprenoid
alcohols, but did not correspond to the alcohols of the allyl pyrophosphates which are known intermediates in the biosynthesis of squalene. Material having the chromatographic properties of C\textsubscript{30} and C\textsubscript{27} monohydroxy sterols was detected in small amounts in these incubations, but cholesterol represented only a minor fraction of this material. Isoprenoid acids (primarily C\textsubscript{20} isomers, with lesser amounts of C\textsubscript{15} isomers) were formed as the major products of these incubations. In addition to material having the properties of the all-trans isomers of farnesolic acid and geranylgeranoic acid, the biosynthesis of compounds having the chromatographic properties of the cis,cis- and cis,trans- (or trans,cis-) isomers of farnesolic acid was noted. Also, relatively large amounts of a C\textsubscript{20} isomer of geranylgeranoic acid (apparently having at least one cis double bond) were recovered from such incubations. These isoprenoid acids were not detected as endogenous components of the retina.

In an in vivo experiment, intraocularly-injected \textsuperscript{14}C-labelled mev-alonic acid was taken up and metabolized to nonsaponifiable lipids by several ocular tissues (predominantly by the retina) over a period of up to 2 hours. In each tissue, the major labelled nonsaponifiable lipid was squalene, with lesser amounts of material having the chromatographic properties of lanosterol. Only a few per cent of the retina nonsaponifiable products behaved chromatographically like C\textsubscript{27} monohydroxy sterols.

It was inferred from these results that the bovine retina has a very limited capacity for de novo sterol biosynthesis and must rely on alternate sources of cholesterol for the biogenesis of ROS membranes.
ACKNOWLEDGEMENTS

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<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CoA</td>
<td>coenzyme A</td>
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<td>CoQ_{10}</td>
<td>coenzyme Q_{10}</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>G-6-P</td>
<td>glucose-6-phosphate</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
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<tr>
<td>NAD (DPN)</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADP (TPN)</td>
<td>nicotinamide adenine dinucleotide 2'-phosphate</td>
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<td>ROS</td>
<td>rod outer segment(s)</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethyl-1,3-propanediol</td>
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<tr>
<td>MW</td>
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<td>HMG</td>
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<td>GLC</td>
<td>gas-liquid chromatography</td>
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<td>IR</td>
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<td>NMR</td>
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<td>R_f</td>
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I. INTRODUCTION

Cellular Organization of the Vertebrate Retina

The vertebrate retina is a peripheral extension of the central nervous system (specifically, the forebrain), being derived from the embryological invaginations of the primitive neural tube which form the optic cup (Cajal, 1960; Cohen, 1963; Coulombre, 1965). The interior of the optic cup and the ventricles of the brain are confluent during early embryological development but the progressive collapse of the optic cup during development forms an apposition of the two neural epithelial layers which are destined to become the neural retina and the retinal pigmented epithelium. A schematic cross-sectional diagram depicting the spatial relationship of the retina to the other ocular components in a human eye is shown in Figure I-1. The retina is a thin (0.1-0.3 mm in thickness) tissue which forms a spherical lining resembling a narrow-mouthed goblet on the interior of the eye. Only the posterior two-thirds of the retina (the "pars optica") is optically sensitive, while the anterior third is photo-insensitive (the "pars ciliaris"); the two portions of the retina are physically as well as functionally distinct, being separated along the interior circumference of the eye by the "ora serrata". Functionally, the retina may be thought of as consisting of two layers: the sensory retina (containing the photoreceptor cells) and the neural retina (containing the remainder of the retinal cells). The classic work of Cajal described the cellular elements of the retina and their connections in a variety of species (Cajal, 1893, 1933). There are five basic neural cell types in the retina: photoreceptor cells (rods
FIGURE I-1  Generalized hemisectical view of a vertebrate right eye, illustrating the spatial relationships between the retina and the other ocular tissues.
and cones), bipolar cells, horizontal cells, amacrine cells and ganglion cells. In addition, the space surrounding the neural cells is virtually occluded by non-neuronal cells (the Müller cells and accessory glial cells). All the cells of the retina, neuronal and non-neuronal, are derived from a common embryological origin, the neural epithelium or neural "ectoderm". Histologically, there are ten layers into which the retina is conventionally divided: (1) the retinal pigmented epithelium (RPE); (2) the photoreceptor cell layer; (3) the "outer limiting membrane" (a network of junctional complexes which binds the basal portion of the photoreceptor cells at the level of the "inner segments" to the apical portion of the Müller cells); (4) the "outer nuclear layer" (composed of the photoreceptor cell nuclei); (5) the "outer plexiform layer" (the level of synaptic contacts between photoreceptor cells, bipolar cells and horizontal cells); (6) the "inner nuclear layer" (perikarya of the bipolar cells, horizontal cells and amacrine cells); (7) the "inner plexiform layer" (level of synaptic contacts between bipolar cells, amacrine cells and ganglion cells); (8) the ganglion cell layer; (9) the "optic fiber layer" (collective processes of the ganglion cells which eventually form the "optic nerve"); (10) the "inner limiting membrane" (basement lamina which forms the vitreal-retinal barrier). A micrograph of the bovine retina which depicts this layered structure is shown in Figure I-2. In contrast to the layered arrangement of the neuronal elements of the retina, the Müller cells span almost the entire thickness of the retina, extending from the inner limiting membrane to the inner segments of the photoreceptor cells.

It is a peculiarity of embryological fate that the photoreceptor
FIGURE I-2

Light micrograph of a representative cross-section of a bovine retina depicting the layered organization of the retina (magnification 1,010 X). Note that the length of a rod cell spans nearly half the thickness of the retina and that the outer plexiform layer and inner nuclear layer are very sparse in this retina. Cone outer segments are seen just above the outer limiting membrane (arrows in photoreceptor layer). A cross-section of a relatively large retinal blood vessel, containing numerous red blood cells, is seen in the lower right corner of this micrograph (arrow). Note the large perikarya of the ganglion cells (ganglion cell layer). (Micrograph courtesy of Ms. Charlotte Levy and Ms. Rekha Mehta, Electron Microscopy Laboratories, Department of Ophthalmology, Baylor College of Medicine.)
cells are disposed most distally to the site of entry of light into the eye, with an orientation which is opposite to that of incident light; thus, light must traverse the cornea, aqueous humor, lens, vitreous humor and all the neural layers of the retina before impinging upon the photoreceptor cells. The retina is anatomically organized for the unidirectional and sequential processing of visual information, with lateral modulation, from photoreceptor cells to bipolar cells to ganglion cells. As an exquisite sensory processing system, the retina displays a property known as convergence, i.e. a relatively large number of initial sensory receptors relays the sensory information to successive processing stages, each of which contains fewer elements than the previous stage. For instance, in the human retina, approximately $10^8$ photoreceptors pass on their visual information by electrochemical propagation to a population of about $10^6-10^7$ bipolar cells which then relay the signal to approximately $10^5-10^6$ ganglion cells. The visual information received by the ganglion cells is then relayed via the optic nerve through the "optic chiasma", where the optic fibers partially decussate and form "optic tracts" which then synapse in the "lateral geniculate body" (in the thalamus) and the "superior colliculus"; higher-order processing is then carried out by the visual cortex.

**Nutrient Supply and Energy Metabolism of the Retina**

As a relatively sequestered tissue, the retina must solve the problems of nutrient supply and gas exchange. This is accomplished with the aid of two independent blood supplies (Walls, 1942; Wolff, 1961). The first is known as the choroidal circulatory system, which is located between the sclera and the pigmented epithelium and consists of an
arborized meshwork of fenestrated capillaries (the "choriocapillaries") which abut the retinal pigmented epithelium, separated from it by a thin endothelial layer and "Bruch's membrane" (Yamada et al., 1958) (see Figure I-2). Adjacent RPE cells are bounded by tight junctions ("zonula occludens") which prevent the flow of blood components between the cells (Cohen, 1968) and therefore restrict the accessibility of this circulatory system to the retina. The photoreceptor layer, which is completely avascular, must rely on diffusion and/or specific transport mechanisms (mediated via the RPE cells) for its supply of nutrients from the choriocapillaries. By a magnificent amplification of its cell surface, the RPE cell facilitates exchange between the choroidal circulation and the sensory retina via an elaboration of basal infoldings (on the choroidal side) and extensive apical processes (into which the outer segments of the photoreceptor cells invaginate) (Yamada, 1961; Dowling and Gibbons, 1962). Most vertebrates also have a sparse, secondary circulatory system (referred to as the "retinal circulation") which consists of a capillary network which penetrates the retina at the level of the inner nuclear layer and ganglion cell layer. These capillaries are not fenestrated, however, and the endothelial linings of the vessels form the so-called "blood-retina barrier" (Cunha-Vas et al., 1966) which restricts exchange to small molecules, ions and gases. Although the retina is practically occluded by Müller cells and assorted other glial cells, there is a finite amount of extracellular space which separates neuronal and glial cells in the form of numerous channels which are 10-20 nm in diameter (Ladman, 1961; Lasansky, 1965; Dowling and Boycott, 1966).

Carbohydrate metabolism and respiration have been extensively
studied in the retina for over 50 years (for a review, see Graymore, 1969, 1970). The experiments of Warburg and his colleagues demonstrated that the retina consumes oxygen more rapidly (per gram of tissue) than most other tissues, including the brain; in addition, the retina converts glucose to lactic acid with extreme facility, even in the presence of oxygen (Warburg et al., 1924; Warburg, 1927). Indeed, the presence of both a marked Pasteur effect (i.e. the stimulation of glycolysis under anaerobic conditions, with depressed respiration) as well as a profound Crabtree effect (i.e. the stimulation of respiration by depressing glycolysis, usually effected by decreasing the level of glucose) has been observed in the retina by many investigators. The rate of glucose utilization (i.e. primarily oxidation to carbon dioxide) by the retina per gram of tissue is higher than for any other tissue so far studied, due to the presence of an extremely active citric acid cycle as well as the pentose phosphate pathway (Cohen and Noell, 1958, 1959, 1960; Futterman and Kinoshita, 1959; cf. Cohen and Noell, 1965; Graymore, 1965, 1969, 1970). Evidence from numerous studies has suggested that the photoreceptor cells are predominantly responsible for the high levels of glycolytic activity and respiration by the retina (Noell and Cohen, 1957; Noell, 1958a, b; Graymore and Tansley, 1959; Graymore et al., 1959; Cohen and Noel, 1960; Graymore, 1960; Noell, 1965). In addition, energy stores in the form of glycogen deposits have been found in nearly every layer of the retina, especially in the inner and outer plexiform layers, and such deposits have been observed to undergo dynamic alterations as a function of light and dark adaptation (Schabadasch and Schabadasch, 1972). Large glycogen deposits have been found in the Müller cells, which are thought to serve both a metabolic and structural role in the retina (Shimizu and Maeda,
1953; Kuwabara and Cogan, 1961).

As previously mentioned, the vitreous body is separated from the retina by the inner limiting membrane, a "molecular sieve" which excludes molecules larger than 70 Å in diameter. The vitreous body is predominantly composed of water (about 99%, by weight) and the only cells present are an extremely sparse population of mononuclear phagocytes (hyalocytes) whose major role is the synthesis and deposition of mucopolysaccharides, collagen and certain structural proteins (Balazs, 1960, 1961, 1968; cf. Pirie, 1969; Berman and Woaden, 1970). These cells are found only in the extreme periphery of the vitreous body, an area designated as the "cortical tissue layer" (Balazs, 1961); in general, this peripheral layer of the vitreous body is considerably more fluid than the quasi-elastic gel which constitutes the bulk of the tissue. Considering the nature and properties of the vitreous body, it is generally believed that this avascular and transparent tissue serves primarily the mechanical roles of maintaining ocular shape, buffering the interior of the globe from various traumas and providing an optically clear path for the unimpeded travel of light between the lens and the retina. The contribution of the vitreous body to the metabolic maintenance of the retina is therefore believed to be minimal, if not totally negligible.

There exists a very special and integral relationship between the retinal pigmented epithelium and the photoreceptor cells. This relationship will be discussed in more detail later in this chapter.

**Lipid Composition of the Vertebrate Retina**

Lipids were first recognized as constituents of the retina by Kühne
(1879) during his pioneering studies on the visual pigment rhodopsin. Although his investigation of retina lipids was only cursory, Kühne did identify lecithin (phosphatidylcholine) in ethanol-ether extracts of frog and cattle retinas. The first attempt to separate and quantitate retinal lipids was reported by Cahn (1881) for cattle, horse and porcine retinas; he found the "moist" cattle retina to contain "0.71% cholesterol, 2.48% lecithin and 0.40% fat" (as cited by Krause, 1938). Later, Lo Cascio (1923) reported further separation and quantitation of bovine retinal lipids; he found "25.7% total lipids, 1.6% cholesterol and 13.7% phospholipids", on a dry weight basis (as cited by Krause, 1938). The first true separation of some of the major lipid classes and partial quantitation of the components was reported by Leinfelder and Salit (1934), who compared the lipid composition of bovine retina, whole brain (including approximately equal amounts of white and gray matter) and blood. The tissues were extracted with absolute ethanol-ether (3:1, by volume), essentially by the method of Bloor (1916), and the lipid classes were separated by differential solubility in organic solvents and quantitated by gravimetric and colorimetric methods. Cholesterol was assayed by taking a portion of the ethanol-ether extract to dryness, extracting the residue with chloroform and assaying aliquots of the extract colorimetrically by a variation of the Liebermann-Burchard method (i.e. with acetic anhydride and concentrated sulfuric acid), as described by Bloor (1916). In addition, these researchers determined the iodine number for the total lipids of each tissue sample to assess the relative content of olefinic compounds, which was considered to be an indicator of the general biological activity of the tissue. A partial tabulation of their results is presented in Table I-1, along with the results subsequently reported
by Krause (1938) and Brante (1949). This early work revealed that lecithin and cephalin (phosphatidylethanolamine) are the major phospholipid classes in the retina, although the absolute amounts of the lipid constituents found by each of these investigators showed considerable variation. Leinfelder and Salit (1934) reported that the water content of bovine retina, brain and blood was 89.0, 78.0 and 79.0 per cent of the wet weight, respectively; the iodine numbers were found to be 22.9, 24.2, and 13.4, respectively, for these tissues. One may calculate the retina water content from the data of Krause (1938) and Brante (1949), whose data suggest values of 87.0% and 84.6%, respectively. Due to differences in methodology, quantitative aspects of the data are difficult to compare. The data of Krause (1938) and Brante (1949) are in closer agreement with each other than they are with the data of Leinfelder and Salit (1934). In particular, Leinfelder and Salit (1934) reported a higher total lipid content, lower cephalin content and much higher fat content for the retina than reported by other researchers (cf. Lo Cascio, 1923; Krause, 1938; Brante, 1949). However, all these investigators noted the very low level of "cholesterol" in the retina, especially when compared with brain tissue. Krause (1938) determined cholesterol gravimetrically as the digitonide, before and after saponification, by the method of Man and Peters (1933). Brante (1949) quantitated cholesterol by precipitating the nonsaponifiable material with digitonin and then assaying the digitonides colorimetrically, as described by Schoenheimer and Sperry (1934). Krause (1938) noted that all the cholesterol in the retina was in the free form (i.e. no cholesteryl esters were detected); in addition, he noted that the total amount of nonsaponifiable material was very small and speculated that the non-sterol portion might contain aliphatic
Table I-1
The Lipid Composition of Bovine Retina and Brain Cortex

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>Leinfelder &amp; Salit (1934)</th>
<th>Krause (1938)</th>
<th>Brante (1949)</th>
<th>Brante (1949)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% DRY WEIGHT</td>
<td>% TOTAL LIPID</td>
<td>% DRY WEIGHT</td>
<td>% TOTAL LIPID</td>
</tr>
<tr>
<td>Total Lipids</td>
<td>30.9</td>
<td>100.0</td>
<td>19.7</td>
<td>100.0</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>10.0</td>
<td>32.4</td>
<td>13.7</td>
<td>69.5</td>
</tr>
<tr>
<td>Lecithin</td>
<td>8.4</td>
<td>27.4</td>
<td>7.0</td>
<td>35.6</td>
</tr>
<tr>
<td>Kephalin</td>
<td>3.5</td>
<td>11.2</td>
<td>4.8</td>
<td>24.3</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>---</td>
<td>---</td>
<td>1.9</td>
<td>9.6</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2.0**</td>
<td>6.5**</td>
<td>2.0**</td>
<td>10.1**</td>
</tr>
<tr>
<td>Cerebroside</td>
<td>1.8**</td>
<td>5.9**</td>
<td>0.8**</td>
<td>4.1**</td>
</tr>
<tr>
<td>Fats ***</td>
<td>12.7</td>
<td>41.0</td>
<td>2.5</td>
<td>12.9</td>
</tr>
</tbody>
</table>

* Values for "% Total Lipid" calculated from the original data of the author.

** Value reported as "glyolipid" in the original literature.

*** Unidentified glycerides.
alcohols such as cetyl alcohol (although he offered no data to support this speculation). Brante (1949) presented an extensive study of the lipid composition of adult nervous tissues from a variety of animals, including human, bovine, rabbit, horse and rat, and the composition for various anatomical sections of brains was reported, including grey matter (cortex, containing cell bodies and unmyelinated processes) and white matter (nerve fibers and myelinated processes). Thus, comparing bovine brain cortex and bovine retina (see Table I-1), the total phospholipid content is fairly comparable whereas the brain cortex has a higher complement of cholesterol and cerebroside. Considering the differential development of these two tissues, it is not surprising to find them to be biochemically as well as anatomically distinct, even though they share a common embryological origin. Collins et al. (1952), in a comparative study of bovine and frog retina composition, reported that the phospholipid content of dry retinas was 16.4% (bovine) and 12.9% (frog), respectively. A comparative study of the lipids of bovine ocular tissues was performed by D'Asaro et al. (1954). They found that choline-containing phospholipids (lecithins and sphingomyelins) represented 51% of the total retinal phospholipids, phospholipids accounted for 82% of the total retina fatty acids, and cholesterol (determined by the method of Sperry and Webb, 1950) existed only in the free form and accounted for 0.65% of the retina dry weight. Each of the ocular tissues studied (i.e. lens, cornea, retina, iris and ciliary body) was distinct in its composition and lipid content; all the tissues contained low levels of cholesterol, with the lowest amount found in lens (0.26%, by dry weight) and the highest in the ciliary body (0.76%, by dry weight).
Using a histological staining technique which was based upon the Libermann-Burchard colorimetric reaction and was claimed to be specific for cholesterol (Schültze, 1924, 1925), Francis (1955) reported that cholesterol is maximally concentrated in the inner plexiform layer of various vertebrate retinas. Due to limitations in the sensitivity of the technique, Francis was not able to detect cholesterol in the photoreceptor layer. Lowry et al. (1956) performed the remarkable feat of dissecting the monkey retina into all its histologically distinct layers and assayed the total lipid content of each layer, on a dry weight basis. They found that the outer nuclear layer contained the lowest proportion of lipids (12%) whereas the optic fiber layer contained the highest (34%). By their estimates, the "outer retinal layer" contained only 20-25% lipid.

The advent of more sophisticated methods for the extraction, separation and analysis of lipids in the late 1950's and early 1960's (especially chromatographic techniques) provided researchers with the tools required for a more detailed understanding of the lipid composition of biological materials. Since the phospholipids represented the major lipids of the retina, most of the research on retina lipids centered on the analysis of phospholipid class composition and the distribution of fatty acids in each class. Bartley et al. (1962) studied the fatty acid composition of various bovine ocular tissues by saponifying the tissues, extracting the fatty acids, preparing their methyl ester derivatives and analyzing the methyl esters by gas-liquid column chromatography. Quantitatively the methyl esters by measurement of the relative peak areas on the chromatogram, they reported that the major fatty acids of whole bovine retina were represented by palmitic acid ($C_{16:0}$; 24.9%), stearic acid ($C_{18:0}$; 17.3%),
oleic acid \( (C_{18:1}, 17.3\%) \) and a 22-carbon polyunsaturated acid \( (22.9\%) \) which was later identified as all-cis docosahexaenoic acid \( (C_{22:6\omega3}) \) by Hands and Bartley (1963). Unsaturated fatty acids represented 51.1% of the total fatty acids of retina, of which over 60% was accounted for by polyunsaturated acids. These authors also noted that the fatty acid composition of bovine retina more closely resembled that of brain cortex than that of whole brain and that each ocular tissue had its own distinct fatty acid composition, with the highest amount of polyunsaturates (especially \( C_{22:6} \)) being present in the retina. By similar methods, Futterman et al. (1971) examined the fatty acid composition of all the major tissues of male Sprague-Dawley rats, including the retina and brain; they reported the following fatty acid composition (in m ole per cent) for rat retina: palmitate \( (C_{16:0}), 19.3\% \); palmitoleate \( (C_{16:1}), 1.3\% \); stearate \( (C_{18:0}), 22.5\% \); oleate \( (C_{18:1}), 9.6\% \); linoleate \( (C_{18:2}), 1.2\% \); arachidonate \( (C_{20:4}), 9.4\% \); other long-chain polyunsaturates (sum of \( C_{22:4}, C_{22:5} \) and \( C_{22:6} \)), 36.7%. Futterman and Andrews (1964) reported the fatty acid composition of whole human retinas. They found the major fatty acids (representing about 83.5% of the total fatty acids) to be palmitate (16.7%), stearate (18.2%), oleate (15.6%), arachidonate (11.8%), and docosahexaenoate (21.2%). The relative fatty acid composition did not appear to be a function of the age or sex of the individual from whom the eyes were obtained.

The phospholipid class composition of vertebrate retinas has been investigated in detail by several researchers. Eichberg and Hess (1967) reported the phospholipid content, class composition and cholesterol content of frog retinas. In this case, cholesterol was quantitated by a
Table I-2
Major Phospholipid Classes of Vertebrate Retina

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Frog (^a)</th>
<th>Calf (^b)</th>
<th>Cow (^c)</th>
<th>Rabbit (^c)</th>
<th>Pig (^c)</th>
<th>Human (^c)</th>
<th>Dog (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lyso PC</td>
<td>—</td>
<td>0.1 ± 0.04</td>
<td>0.2 ± 0.2</td>
<td>2.6 ± 0.7</td>
<td>0.3 ± 0.3</td>
<td>0.3 ± 0.3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Sph</td>
<td>12.7</td>
<td>5.8 ± 0.32</td>
<td>2.1 ± 0.0</td>
<td>4.4 ± 0.7</td>
<td>3.7 ± 0.2</td>
<td>4.3 ± 0.5</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>PC</td>
<td>52.7</td>
<td>40.1 ± 1.00</td>
<td>43.2 ± 0.9</td>
<td>43.9 ± 2.7</td>
<td>47.1 ± 0.6</td>
<td>47.8 ± 3.7</td>
<td>46.8 ± 1.5</td>
</tr>
<tr>
<td>PE</td>
<td>18.8</td>
<td>33.8 ± 0.83</td>
<td>34.1 ± 2.0</td>
<td>34.7 ± 1.9</td>
<td>32.3 ± 0.4</td>
<td>31.7 ± 2.1</td>
<td>30.0 ± 0.4</td>
</tr>
<tr>
<td>PS</td>
<td>9.6</td>
<td>10.0 ± 0.35</td>
<td>10.0 ± 1.1</td>
<td>7.4 ± 2.0</td>
<td>8.1 ± 1.0</td>
<td>8.6 ± 1.0</td>
<td>8.9 ± 1.1</td>
</tr>
<tr>
<td>PI</td>
<td>1.6</td>
<td>6.4 ± 0.18</td>
<td>5.6 ± 0.8</td>
<td>4.3 ± 0.6</td>
<td>5.5 ± 0.4</td>
<td>4.4 ± 0.3</td>
<td>4.6 ± 0.3</td>
</tr>
</tbody>
</table>

* Phosphorous analysis after two-dimensional thin-layer chromatography, expressed as mole per cent of total lipid phosphorous, ± S.D. (n = 6).

\(^a\) Eichberg and Hess (1967).
\(^b\) Broekhuysen (1968).
\(^c\) Anderson (1970).
\(^+\) Abbreviations: lyso PC, lyso phosphatidylcholine; Sph, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.
modified Liebermann-Burchard assay after saponification of the total lipid extract and was also chromatographically identified in the nonsaponifiable extract by co-migration with a standard of cholesterol on Silica Gel G thin-layer plates. A value of 0.33 was reported for the cholesterol:l lipid phosphorous mole ratio. Subsequently, Broekhuysse (1968) reported the phospholipid class composition of various bovine ocular tissues, including the retina. A much more extensive investigation of the retina phospholipid class composition and fatty acid composition of each phospholipid class was reported by Anderson and co-workers (Anderson, 1970; Anderson et al., 1970) for several vertebrate species. Table I-2 lists the results of the studies cited above concerning the phospholipid class composition of vertebrate retinas. Remarkably, over a wide species range, the phospholipid class composition of the retina and the fatty acid species distribution within each class are strikingly uniform. In addition, the overall phospholipid class composition of the retina is quite similar to that of a variety of other tissues. The major phospholipid classes are represented by phosphatidylcholine (PC; 45.9 ± 4.0%) and phosphatidylethanolamine (PE; 30.8 ± 5.5%), with lesser amounts of phosphatidylserine (PS; 8.9 ± 1.0%), phosphatidylinositol (PI; 4.6 ± 1.5%) and sphingomyelin (Sph; 5.3 ± 3.4%). Only traces of lysophosphatidides were detected in each case. Small amounts of cardiolipin and phosphatidic acid, moderate amounts of ethanolamine plasmalogens (i.e. 13.8% of the total ethanolamine phospholipid) and high amounts of alkyl ether phospholipids (up to 12.7% of the total phospholipid, including the contribution of sphingomyelins) were reported for frog retinas (Eichberg and Hess, 1967). Small amounts of choline plasmalogen, phosphatidic acid
and phosphatidyl glycerol, moderate amounts of cardiolipid and relatively high levels of ethanolamine plasmalogens were found in all bovine ocular tissues (Broekhuyse, 1968); in the bovine retina, ethanolamine plasmalogens represented 32.6% of the total ethanolamine phospholipid. A similar phospholipid composition was determined for the rat retina (Dreyfus et al., 1971), where ethanolamine plasmalogens may represent between 26.6% and 32.0% of the total ethanolamine phospholipid content and choline plasmalogens may account for 4.9-6.4% of the total choline phospholipid content, depending on the state of illumination of the tissue. As a phospholipid class, PC contains primarily saturated fatty acids (mostly palmitic and stearic acid), moderate levels of oleic acid and very minor amounts of polyunsaturates; in contrast, polyunsaturated fatty acids predominate in the other phospholipid classes, with PE and PS containing very high levels (20-30 mole %) of docosahexaenoic acid and PI containing predominantly arachidonic acid (Anderson, 1970; Anderson et al., 1970). Sphingomyelin is unique in comparison, containing predominantly saturated (palmitic and stearic) acids and no detectable polyunsaturates. For a given species, the phospholipid class composition and fatty acid distribution within each class was comparable to data previously reported for the lipid composition of vertebrate brain cortex (O'Brien and Sampson, 1965). The trends in the phospholipid class composition and fatty acid distribution described above for whole retina are also reflected in the subcellular components of the retina. Fractionation of the bovine retina into rod outer segments (ROS) mitochondria, microsomes and nuclei, and analysis of the phospholipid class composition and fatty acid composition of each class revealed
that the ROS contained lesser amounts of PC, PI and Sph and larger
amounts of PE and PS than the other retinal fractions (Anderson, et al.,
1975). The trace levels of choline plasmalogen and moderate levels of
ethanolamine plasmalogen reported previously were localized mostly to
the microsomal and mitochondrial fractions, with lesser amounts in the
nuclear fraction and no detectable plasmalogens in the ROS fraction.

Quantitative studies on the diglyceride composition of the toad
retina and brain (Avelaño and Bazán, 1972, 1973) revealed that the
diglyceride content of the amphibian retina is several-fold higher than
that of brain, and that the fatty acid composition of the retinal digly-
cerides resembles that of retinal phospholipids more closely than that
of brain diglycerides. The results of a comparative study of mammalian
and amphibian retinas (Avelaño and Bazán, 1974) revealed moderately
high levels of free fatty acids, with bovine retinas containing the
largest amounts of C_{20:4} and C_{22:6} acids. The triglycerides of bovine,
rabbit and toad retinas contain predominantly palmitic, stearic and
oleic acids; the level of docosahexaenoic acid in the triglycerides of
cattle and toad retinas was relatively high, whereas the rabbit retina
triglycerides (which contain C_{22:4} as the major acid) were very low in
docosahexaenoic acid. In mammalian retinal diglycerides, arachidonic
acid predominates as the major acyl species (ca. 25 mole %), whereas
docosahexaenoic acid predominates (ca. 42 mole %) in amphibian retinal
diglycerides; unlike amphibia, however, mammalian retina phospholipids
and diglycerides are dissimilar in their fatty acid composition, with a
much higher level of C_{22:6} in the phospholipids than in the diglycerides
The early studies on retina lipid composition reported previously (Leinfelder and Salit, 1934; Krause, 1938; Brante, 1949; Eichberg and Hess, 1967) indicated that the vertebrate retina contains only moderate amounts of glycolipids. In contrast, glycolipids (especially the gangliosides) are major constituents of other neural tissues, including the cerebral cortex (Svennerholm, 1964; Suzuki, 1965a, b; Prensky and Carr, 1969; Hakomori and Saito, 1969; Samuelsson, 1971a, b). A number of reports have appeared which describe the gangliosides of vertebrate retinas (Handa and Burton, 1969; Kostic et al., 1969; Dreyfus et al., 1971, 1974b; Holm et al., 1972; Edel-Harth et al., 1973; Urban et al., 1973, 1975; Holm and Mansson, 1974). In the mammalian retina, ganglioside \( G_{D3} \) is the major acidic glycosphingolipid (Holm et al., 1972; Edel-Harth et al., 1973), whereas in the avian retina \( G_{DLa} \) is apparently the major ganglioside (Edel-Harth et al., 1973; Urban et al., 1975). The predominance of \( G_{D3} \) in the mammalian retina is peculiar, since this ganglioside is only found in relatively minor amounts in other neural tissues. Not only does \( G_{D3} \) account for over 50 mole % of the gangliosides of mammalian retinas, but its sphingosine and fatty acid composition is quite distinct from that of \( G_{DLa} \), \( G_{DLb} \) and \( G_{TL} \) (the other major gangliosides of the retina, each of which represents about 10-15 mole % of the total gangliosides) (Holm et al., 1972; Holm and Mansson, 1974).

Also, whereas the fatty acid composition of the three lesser gangliosides of the mammalian retinas was very similar to that of the corresponding brain gangliosides, the fatty acid composition of retinal \( G_{D3} \) was distinct from that of brain \( G_{D3} \) (Holm et al., 1972). In bovine retina, the concentration of total ganglioside N-acetylneuraminic acid is about
0.4 μmole/gram fresh tissue weight (i.e. about 29 nmol/μmole of lipid phosphorous), which corresponds to about 10-12% of the value determined for brain (Vanier et al., 1971; Holm et al., 1972). It has also been reported that exposure of the bovine retina to light causes a large (ca. 40%) increase in the ganglioside content of the rod outer segments, with no significant change in the absolute amount of gangliosides in the whole retina and no apparent alteration of the relative concentrations of the individual ganglioside species in either whole retina or ROS (Dreyfus et al., 1974b). The overall ganglioside composition of bovine ROS closely resembles that of the whole retina, but the ROS ganglioside N-acetylmuraminic acid content only represents about 1% of the whole retina ganglioside content (Dreyfus et al., 1974b). ROS ganglioside content will be discussed in more detail in a section of this chapter dealing with the lipid composition of ROS membranes.

**Structural Organization of the Vertebrate Photoreceptor Cells**

The structural organization and physiology of the vertebrate photoreceptor cells has been reviewed in detail elsewhere (Cohen, 1963, 1969, 1972; Dowling, 1967; Young, 1969, 1976; Daemen, 1973; Ebrey and Honig, 1975; Montal and Korenbrot, 1976). Therefore, only a brief presentation of the more salient features of visual cell structure will be given here. There are two morphologically and physiologically distinct visual cell types: the rods and the cones. Rods are elongated and cylindrical in shape (hence their name), whereas cones are relatively shorter and generally possess a conical-shaped distal portion (outer segment). Functionally, the rod cells are dim light receptors (i.e. they provide visual acuity), whereas the cones are exclusively color receptors. With
few exceptions, rods predominate in gross excess over cones in a given retina, and in certain species (notably the rat and other nocturnal animals) very few cones are found. The following discussion will be restricted to the vertebrate rod cell, since it is the most extensively characterized visual cell type and the cones are not directly relevant to this thesis.

A schematic diagram which depicts the typical (although highly stylized) features of a rod cell is shown in Figure I-3. The rod cell may be divided into four anatomically and functionally separate parts: (1) the rod outer segment (ROS); (2) the rod inner segment (RIS); (3) the nuclear region; and (4) the synaptic pedicle. The early light-microscopic studies of Schültze (1866, 1973) revealed that the ROS could be mechanically disrupted and separated from the rest of the rod cell, usually fracturing the outer segment at right angles to its long axis; the fragments so formed appeared striated and these striations were interpreted as "disks". Sjöstrand (1948, 1949, 1953, 1961) confirmed the lamellar organization of the rod outer segment in a series of electron-microscopic studies of vertebrate retinas. The ROS is a highly-specialized appendage of the rod cell composed of a rigidly-ordered stack of 500-2000 disk-shaped membranes (depending on the species) enclosed by the plasma membrane of the cell. More recent studies have shown that, with the exception of the first several disks at the base of the outer segment (the so-called "basal disks", "open disks" or "basal infoldings"), the disk membranes are "free-floating", i.e. they are not in physical contact either with each other or with the plasma membrane (Dowling, 1967; Cohen, 1968, 1972; Laties and Liebman, 1970; Laties et
FIGURE I-3

Schematic representation of a longitudinal section of a rod cell, illustrating the subcellular compartmentation and various anatomical elements of this cell type. Note that the overwhelming majority of disks are the "free-floating" type (i.e. not contiguous with adjacent disks or with the plasma membrane of the cell).
(Ruppel and Hagins, 1973). The frog ROS is about 5-7 μm in diameter, 35-50 μm in length and contains about 1800-2000 disks (Nilsson, 1965; Cohen, 1973). The bovine ROS contains nearly 1000 disks and has the approximate dimensions 2 μm x 22 μm (Bonting, 1969). The rat ROS has the approximate dimensions 2 μm x 24 μm, whereas the human ROS measures about 2 μm x 40-60 μm and contains approximately 1000 disks (Cohen, 1972, 1973).

The ROS is connected to the inner segment via a thin ciliary neck, a cytoplasmic bridge via which the exchange of material takes place between the two cellular compartments. Unlike the motile cilia, the cilium in the rod cell contains only the outer ring of nine doublet filaments (but lacks the inner pair of filaments commonly found in other cilia) (De Robertis, 1960). The rod inner segment (RIS) has two regions: (1) the ellipsoid, which is the portion of the RIS closest to the outer segment and contains numerous densely-packed mitochondria; and (2) the myoid which houses the network of smooth and rough endoplasmic reticulum and the Golgi apparatus. The presence of these organelles in the inner segment indicates that the RIS is the cellular compartment where the bulk of the cellular metabolism takes place, in contrast to the notably organelle-devoid ROS; however, the ROS possesses a variety of enzymatic capabilities of its own, as will be discussed in further detail later.

The nucleus of the cell lies just below the myoid region and is not remarkable in any respect. An axonal process connects the RIS and nuclear regions with the synaptic pedicle, which contains numerous synaptic vesicles (containing neurotransmitters and other components)
and a unique structure known as the "synaptic ribbon".

**Composition of the Rod Outer Segment**

Due to the fragility of the connecting cilium, the ROS may be separated from the rest of the rod cell by relatively gentle mechanical agitation of the retina in a sucrose solution, followed by differential centrifugation (Saito, 1938; Wald and Brown, 1951-52; Matthews et al., 1963). The purity of the ROS preparation is enhanced greatly by subjecting the crude preparation (i.e. the supernatant obtained by differential centrifugation) to either continuous or discontinuous density gradient centrifugation (Collins et al., 1952; McConnell, 1965; Shichi et al., 1969; Lolley and Hess, 1969; Borggraeven et al., 1970; Bownds et al., 1971; Papermaster and Dreyer, 1974). If one exercises caution in the gentle homogenization of the retinas and takes care in the preparation and handling of the sucrose density gradients (cf. Papermaster and Dreyer, 1974), the material which floats at a density of approximately 1.11 g/cc represents a population of homogeneous and relatively intact ROS. The disk membranes may be further isolated as single-walled vesicles by hypotonic lysis of the purified ROS material (Raubach et al., 1974), followed by floatation of the intact disks on a cushion of Ficoll (Smith et al., 1975). After washing away residual sucrose or Ficoll, the ROS material may be used for various chemical and physical analyses.

Using ROS material prepared as described above, several laboratories have reported the composition of outer segments, primarily from bovine and frog retinas (Daemen, 1973; Abrahamson and Fager, 1973). A partial tabulation of the existing data is given in Table I-3. The data show some degree of variation (probably due to differences in the methods of
preparation and extraction of the ROS material, as well as differences in the analytical techniques employed), but indicate that the overall composition of frog and bovine ROS is quite similar. In general, on a dry weight basis, the ROS is composed of approximately 40-50% lipid and 50-60% protein; phospholipids account for 80-90% of the total lipid in bovine ROS and at least 65-75% of frog ROS lipid. Carbohydrate accounts for up to 4% of the ROS dry weight (DeGrip et al., 1973), but the absolute amount and its origin have been disputed (Daemen, 1973). The extra disk space in the ROS apparently contains mucopolysaccharide (Cohen, 1972). About 2% of the ROS dry weight can be attributed to carbohydrate associated with rhodopsin, which contains at least 5 moles of N-acetyl glucosamine and 9 moles of mannose per mole (Plantner and Kean, 1976; cf. Heller and Lawrence, 1970). Glycolipids have been reported to account for ~10% of the total ROS dry weight and ~25% of the ROS total lipid (by weight) in the frog retina (Eichberg and Hess, 1967; Mason et al., 1973). The ganglioside composition of frog ROS (Hess et al., 1971; Edel-Harth et al., 1973) and bovine ROS (Dreyfus et al., 1974b) has also been reported. However, it is not clear whether these glycolipid constituents represent true endogenous components of the ROS or whether they arise by contamination of the ROS preparation with glycolipid-rich subcellular elements of the retina (or even incomplete washing of the membranes, which would result in residual sugars).

The cholesterol content of the ROS is of direct pertinence to this thesis. Although all the reported values indicate that the cholesterol content of the ROS is very low, there is considerable variation in the data. This variation may be attributed to differences in the methodology
### TABLE I-3

Composition of Bovine and Frog Rod Outer Segment Membranes

<table>
<thead>
<tr>
<th>TOTAL LIPID</th>
<th>PHOSPHOLIPID A</th>
<th>PHOSPHOLIPID B</th>
<th>CHOLESTEROL A</th>
<th>CHOLESTEROL B</th>
<th>TOTAL PROTEIN</th>
<th>RHODOPSIN</th>
<th>INVESTIGATORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>61.5</td>
<td>27.7</td>
<td>45.0*</td>
<td>---</td>
<td>---</td>
<td>38.5</td>
<td>14</td>
<td>Collins et al. (1952)</td>
</tr>
<tr>
<td>38.8</td>
<td>31.5</td>
<td>81.2*</td>
<td>0.9</td>
<td>2.3*</td>
<td>61.2</td>
<td>---</td>
<td>Sjöstrand (1959)</td>
</tr>
<tr>
<td>59.8</td>
<td>53.3</td>
<td>89.1*</td>
<td>3*</td>
<td>5</td>
<td>40.2</td>
<td>---</td>
<td>Fleischer and Mc Connell (1966)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>38.9</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>Adams (1969)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>35.3</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>Poincelot and Zull (1969)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>27.7</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>Anderson and Maude (1970)</td>
</tr>
<tr>
<td>39.0</td>
<td>31.5</td>
<td>80.8*</td>
<td>3.1</td>
<td>7.9*</td>
<td>---</td>
<td>22</td>
<td>Borggreven et al. (1970)</td>
</tr>
<tr>
<td>50</td>
<td>43*</td>
<td>86</td>
<td>4*</td>
<td>8</td>
<td>36</td>
<td>31</td>
<td>De Grijp et al. (1973)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>1.3*</td>
<td>3.2*</td>
<td>---</td>
<td>---</td>
<td>Hendriks et al. (1976)</td>
</tr>
<tr>
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<td>26.6</td>
<td>65.5</td>
<td>1.7</td>
<td>4.3</td>
<td>59.4</td>
<td>---</td>
<td>Eichberg and Hess (1967)</td>
</tr>
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<td>74.5*</td>
<td>2.2</td>
<td>5.5*</td>
<td>60.4</td>
<td>27.0</td>
<td>Mason et al. (1973)</td>
</tr>
<tr>
<td>50</td>
<td>37</td>
<td>74*</td>
<td>---</td>
<td>---</td>
<td>50</td>
<td>---</td>
<td>Hall et al. (1973)</td>
</tr>
</tbody>
</table>

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**a** Values expressed as dry weight per cent; A, per cent of membrane dry weight; B, weight per cent of total lipid.

* Values calculated from the original data of the authors.
used for the preparation of the ROS material, the methods used for determining the cholesterol content of the membranes, lipid extraction and/or saponification procedures, or a combination of these factors. Cholesterol was first reported as a component of the bovine ROS by Sjöstrand (1959), but he gave no details regarding the methods used for identification and quantitation of the sterol content. Fleischer and McConnel (1966) estimated the cholesterol content of a bovine ROS preparation by thin-layer chromatography (in comparison with a standard of cholesterol) after gravimetric analysis of the neutral lipids isolated by silicic acid column chromatography of the total lipid extract. They also noted the presence of several redox constituents in the neutral lipid fraction, but demonstrated by TLC that these unidentified components did not co-migrate with Coenzyme Q₁₀ or some other related derivatives. Other researchers (Eichberg and Hess, 1967; Borggreven et al., 1970; De Grijp et al., 1973; Mason et al., 1973; Hendriks et al., 1976) prepared total lipid extracts of the ROS preparations and then quantitated the cholesterol content of the nonsaponifiable material prepared from the total lipid extract by a modified Liebermann-Burchard colorimetric assay. The sterol was also identified by thin-layer chromatography of the nonsaponifiable material (Eichberg and Hess, 1967; Mason et al., 1973).

The values reported for the cholesterol content of bovine ROS membranes range from 0.9% to 4% (per cent of total ROS dry weight), which corresponds to between 2.3% and 8%, by weight, of the total ROS lipid. The range of values reported for the cholesterol content of frog ROS membranes is much smaller, with cholesterol reportedly representing 1.7-2.2% of the membrane dry weight and 4.3-5.5% of the total lipid, by weight.
Rhodopsin is the visual pigment most common in vertebrates (Wald, 1968; Bridges, 1970, 1972, Morton, 1972). In intact or partially fragmented ROS, rhodopsin represents about 80-90% of the total protein (Hall et al., 1969; Heitzman, 1972; Robinson et al., 1972; Papermaster and Dreyer, 1974). In extensively-washed ROS membrane preparations (in which cytoplasmic and "peripheral" proteins have been removed), rhodopsin may account for 97-99% of the total protein, by weight. The only other readily apparent protein is a very large component (ca. 238,000 daltons in bovine ROS, ca. 290,000 daltons in frog ROS membranes) which accounts for the remaining 1-3% of the total protein (0.1-0.4 mole per cent) and is specifically located in the disk margins and incisures of the ROS (Papermaster and Dreyer, 1974; Papermaster et al., 1976, 1978; Krebs and Kuhn, 1977). In addition, a variety of enzymatic activities have been ascribed to the ROS, including adenylate cyclase (Bitensky et al., 1972), guanylate cyclase (Pannbacker, 1973; Bensing er et al., 1974), cyclic nucleotide phosphodiesterases (Pannbacker et al., 1972; Schmidt and Lolley, 1972; Chader et al., 1974; Miki et al., 1975), protein kinases (Kühn and Dreyer, 1972; Bownds et al., 1972; Frank and Bensing er, 1974; Miller and Paulsen, 1975), various adenosinetriphosphatases (Frank and Goldsmith, 1965; Ostwald and Heller, 1972; Hemminki, 1974, 1975; Sack, 1977), retinol dehydrogenase (Wald and Hubbard, 1949; Futterman, 1963; Futterman et al., 1970; Zimmerman et al., 1975, 1976) and the phospho-gluconate pathway enzymes (Lowry et al., 1961; Futterman, 1963). It has been reported that the enzymes required for de novo biosynthesis of phospholipids are not present in the ROS (Swartz and Mitchell, 1970). In the frog ROS, rhodopsin has a concentration of about 2.5 mM (Liebman,
1962, 1972), and similar concentrations are apparently present in other vertebrates studied (Daemen, 1973).

The phospholipid class composition as well as the fatty acid composition of the phospholipids for bovine, frog and rat ROS membranes have been extensively investigated (Eichberg and Hess, 1967; Poinceliot and Zull, 1969; Anderson and Maude, 1970, 1972; Borggreven et al., 1970; Nielsen et al., 1970; Poinceliot and Abrahamson, 1970; Mason et al., 1973; Hall et al., 1973; Anderson and Risk, 1974; Dreyfus et al., 1974a; Hendriks et al., 1976; Miljanich, 1978). A partial compilation of the data concerning the phospholipid class composition and the fatty acid composition is given in Tables I-4 and I-5, respectively. The major phospholipid species are phosphatidylethanolamine and phosphatidylcholine (which occur in roughly equal amounts), with lesser amounts of phosphatidylserine, phosphatidylinositol and sphingomyelin. The compositional data are, in most respects, rather uniform for bovine, frog and rat ROS membranes. The most striking feature of the ROS lipid composition is the very high level of long-chain polyunsaturated fatty acids (i.e. roughly 50% of the total acyl pool), with docosahexaenoic acid accounting for 30-40% of the total fatty acid content. The three major phospholipids (PE, PC and PS) are found in the ratio 40:40:10 (i.e. accounting for 90% of the total phospholipid); the $C_{22:6}$ fatty acid comprises 39%, 23% and 45%, respectively, of the total acyl chains of these phospholipids. The distribution of the $C_{22:6}$ acid is more similar in the phospholipids of rat and frog ROS membranes than for bovine ROS phospholipids, whereas palmitic acid is distributed similarly in all three animal species and stearic acid is notably higher in the rat
<table>
<thead>
<tr>
<th>SPECIES</th>
<th>PHOSPHOLIPID CLASS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TOTAL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>INVESTIGATORS</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PE</td>
<td>PC</td>
<td>PS</td>
</tr>
<tr>
<td>Bovine</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>38.5</td>
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<td>37.0*</td>
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<td></td>
</tr>
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<tr>
<td></td>
<td>36.6</td>
<td>41.0</td>
<td>12.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values expressed as mole per cent of total lipid phosphorus.

<sup>b</sup>Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; Sph, sphingomyelin

*Value includes plasmalogen.
<table>
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<th></th>
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</thead>
<tbody>
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<td>Bovine</td>
<td>2.5</td>
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<td>1.3</td>
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<td>5.4</td>
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<td>3.9</td>
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<td>7.0</td>
<td>0.8</td>
<td>3.6</td>
<td>5.6</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
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<td>10.0</td>
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<td>29.4</td>
<td>9.5</td>
<td>0.3</td>
<td>5.3</td>
<td>1.3</td>
<td>35.0</td>
</tr>
</tbody>
</table>

**Values expressed as mole per cent of total fatty acid.**

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*a* Values calculated from the original data of the authors.

*b* Values calculated by Daemen (1973), from the original data of the authors.

**c** Values as calculated by Daemen (1973), from the original data of the authors.
phospholipids than in those of frog or bovine ROS. Anderson and Sperling (1971) determined the positional distribution of each fatty acid type in each of the major phospholipid classes of bovine ROS and frog ROS (Anderson and Risk, 1974) and found that there is a marked preference of saturated acids for the 1-position of the glycerol moiety, whereas the polyunsaturates predominate at the 2-position. The exception to this finding is the presence of C\(_{24:4}\) and C\(_{24:5}\) acids at the 1-position of bovine ROS phosphatidylserine (Anderson et al., 1975); these acids were not found in rat or frog ROS membranes and were also notably absent from the microsomal, mitochondrial and nuclear subcellular fractions of the bovine retina (Anderson et al., 1975). The presence of plasmalogens has been reported for bovine (Borggreven et al., 1970; Dreyfus et al., 1974a) and frog (Eichberg and Hess, 1967) ROS membranes. Borggreven et al. (1970) reported that ethanolamine plasmalogens accounted for about 3.8% of the total lipid phosphorous (ca. 9.7% of the total ethanolamine phospholipid) in bovine ROS membranes, whereas choline plasmalogens were undetected and very little phosphatidic acid (0.4% of total lipid phosphorous) and cardiolipin (0.2% of total lipid phosphorous) was found. Dreyfus et al. (1974a) found about 8% of the total ethanolamine phospholipid was due to ethanolamine plasmalogens, while choline plasmalogens represented about 3% of the total choline phospholipid of bovine ROS membranes. In contrast, Eichberg and Hess (1967) found the following additional lipid components in frog ROS membranes (expressed as per cent of total lipid phosphorous): ethanolamine plasmalogen, 0.6%; alkyl ether phospholipids, 3.5%; phosphatidic acid, 3.0%; cardiolipin, 1.3% (only trace amounts of choline plasmalogens were found). The presence
of these additional ROS phospholipid components (especially plasmalogens) has not been confirmed by other investigators (cf. Anderson and Maude, 1970, 1972; Anderson and Sperling, 1971; Anderson et al., 1975), although such components have been found in other retinal subcellular fractions (Anderson et al., 1975). Very recently, it has been reported that the phospholipid classes of ROS membranes may be further subfractionated by thin-layer chromatography (and by silicic acid column chromatography, with less resolution) on the basis of the 1,2-acyl distribution of each phospholipid class (Miljanich et al., 1979). Using this technique, Miljanich et al. (1979) have reported that bovine ROS phospholipids contain the following complement of fatty acids (in mole per cent): PS contains 43% diunsaturated species and 57% saturated-unsaturated species; PC contains 24% diunsaturated species, 58% saturated-unsaturated species and 18% disaturated species; PE contains 24% diunsaturated species, 75% saturated-unsaturated species and 1% disaturated species. In each case, the unsaturated acids were predominantly polyunsaturated.

Recently, the neutral lipid fraction of frog ROS membrane lipids has been investigated (Wiegand and Anderson, 1977). Gravimetric and gas-liquid chromatographic analyses revealed that neutral lipids account for about 10%, by weight, of the total lipid content; the major components were cholesterol, free fatty acids and diglycerides, with a molar ratio of 50:30:15, respectively. Over 50% of the free fatty acid content is composed of palmitic acid. Strikingly, the diglyceride pool consists almost entirely of just two species: a C_{38} species (having predominantly palmitic acid in the 1-position and docosahexaenoic acid in the 2-position)
and a C\textsubscript{40} species (having predominantly stearic acid in the 1-position and docosahexaenoic acid in the 2-position). Triglycerides were not detected.

Both the free and esterified forms of retinol (vitamin A) have been identified and quantitated in frog retina and ROS membranes (Bridges, 1976a). In the ROS, the free alcohol predominates (ca. 82\% of the total retinol species), with the ratio of the 11-cis isomer to the all-trans isomer approximately 1:1; in the rest of the retina this situation is reversed, with the esterified form predominating (ca. 82\%) and the isomeric ratio being almost 2:1 in favor of the all-trans isomer. The value of the total retinol content of frog ROS membranes was expressed as 4.2 mole per cent (per mole of rhodopsin). There is little or no free retinal-dehyde present in the ROS. Using the known composition of frog ROS membranes as well as the known concentration and molecular weight values for frog rhodopsin (cf. Daemen, 1973), one may calculate a value of about 0.01\%, on a dry weight basis, for vitamin A in frog ROS membranes (ca. 0.02\%, by weight, of the total ROS lipid). It has also been reported that α-tocopherol (a member of the vitamin E family) is a constituent of ROS membranes, and occurs in the molar ratio of 1:10 with respect to rhodopsin (Dilley and McConnell, 1970). Since tocopherol is known to inhibit the autooxidation of polyunsaturated fatty acids and retinol (Zalkin and Tappel, 1960) and dietary vitamin E deficiency is known to cause peroxidation of lipids in vivo (Dam, 1962; Farnsworth et al., 1979), it has been suggested that vitamin E in the retina may protect the polyunsaturated acids from peroxidation (Farnsworth and Dratz, 1976; Farnsworth et al., 1979). Considering the high oxygen tension across the retina, such a
TABLE I-6

Lipid-Rhodopsin "Unit Cell" Composition of Bovine and Frog ROS Membranes

<table>
<thead>
<tr>
<th>MOLECULAR COMPONENT</th>
<th>MOLECULES/UNIT CELL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BOVINE</td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>1.0</td>
</tr>
<tr>
<td>Total Phospholipid</td>
<td>74.7</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>31.6</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>29.3</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>10.7</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>1.4</td>
</tr>
<tr>
<td>Sphingomyelin</td>
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</tr>
<tr>
<td>Other</td>
<td>0.8</td>
</tr>
<tr>
<td>Total Neutral Lipid</td>
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<tr>
<td>Cholesterol</td>
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<tr>
<td>Free Fatty Acids</td>
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</tr>
<tr>
<td>Diglycerides</td>
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<tr>
<td>Monoglycerides</td>
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</tr>
<tr>
<td>Triglycerides</td>
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<tr>
<td>Vitamin E</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Glycolipids</td>
<td>T</td>
</tr>
</tbody>
</table>

*a Adapted from Miljanich (1978)

* Abbreviations: T, trace; ND, not determine.
protection mechanism would be particularly valuable to this tissue.

A detailed study of the total composition of bovine and frog ROS membranes has been performed by Miljanich (1978). In this study, he proposes the novel concept that the ROS membrane may be considered in terms of a "unit cell", with each type of lipid molecule being quantitatively related to a single molecule of rhodopsin and forming a repeating structural unit. The composition of bovine and frog ROS membranes, as determined by Miljanich (1978), is given in Table I-6. In this case, cholesterol was determined by gas-liquid chromatography, in comparison with an authentic cholesterol standard. By this analysis, the bovine ROS membrane phospholipid:rhodopsin molar ratio is 75:1 and the cholesterol:rhodopsin molar ratio is 8:1; phospholipid accounts for about 83 mole % of the total membrane lipid, and the cholesterol:phospholipid molar ratio is 0.11. For frog ROS membranes, the phospholipid:rhodopsin molar ratio is 65:1, the cholesterol:rhodopsin molar ratio is 9:1, phospholipid represents about 80% of the total lipid and the cholesterol:phospholipid molar ratio is about 0.13. It should be noted that plasmalogens, triglycerides and glycolipids were essentially undetected in these membrane preparations, and the phospholipid and neutral lipid compositions were in reasonable agreement with the data previously cited in this chapter.

**Rhodopsin: Chemical and Physical Properties**

The chemistry of the visual process has been extensively studied over the past century, yielding substantial information on the chemical and physical properties of the visual pigments and the relationship of these properties to the overall process of phototransduction by the
retina. The subject has been reviewed extensively (see Wald, 1953, 1968, 1974; Abrahamson and Ostroy, 1967; Bridges, 1970, 1972; Bonting, 1969; Ebrey and Honig, 1975; Hagins, 1972; Cone, 1974; Montal and Korenbrot, 1976; Ostroy, 1977). This section will present a brief review of the properties of rhodopsin which are pertinent to this dissertation as a prerequisite for understanding the discussions which follow in subsequent sections.

Rhodopsin is an unusual molecule composed of two distinct portions: a protein (opsin) and a chromophoric prosthetic group (11-cis retinal or 11-cis-3-dehydroretinal). The protein portion is actually a glycoprotein (Heller, 1968, 1969; Shich et al., 1969; Heller and Lawrence, 1970), containing 305 amino acids and at least 5 moles of glucosamine (N-acetylated) and 9 moles of mannose per mole of protein (Plantner and Kean, 1976). The amino-terminus of the protein is blocked and the primary sequence of this end of the molecule (the first 21 residues) has been determined and found to contain all the sugar residues of the protein (Hargrave, 1977; Hargrave and Fong, 1977). The sugars are attached at two distinct sites on the N-terminal peptide (Asn-2 and Asn-15), and the structural arrangement of the oligosaccharide has been elucidated recently (Fukada et al., 1977). A comparative study of frog, rat and bovine rhodopsin revealed that the protein contains approximately equal proportions of hydrophobic and hydrophilic amino acids and that there is little species variation in the amino acid composition and in absorbance characteristics of the protein (Heller, 1968, 1969; Shich et al., 1969). The protein is very unusual in that it contains 10 cysteine residues, 6 of which apparently exist in the free sulfhydryl form while the remaining
4 form two disulfide bridges (Bonting et al., 1974). The exposure of certain cysteines to the aqueous environment changes upon illumination of the native photopigment (Wald and Brown, 1951-52, 1953-54; De Grip et al., 1975). The chromophore, 11-cis-12-s-cis-retinaldehyde (or its 3-dehydro derivative), is covalently bound to the native protein via a Schiff-base linkage to the ε-amino group of a lysine residue (Bownds and Wald, 1965; Bownds, 1967; Akhtar et al., 1967). In the native protein, the Schiff-base linkage is not accessible to hydrophilic reducing agents, such as sodium borohydride (Bownds and Wald, 1965; Heller, 1969), but may be reduced with hydrophobic agents such as cyanoborohydride (Fager et al., 1972). However, upon exposure to light, reduction with sodium borohydride is possible (Bownds and Wald, 1965; Heller, 1969). These results suggest that the chromophore residues in a hydrophobic pocket in the native photopigment which opens and exposes the ops-in-retinal bond upon illumination.

Rhodopsin is completely insoluble in aqueous buffers and may be extracted from the retina and solubilized only with the aid of various detergents (which demonstrates that it is an "integral" membrane protein, firmly bound in a hydrophobic matrix). The visual pigment was first solubilized by Kühne (1878), who used a solution of bile salts to extract frog retinas. Subsequently, it was found that digitonin was very effective in solubilizing rhodopsin (Tansley, 1931), and aqueous digitonin solutions were used routinely from the 1930's through the 1960's for the preparation of visual pigment extracts used to study the spectroscopic and chemical properties of rhodopsin in solution (Hubbard et al., 1965; Wald, 1968; Kropf, 1972). A variety of other detergents have been used
successfully for the solubilization of rhodopsin, including cationic quaternary ammonium salts (i.e. dodecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide) (Bridges, 1957; Heller, 1968; Hong and Hubbell, 1972, 1973), nonionic detergents (i.e. Tween-80, Emulphogene, Triton X-100 and alkylglucosides) (Zorn and Futterman, 1973; Shichi et al., 1969; Osborne, et al., 1974; Stubbs et al., 1976) and zwitterionic detergents (i.e. Ammonyx-LO, N,N-dimethyldodecylamine oxide) (Ebrey, 1971; Applebury et al., 1974; Raubach et al., 1974a). In most of these detergents, rhodopsin is thermally unstable and denatures irreversibly, with the exception of the alkylglucosides and (to a lesser extent) digitonin (Stubbs et al., 1976; Plante et al., 1977).

The molecular weight of detergent-solubilized rhodopsin (primarily from bovine and frog retinas) has been a matter of some controversy (Daemen, 1973; cf. Kossi et al., 1977). Various techniques have been employed for molecular weight determination, including gel filtration, ultracentrifugation, polyacrylamide gel electrophoresis in sodium dodecylsulfate, amino acid composition and even the Langmuir-film monolayer method (Hubbard, 1954; Shield et al., 1967; Heller, 1968, 1969; Shichi et al., 1969; Daemen et al., 1972; Heitzman, 1972; Robinson et al., 1972; Papernaster and Dreyer, 1974; Lewis et al., 1974; Frank and Rodbard, 1975; Kossi et al., 1977). The values range from 26,400 to about 42,000 daltons, with the majority of values in the range of 35,000–39,000 daltons. Solubilized rhodopsin from cattle, rat and frog (red rods) displays a native absorption spectrum (i.e. visible and near-ultraviolet spectrum, in the dark) with absorbance maxima at 498-502 nm (species-dependent), \(\sim 340\) nm and 278 nm; the maxima near 500 nm and
340 nm are due to the retinaldehyde moiety of the visual pigment (in the 11-cis configuration), whereas the absorbance at 278 nm is due to the aromatic amino acid residues of opsin (Hubbard et al., 1965; Wald, 1968; Bridges, 1970, 1972; Kropf, 1972). The extinction coefficient for the absorbance near 500 nm has also been a matter of contention. For bovine rhodopsin, the reported values range from 38,300 to 43,250 (depending on the detergent used for solubilization and other undetermined factors) with the majority of values centered around 41,000 (Wald and Brown, 1953-54; Matthews et al., 1963; Shichi et al., 1969; Shichi, 1970; Zorn and Futterman, 1971; Bridges, 1971; Rotmans et al., 1972; Daemen et al., 1972).

It has been known for some time that the absorption of light by the chromophore of rhodopsin leads to the photoisomerization of the retinal moiety from the 11-cis to the all-trans configuration (Hubbard and Kropf, 1958; Hubbard et al., 1965; cf. Wald, 1968). Photoisomerization is the immediate consequence of photon absorption; however, this process leads to a series of sequential transitions in the rhodopsin molecule ("dark reactions") which results in the formation of spectroscopically and conformationally distinct entities (photoproducts) which are in thermal equilibrium with each other (all of which contain covalently-bound retinaldehyde in the all-trans configuration, but differ in the conformational state of the opsin and the particular microenvironment in which the chromophore lies). After the process of photon absorption and subsequent photoisomerization, the absorbance maximum near 500 nm of the native rhodopsin molecule is lost, a process known as "bleaching". A schematic outline of the bleaching process which lists the photoproducts, their
characteristic absorbance maxima ($\lambda_{\text{max}}$), their thermal stability limits and approximate lifetimes near room temperature (ca. 20° C) is shown in Figure I-3 (cf. Abrahamson and Fager, 1973; Montal and Korenbrot, 1976; Ostroy, 1977). From this scheme, it is apparent that the actual loss of color of the rhodopsin takes place in the transition from metarhodopsin I$_{480}$ to metarhodopsin II$_{380}$, and that the processes involved in visual excitation (which occur within a few milliseconds of photon absorption; Tomita, 1970; Hagins, 1972) must proceed within the time it takes to form metarhodopsin II$_{380}$. Hydrolysis of the Schiff-base linkage which binds all-trans retinal to ops in the metarhodopsin II$_{380}$ intermediate leads to the release of free retinal from the protein, completing the bleaching sequence. Up to the stage of metarhodopsin II$_{380}$, the absorption of a second photon by any of the spectral intermediates can lead to the re-isomerization of the all-trans chromophore to the 11-cis configuration and result in the "regeneration" of rhodopsin (Wald, 1968). Once the all-trans retinal is released, the only way to regenerate native rhodopsin is to supply the ops in with 11-cis retinal (i.e. in the dark). This sequence of transitions in the visual pigment has been observed in intact isolated retinas and ROS suspensions as well as in detergent solutions of rhodopsin, and the absorbance spectra of the products in both the membrane environment and in detergent solution are virtually identical (Hubbard et al., 1965; Cone and Cobbs, 1969; Kropf, 1972; Abrahamson and Fager, 1973). The existing body of data strongly supports the prevailing concept that the absorption of a photon by rhodopsin is the initial step in the processes involved in visual excitation, and that the chemical and physical changes which are entailed in
FIGURE I-4

Schematic outline of the "visual cycle", illustrating the sequential intermediates of the "dark reactions" which follow the absorption of a photon of rhodopsin. Wavy lines denote "light reactions" (requiring the absorption of a photon), while straight lines denote "dark reactions". The approximate half-life (☉) and thermal stability limit (in °C) for each intermediate are given, along with wavelength of maximal absorbance ($\lambda_{\text{max}}$).
\[ \tau \approx 6 \times 10^{-12} \text{s} \quad \xrightarrow{\text{hv}} \quad \text{Rhodopsin (498 nm)} \]
\[ \tau \approx 3 \times 10^{-8} \text{s} \quad \xrightarrow{\geq -140^\circ} \quad \text{Prelumirhodopsin (543 nm)} \]
\[ \tau \approx 10^{-6} \text{s} \quad \xrightarrow{\geq -40^\circ} \quad \text{Lumirhodopsin (497 nm)} \]
\[ \tau \approx 10^{-3} \text{s} \quad \xrightarrow{\text{H}^+ \geq -15^\circ} \quad \text{Metarhodopsin I (478 nm)} \]
\[ \tau \approx 10^2 \text{s} \quad \xrightarrow{\text{H}_2\text{O} \geq 3^\circ} \quad \text{Metarhodopsin II (380 nm)} \]
\[ \tau \approx 10^2 \text{s} \quad \xrightarrow{\text{H}_2\text{O} \geq 3^\circ} \quad \text{Metarhodopsin III (465 nm)} \]
\[ \text{Opsin} + \quad \xleftarrow{\text{II-cis Retinal}} \quad \text{All-trans Retinal (387 nm)} \]

enzymatic isomerization
the bleaching sequence are causally related to the sequelae of transient electrical changes which are associated with the generation of a nerve impulse in the visual pathway. The details of the mechanism by which the energy of a photon is converted into the electrochemical energy of the nerve impulse generated in the photoreceptor cell remain yet elusive, although hypotheses concerning the phototransduction process have been formulated and are currently under investigation (Wald, 1968, 1974; Bonting, 1969; Hagins, 1972; Cone, 1974).

The all-trans retinaldehyde released from the fully bleached photopigment does not persist in the retina in vivo or in vitro, but is rapidly reduced to the corresponding all-trans alcohol by a retinal reductase which is present in the ROS (Wald and Hubbard, 1949; Futterman and Saslaw, 1961; Bridges, 1962; Futterman, 1963; Futterman et al., 1970). The alcohol may then be re-oxidized and then isomerized to form 11-cis retinal (Hubbard, 1956) which would recombine with opsin to regenerate rhodopsin, or the all-trans alcohol (either in the free or esterified form) could leave the ROS and be transported to the pigment epithelium (RPE) for storage (Dowling, 1960; cf. Futterman, 1974; Bridges, 1976b). It has been suggested that the rates of reoxidation and re-isomerization of the all-trans retinal in the ROS are too slow to account for the in vivo rate of rhodopsin regeneration. Instead, it is more likely that the stores of retinyl esters in the RPE (either in the all-trans or 11-cis configuration) are transported back to the ROS, where they may be isomerized (in the case of the all-trans species) and/or hydrolyzed to form the 11-cis retinol; the alcohol could then be oxidized (by an 11-cis retinol oxidase) to the corresponding aldehyde, which would then be
available for combination with opsin to reform rhodopsin (Futterman, 1974; Zimmerman, 1974; Bridges, 1976b, 1977). The pathway by which rhodopsin is bleached and then regenerated is known as the "rhodopsin cycle" or "visual cycle" and has been the subject of much research and considerable speculation; as yet, the exact mechanism of the regeneration process remains unresolved.

Molecular Organization of the Vertebrate Photoreceptor Membranes

Studies of the vertebrate photoreceptor by high-resolution electron microscopy revealed that the rod outer segment is a highly-organized, periodically-arranged structure consisting of an ordered stack of disk-shaped membranous sacs enveloped by a plasma membrane (Sjöstrand, 1949, 1953, 1961; De Robertis and Lasansky, 1958; Moody and Robertson, 1960; cf. Cohen, 1972). The osmotic sensitivity of the disks to fixation conditions suggested that the closely-apposed membranes which formed each disk were separated by an "intradisk space" (De Robertis and Lasansky, 1961), and each disk was a separate, osmotically active unit. X-ray diffraction studies have revealed that, in several species, each disk is about 150 Å thick and is composed of two adjacent membranes (each about 65 Å thick) separated by a 10-20 Å wide intradisk space (Corless, 1972; Blaurock and Wilkins, 1972; Korenbrot et al., 1973; cf. Worthington, 1974). The center-to-center, disk-to-disk repeat distance is roughly twice the disk thickness, or about 300 Å (Korenbrot et al., 1973). The ratio of disk membranes to ROS plasma membrane, by weight, varies from 40:1 to 100:1, depending on the species. Thus, in conjunction with the known composition of ROS membranes, it is obvious that the majority of the rhodopsin resides in the disk membranes; however, rhodopsin has
also been located in the plasma membrane of the outer segment (Dewey et al., 1969; Jan and Revel, 1974; Basinger et al., 1976). The results of early studies on the intrinsic birefringence of ROS membranes suggested that the membranes which form each disk are arranged in a bilayer, with the lipid hydrocarbon chains oriented perpendicular to the plane of the membrane (Schmidt, 1935; cf. Liebman et al., 1974). The "lipid bilayer" structure of photoreceptor membranes has been confirmed by x-ray diffraction (Blaurock and Wilkins, 1969, 1972; Corless, 1972; Webb, 1972; cf. Worthington, 1974) and is consistent with data obtained by freeze-fracture electron microscopy (Clark and Branton, 1968; Leeson, 1971; Chen and Hubbell, 1973; Corless et al., 1976). These studies and others also demonstrated that rhodopsin is at least partially submerged into the bilayer, although its exact position in the membrane and the extent to which it penetrates the lipid phase remain a matter of considerable dispute (see below).

The results of chemical labelling studies, employing putatively membrane-impermeable agents, have suggested that the phospholipids of the disk membranes are asymmetrically distributed across the lipid bilayer, with about 70% of the amino phospholipid localized to the outer (extra-diskal) half of the bilayer and the bulk of the choline phospholipid presumably distributed in the inner (intradiskal) half of the bilayer (Raubach et al., 1974b; Litman, 1974; Smith et al., 1977). Other results of chemical modification studies as well as the results of the exposure of disk membranes to phospholipase C have indicated that phosphatidylserine is relatively resistant to both chemical modification and lipolytic cleavage, and that PS may be closely associated with
rhodopsin in the native membrane (Borggreven et al., 1971; De Grip et al., 1973). Recently, the results of a study which employed both chemical labelling techniques as well as chemical cross-linking (with a membrane-permeable probe) have demonstrated phosphatidylethanolamine is distributed asymmetrically in the bilayer, with 63-72% located on the outer disk surface, 18-27% located on the inner disk surface and 6-14% not accessible to the probes, while phosphatidylserine is distributed approximately equally on both sides of the bilayer (25-35% on each half) with 35-50% of the PS inaccessible to chemical labelling (Crain et al., 1978). Analysis of the lipids after chemical cross-linking revealed that, under conditions of maximal cross-linking, 72% of the total membrane PE became cross-linked to other PE molecules, 43% of the total PS became cross-linked to PE molecules, and 6% of the total PE and 37% of the total PS were cross-linked to membrane protein (mostly rhodopsin, presumably). The close agreement in the values determined for the percentage of PE and PS which are both resistant to chemical labelling and which become cross-linked to protein in the membrane suggest a specific interaction of the membrane protein (most likely rhodopsin) with a discrete population of phospholipid molecules. Fatty acid analysis of the membrane phospholipids before and after cross-linking demonstrated that there was no preferred fatty acid composition for phospholipids associated more closely with protein than for "free" (i.e. randomly distributed) phospholipid, and also indicated that most of the PE is randomly distributed within the plane of the membrane. The concept of a "boundary layer" or annulus of relatively immobilized lipid which surrounds integral membrane proteins has received some attention, primarily due to studies
employing spin-labelled lipid probes (Jost et al., 1973; Griffith et al., 1973; Hesketh et al., 1976; Lee, 1976). However, although it is apparent from NMR and ESR measurements that there are significant protein-lipid interactions in ROS membranes and rhodopsin-phospholipid recombinant membranes (Hong and Hubbell, 1972, 1973; Brown et al., 1976, 1977), it is not clear that a discrete annulus of "immobilized" lipids is associated with rhodopsin (Brown et al., 1977); the possibility that a heterogeneous population of motionally-discrete, short-lived lipid clusters exist in equilibrium with each other in the membrane cannot be ruled out (cf. Lee et al., 1974). In view of the fact that there is apparently no preferred phospholipid head-group for recombination of rhodopsin in defined phospholipid bilayers (Hong and Hubbell, 1972) and that the fatty acid composition of the reacted and unreacted phospholipids in the cross-linking experiment did not differ appreciably (Crain et al., 1978), the results of the chemical labelling and cross-linking experiments with respect to the apparent preferential proximity and association of phosphatidylserine with ROS membrane protein are not understood at this time.

Until recently, there were no reports concerning the transbilayer distribution or topological arrangement of cholesterol in photoreceptor membranes. Using the freeze-fracture technique, it was found that filipin (a polyene antibiotic which forms a specific complex with 3β-hydroxy sterols) produced lesions in "particle-free patches" of disk membranes and plasma membranes of both mouse and frog ROS, under certain conditions, as well as lesions in the particle-populated apical portion of the inner segment plasma membrane (Andrews and Cohen, 1979). Treatment of the membranes with saponin (which also interacts specifically
with certain sterols) leads to the loss of the particle-free patches; in addition, it was observed that such particle-free patches were apparent only in the more basal disks but not in the more slerally displaced disks. These results were interpreted to suggest that cholesterol is preferentially located in the particle-free patches of the ROS membranes (such areas presumably devoid of rhodopsin), and that either the cholesterol content and/or the organization of lipids and protein in the disk membranes is altered as one proceeds from the basal to the apical portion of the ROS.

Attempts to ascertein the location of rhodopsin in the disk membrane have yielded conflicting results, and the controversy has yet to be settled. Using electron microscopy and low-angle x-ray diffraction techniques, in combination with the use of antibodies raised against rhodopsin, Blasie and co-workers correlated the particles observed on the granular surface of the disk membranes (as seen by electron microscopy) with the electron-density profile obtained by x-ray diffraction of the membranes and interpreted the results as indicating that rhodopsin is partially buried in the lipid bilayer matrix and partially exposed to the aqueous space (Blasie et al., 1965, 1969; Blasie and Worthington, 1969). The concept was forwarded that the disk membrane exists as a two-dimensional fluid, with rhodopsin floating in the lipid matrix (Blasie and Worthington, 1969). Further studies by Blasie (1972a, b) using x-ray methods were performed in order to ascertain the extent to which the protein was imbedded in the bilayer. The results of the first study (Blasie, 1972a) were interpreted to suggest that, in the dark, rhodopsin (which was equated with the apparently spheroidal particles of 40 Å diameter seen in electron
micrographs of negatively-stained disk membranes) is submerged to the extent of about one-third of its diameter into the membrane while the remaining two-thirds is exposed to the aqueous environment; upon bleaching, the protein appeared to sink into the lipid matrix about one-sixth of a diameter further. Unfortunately, these results have not been confirmed by subsequent studies and are currently thought to be artifactual. By varying the pH, osmotic strength and density of the medium in which the ROS samples were placed, Blasie (1972a) was able to demonstrate that the portion of the visual pigment which was exposed to the aqueous space was negatively charged, and he suggested that rhodopsin has a distinct "polarity" in the membrane (i.e. vectorial orientation). Considering all of his results, Blasie proposed that rhodopsin is located only on the outer half of the bilayer, facing the extradisk space. However, Vanderkooi and Sundaralingam (1970) proposed that rhodopsin is partitioned on both the extradisk and intradisk faces of the membrane, but does not penetrate the membrane extensively. Worthington (1971, 1974) has reviewed the x-ray data reported by his lab and others and has proposed that rhodopsin is buried only in the luminal (intradisk) membrane half, whereas Blaurock and Wilkins (1969, 1972) suggest that rhodopsin is symmetrically distributed on both halves of the bilayer and lies only on the membrane surface. The results of a freeze-fracture electron microscopic study by Corless et al. (1976) support the model proposed by Blasie (1972a), i.e. that rhodopsin is restricted to the cytoplasmic (extradisk) half of the bilayer. However, the model proposed by Dratz et al. (1972), which states that rhodopsin is primarily confined to the hydrocarbon interior of the membrane, has been supported by neutron diffraction studies (Saabil et
al., 1976). Jan and Revel (1974), using peroxidase-coupled antibodies to rhodopsin, noted heavy staining in electron micrographs of both sides of the ROS plasma membrane and the cytoplasmic surface of the disk membranes, with lesser staining on the lumenal surface of the disks. They suggested that rhodopsin exposes antigenic sites of differing reactivities to both sides of the membrane, indicating that rhodopsin spans the membrane. Other studies by freeze-fracture and freeze-etch electron microscopy have also suggested that rhodopsin spans (or nearly spans) the membrane bilayer (Chen and Hubbell, 1973; Krebs and Kühn, 1977). Exposure of ROS membranes to proteases (under limited proteolytic conditions) results in the cleavage of up to 35-45% of the rhodopsin molecule without loss of the characteristic absorbance near 500 nm and without loss of the carbohydrate-containing portion of the polypeptide from the membranes (Daemen et al., 1974; Saari, 1974; Trayhurn et al., 1974). The carboxy terminal sequence for bovine rhodopsin has been determined and short-term exposure of the membranes to thermolysin can release a polypeptide fragment from the membranes which contains the C-terminal 12 amino acids of rhodopsin (Hargrave and Fong, 1977). The use of a ferritin-conjugated lectin (concanavalin A) has led to the cytochemical localization of the carbohydrate moiety of rhodopsin on the lumenal surface of the disk (Rolich, 1976). These results are in conflict with those of Steinemann and Stryer (1973), who reported that intact ROS membranes will bind water-soluble concanavalin A stoichiometrically (i.e. one Con A molecule per molecule of rhodopsin) and specifically, suggesting that the carbohydrate moiety was located on the cytoplasmic surface of the disk. Surprisingly, it was found that the fragments formed by limited proteolysis of rhodopsin
were no more hydrophilic than the overall amino acid composition of intact opsin (Saari, 1974; Hargrave and Fong, 1977). With the exception of the results of Steinemann and Stryer (1973), the above results suggest that rhodopsin is vectorially oriented in the membrane, with its carboxy-terminal region exposed to the extradiskal space and its amino-terminal region (which contains the oligosaccharide moiety) exposed to the intradisk lumen, thus spanning the disk membrane. The results of energy transfer experiments have been interpreted to imply that rhodopsin is a cigar-shaped molecule, 70–75 Å in length, with the chromophore located about 65 Å from the cytoplasmic surface of the disk (Wu and Stryer, 1972; Steinemann et al., 1973); this is consistent with the ability of the molecule to span the membrane. Finally, the high degree of linear dichroism exhibited by the ROS when illuminated with light (500 nm) incident perpendicular to the long axis of the rod and polarized parallel to the plane of the disks has indicated that the chromophore (and therefore the whole visual pigment) is highly oriented in the plane of the disk membrane (Denton, 1959; Wald et al., 1963; Liebman, 1962, 1972). The dichroic ratio can be as high as 7 when incident light perpendicular to the rod axis is polarized parallel to (instead of perpendicular to) the plane of the disk; however, when the incident light is parallel to the long axis of the rod, the dichroic ratio is essentially one.

Given the compositional data for vertebrate ROS membranes and assuming reasonable values for the average molecular weights of rhodopsin, phospholipid and the known molecular weight of cholesterol (see previous sections), it may be calculated that the molar ratio of phospholipid: rhodopsin varies from about 65:1 to 100:1 whereas the molar ratio for
cholesterol:rhodopsin may range from 4:1 to 10:1 (Daemen, 1973; Abrahamson and Fager, 1973; Miljanich, 1978), depending on the species and the variation in the compositional data. The frog ROS has the approximate dimensions 5-7 μm x 35-50 μm, contains 1800-2000 disks, and also contains about $10^9$ molecules of rhodopsin or $5-6 \times 10^5$ rhodopsins per disk (Nilsson, 1965; Cohen, 1972; Liebman, 1972). This corresponds well with the rhodopsin concentration of 2.5 mM, as measured by microspectrophotometry (Liebman, 1972). In contrast, it has been reported that the bovine ROS has the dimensions of 2 μm x 22 μm and contains 990 disks (Bonting, 1969). Using Bonting's values for the dimensions of bovine ROS and assuming that the packing density of rhodopsin is the same in bovine disks as it is for frog, it may be calculated that there are approximately $6-7 \times 10^6$ rhodopsin molecules per disk in the bovine ROS.

The Biogenesis and Renewal of Rod Outer Segment Membranes

During the cellular differentiation of the photoreceptor cells, the elaboration of disk membranes appears to proceed by the progressive invagination of the plasma membrane from a locus opposite that of the connecting cilium, at the base of the newly forming outer segment (Nilsson, 1964). Based purely on morphology and ultrastructure, the concept was proposed that the free-floating disks of the ROS are formed from basal disks which fuse at one end and "pinch off" from the plasma membrane (Sjöstrand, 1961). It has been suggested that there is a discrete zone of active membrane assembly at the base of the outer segment, and that new membrane is formed by addition to pre-existing membrane at this locus (Young, 1974).

Upon examination of the retina by microscopy, one may observe a
rather stereotypic arrangement of the cells and uniformity in the length
of the outer segments of the photoreceptors. From the results of a
series of elegant autoradiographic studies primarily by Young and associa-
tes, dynamic and novel aspects of photoreceptor cell processes have been
documented (for a review, see Young, 1976). It was reported that mice
and rats which had been injected with radioactively labelled amino acids
incorporated the substrates into proteins (correlated with the deposition
of silver grains in autoradiographs) in the inner segments of the photo-
receptor cells; at a later time, the labelled material was found to be
present in the outer segments (Droz, 1963). Subsequently, similar but
more detailed studies with rats and frogs revealed that most of the
labelled protein which initially concentrated in the myoid region (within
15 minutes after injection of the substrates) migrated sequentially from
the rough endoplasmic reticulum to the Golgi complex and then on past
the ellipsoid region through the connecting cilium to the base of the rod
outer segment, where it was concentrated (within 1–2 hours after in-
jection) in the basal disks in the form of a discrete band of activity
(observed as deposited silver grains in autoradiographs) (Young, 1967,
1968; Young and Droz, 1968). Over a period of time (about 9–10 days in
the rat, and about 8–9 weeks in the frog), the band of radioactivity was
progressively displaced sclerally toward the apical end of the outer seg-
ment and was later observed associated with a discrete packet of mem-
branous lamellae in the adjacent pigment epithelium. It was shown sub-
sequently that the band of labelled material, associated with a group of
25–50 adjacent disks, was actually released from the tip of the ROS
into the subretinal space (a process known as "shedding") as a packet of
membranes enveloped by a detached portion of the plasma membrane (in a process similar to that initially used for the formation of the disks), and that the material was immediately phagocytized and degraded by the pigment epithelium (Young and Bok, 1969; Ishikawa and Yamada, 1970). During these studies, it was also observed that a small portion of the total label incorporated into proteins became diffusely distributed in the outer segment of rods, whereas in the cone outer segments there was no discrete band of label and all the silver grains were diffusely distributed. It was suggested that differences in both rod and cone ultrastructure as well as membrane molecular architecture were responsible for the observed divergent labelling patterns in the two types of photoreceptor cells (Young, 1969, 1974). The apparently coupled processes of continual membrane assembly at the base of the ROS and subsequent shedding of "old" membranes intermittently from the apical tip of the ROS have been collectively termed renewal by "membrane replacement", to distinguish them from the process of "molecular replacement" which proceeds simultaneously and continuously (Young, 1974). A careful study of the diffusely distributed protein in both rod and cone outer segments indicated that these components (some of which may represent either soluble or loosely-bound proteins) may experience turnover by the "molecular replacement" mechanism, i.e. a continual exchange of new and old molecules (Bok and Young, 1972), and it was suggested that this mechanism is the only form of renewal available to the cones (Young, 1974). Recent studies have demonstrated that cone outer segment membranes may also be renewed by "membrane replacement" (Anderson and Fisher, 1975, 1976; Anderson et al., 1978; Hogan et al., 1974; Steinberg et al., 1977).
The process of "membrane replacement" was confirmed in adult as well as young animals, demonstrating that the synthesis and renewal of outer segment membranes takes place continually throughout life (Young, 1967; Young and Droz, 1968). It was later demonstrated that 80-85% of the label incorporated into ROS protein was associated with opsin, the visual pigment apoprotein (Hall et al., 1968, 1969). The specific activity of the opsin, as well as the intensity of the band of radioactivity in the ROS, remained constant from about 6 hours to about 8.5 weeks after injection of tritiated amino acids (in the adult Rana pipiens).

After 8.5 weeks, a decline in the specific activity of the visual pigment and a loss of labelled disks from the ROS was observed concomitantly, with the simultaneous appearance of the labelled disks in the pigment epithelium. Similar observations were reported by other researchers (Matsubara et al., 1968; Bargout et al., 1969). These studies demonstrated that opsin, once incorporated into the disk membrane, remains associated with the disk until it is degraded by the pigment epithelium.

In striking contrast to the results described above, autoradiographs of frog retinas examined after injection of tritiated fatty acids revealed almost immediate and heavy labelling of the pigment epithelium cytoplasm (mostly localized to the oil droplets, a known storage site for vitamin A esters) and the ROS, with very minor labelling of the photoreceptor inner segments (Bibb and Young, 1974a). Although the intensity of the labelling increased in the ROS with time, the labelling in the ROS was always much more intense than in the inner segments and at no time did a discrete band of activity concentrate in the outer segments; instead, the silver grains were distributed diffusely over the entire
outer segment and persisted even 8 weeks after injection. However, about 4 days after the injection, there appeared to be a relatively higher concentration of label in the basal disks than in the rest of the rod cell, but the pattern was short-lived. Extraction of the retinas with chloroform-methanol (2:1, v/v) and a cursory examination of the extracts by TLC revealed that most of the label was incorporated into the representative phospholipids of the ROS. It was suggested that there was a direct exchange of lipids between the pigment epithelium and the ROS membranes, with ongoing exchange of new and old intact lipid molecules (i.e., "molecular replacement") in the ROS as well as acyl exchange between individual lipid molecules in the membranes. Similar experiments were performed, using tritiated glycerol as the precursor (Bibb and Young, 1974b). In this case, however, label became concentrated in the myoid region of the inner segments within 15 minutes after injection of the substrate, and within a few hours was incorporated into the basal disks of the outer segment as a discrete band; considerable diffuse labelling of the outer segments and the rest of the photoreceptor cells was also observed. With time, the band of activity was apically displaced toward the outer segment tip and then shed (within 8 weeks after the injection) and phagocytized by the pigment epithelium. During this time, labelling of the pigment epithelial cells and the rest of the retina was also observed. Purified ROS membranes (prepared by sucrose floatation) were extracted with chloroform-methanol (2:1, v/v) or with phosphate-buffered cetyltrimethylammonium bromide; the lipid extracts were assayed for total radioactivity, while the detergent-extracted material was chromatographed on an agarose column (separating lipids from protein) with
continuous monitoring of total radioactivity and absorbance at 280 nm and 500 nm. Some retinas were extracted with chloroform-methanol prior to fixation and the autoradiographs were compared with those from retinas which had not been extracted. About 80% of the label was associated with lipids (presumably phospholipids) in the purified ROS preparations (isolated 1, 7 and 14 days after injection) while the remainder was associated with protein (mostly visual pigment). Extraction of retinas with organic solvents removed almost all the label from the pigment epithelium (mostly from the oil droplets). After 5 weeks, only about 25% of the label was extractable from the band which migrated up the ROS, whereas 50-60% of the diffusely labelled material on either side of the band and from other parts of the retina was extractable. Some of the label was also observed to be associated with glycogen particles in the retina. These results were interpreted to mean that the synthesis of new membranes in the ROS utilizes phospholipids biosynthesized in the inner segment, and that both "membrane replacement" and "molecular replacement" take place simultaneously but that molecular replacement of lipids is more rapid than for proteins. Experiments of this kind should be interpreted cautiously, since it is possible that rearrangement of the original molecular organization of the membrane (especially the lipid matrix) may have taken place during the preparation of the samples for electron microscopy. Stein and Stein (1967) had previously developed methodology for the preparation of liver tissue for electron microscopy with only modest extraction of lipids. Bibb and Young (1974a, b) adapted the methodology for the preparation of retina samples, but did not present evidence to document the claim that
lipids were not appreciably extracted from the retinas during the preparative procedures.

The turnover time for mammalian ROS disk membranes is about 9-12 days (Young, 1967, 1971) and about 8-9 weeks for frogs (Young, 1967; Hall et al., 1969). Early experimental results indicated that exposure of animals to increased temperature or higher intensities of light could result in a more rapid turnover of photoreceptor membranes (Young, 1967). For instance, when Rana pipiens are maintained in incubators having an ambient temperature of 28-29°C instead of 20-22°C, the ROS turnover time decreases from the normal 8-9 weeks to about 4 weeks (Dr. R. E. Anderson, personal communication). It is apparent that the coupled synthesis of new membranes and the shedding of old membranes is normally a synchronized steady-state process, maintaining the length of the outer segment constant throughout life, and that a considerable proportion of the overall energy production of the cell must be diverted and utilized for this process (Young, 1976). Recent results have demonstrated that the process of ROS membrane renewal is under circadian regulation as well as being influenced by changes in light-dark schedules and ambient temperature (Basinger et al., 1976b; La Vail, 1976a, b; Hollyfield et al., 1976, 1977; Besharse et al., 1977a, b; cf. Hollyfield and Basinger, 1978).

The fact that disk addition and disk shedding may be "uncoupled" from one another experimentally by placing animals in abnormal light-dark conditions (i.e. continual light or continual dark) suggests that the processes are mechanistically independent and that a "normal" photoperiod is required to maintain the steady-state. The results of the studies immediately cited above have indicated that, under cyclic lighting con-
ditions, amphibian rods exhibit a "burst" of shedding with the onset of
the light cycle (the light apparently synchronizing the shedding of a
population of the total rods), followed through the light cycle by a
transient increase in the rate of disk addition at the base of the outer
segment, and that both disk shedding and disk addition decline to basal
levels throughout the dark cycle (Basinger et al., 1976; Hollyfield et
al., 1976, 1977; Besharse et al., 1977a, b). In the rat, the process of
disk shedding appears to be strictly circadian, with the largest incre-
ment of shedding occurring in darkness just before the onset of morning
light (La Vail, 1976a, b).

In vitro systems have been described for both bovine (O'Brien et al.,
1972) and frog retinas (Basinger and Hall, 1973) which have been used
successfully to study the synthesis of rhodopsin and the assembly of the
disk membranes. The rate of incorporation of labelled leucine into opsin
and subsequent transport and incorporation of the protein into new disks
in frog retinas in vitro closely paralleled the in vivo rate. It was
observed that opsin was incorporated into rod outer segment membranes in
vitro regardless of whether or not the retinas were dark-adapted or par-
tially bleached, suggesting that the chromophore is not necessary for
incorporation of the visual pigment into the membrane (and that it may
actually be added to the apoprotein after initial assembly of the newly-
formed disks). The typical band of radioactivity previously observed
during the in vivo metabolism of labelled amino acids by photoreceptor
cells was also observed in the in vitro incubations of frog retinas
(Basinger and Hall, 1973). Autoradiographic studies of frog (Young and
Bok, 1970; Hall and Bok, 1974), rat (Sidman and Dowling, 1963; Sherman,
1970) and mouse retinas (Pourcho and Bernstein, 1975) after injection of the animals with labelled vitamin A have failed to localize the cellular site of attachment of retinaldehyde to the apoprotein opsin; only diffuse labelling of the outer segments was observed. The in vitro systems have been used to follow the incorporation of tritiated glucosamine into the visual pigment in both frog (Bok et al., 1974) and bovine retinas (O'Brien and Muellenberg, 1974), and it has been demonstrated that opsin is glycosylated sequentially at the rough endoplasmic reticulum (during protein synthesis) and at the Golgi complex (Bok and O'Brien, 1976). An elegant study of the subcellular distribution of labelled opsin at several time points after in vivo metabolism of labelled amino acids in the frog retina yielded results which suggested that newly synthesized opsin, upon completion of the polypeptide chain, migrates through the inner segment to the outer segment of the rod cell as a water-insoluble, membrane-bound entity (Papermaster et al., 1975). Incubations of intact frog retinas in vitro with both $^{14}$C-leucine and $^3$H-choline in the presence of puromycin (a protein synthesis inhibitor) resulted in an 80% inhibition of the incorporation of rhodopsin into ROS membranes and a 20-30% inhibition of incorporation of phosphatidylcholine into ROS membranes (Hall et al., 1973). These results were interpreted to imply that up to 30% of the PC synthesized by the rod cell is co-incorporated with the visual pigment (or opsin) into the disk membranes.

Biochemical studies of the incorporation of phospholipids and rhodopsin into frog ROS membranes in vivo after injection of $^{14}$C-leucine and $H_3^{33}$PO$_4$ have given results which indicate that the kinetics of the
synthesis, assembly and turnover of the lipid and protein constituents of the disk membranes are quite dissimilar (Hall et al., 1973). Unlike the visual pigment (which is incorporated into disks within a few hours after injection and remains in the outer segment for 8-9 weeks, as previously stated), the major phospholipids (PE, PC, PS and sphingomyelin) increase steadily in specific activity for 3-4 weeks after injection of the labeled precursor and then reach a plateau level for the duration of the experiment (in this case, up to 63 days). In striking contrast, PI increases in specific activity sharply up to about 5 days after injection (to a level considerably higher than any other phospholipid class) and then declines relatively rapidly to a level similar to that of the other ROS phospholipids and remains unchanged after about 2 weeks post-injection. The teleology behind this rapid turnover of PI relative to that of the other phospholipids is not known. It is interesting to note that the time point of maximal specific activity of PI in the ROS corresponds very closely with the formation of the relatively substantial (but ephemeral) accumulation of radioactivity concentrated at the base of the frog ROS after injection of tritiated glycerol (Bibb and Young, 1974b).

Using labelled choline, ethanolamine and phosphorous as lipid precursors, it has been observed that the ROS of frogs retain their subsequently labelled phospholipids at a relatively constant level of specific activity over a time course substantially longer than the turnover time of rhodopsin (Dr. R. E. Anderson and Dr. S. F. Basinger, personal communications). However, it was also observed that the total retina pools of these precursors retained large amounts of label for
at least 28 days; this indicated that the lipids became labelled as a result of a continuous (rather than a discrete) pulse of radioactivity. In contrast, injection of labelled serine or glycerol resulted in a discrete pulse of label. Calculations of ROS phospholipid turnover times indicated that there was an exponential decline in the specific activity of the lipids, with a half-time which closely approximated the turnover time of ROS protein. These data suggest that the newly synthesized phospholipids become diffusely distributed in the ROS and that the turnover of ROS lipid and protein constituents are coincident.

The Importance of Lipids in Normal Retina Function

Comparative studies of a wide variety of biological membranes with regard to their molecular composition and relative biological activity have suggested that high phospholipid content, low cholesterol content and high levels of polyunsaturated fatty acids are characteristics of "metabolically active" membranes, such as mitochondrial and chloroplast membranes, in contrast to "metabolically inactive" membranes, such as nerve myelin (Fleischer and Rouser, 1965; O'Brien, 1967). Based on this system of classification and the compositional data previously presented, it is reasonable to designate the rod outer segment membranes as "metabolically active". Considering the composition of ROS membranes, one would predict that the membrane matrix would be highly "fluid" (Singer, 1971, 1974; Bretschker, 1973; Cherry, 1976). Indeed, a variety of physical measurements have indicated that this is the case (Trauble and Sackman, 1973; Verma et al., 1973; Cone, 1974; Pontus and Delmelle, 1975; Brown et al., 1977). Failure to induce transient photodichroism in rod outer segments when exposing the photoreceptors to a flash of
light polarized perpendicular to the long axis led Hagins and Jennings (1959) to speculate that rhodopsin must have considerable rotational freedom. It was later demonstrated that rhodopsin rapidly rotates about an axis perpendicular to the plane of the disk membrane with a correlation time at 20°C of about 20 μsec. (Brown, 1972; Cone, 1972). Cone (1972) also demonstrated that this rotational mobility could be reduced by increasing the viscosity of the external medium surrounding the photoreceptors (i.e. by adding sucrose to the medium), suggesting that rhodopsin must be in contact with the extracellular space. Subsequently, it was observed that rhodopsin also has considerable translational mobility within the plane of the disk membrane, and microspectrophotometric measurements have indicated that, in the rods and cones of both frogs and mud puppy, visual pigment molecules have a diffusion coefficient of about 5 x 10^{-9} cm^2/sec at 20°C, allowing a root-mean-square displacement of 0.3 μm/sec (Poo and Cone, 1973, 1974; Liebman and Entine, 1974). This behavior is consistent with a membrane viscosity of about 1-2 Poise (i.e. about the consistency of light salad oil) and indicates that rhodopsin has the capability of traversing the mean diameter of a frog disk membrane in about 20 seconds. The functional significance of this dynamic behavior of rhodopsin has not been resolved as yet.

As mentioned previously, the existing evidence suggests that rhodopsin is vectorially oriented within the disk membrane. The oligosaccharide moiety is located at the amino-terminal end of the molecule (apparently exposed to the lumen of the disk), while the carboxyl-terminal end protrudes into the extradiskal space and a substantial portion of the molecule is buried in (and perhaps spans) the lipid bilayer
matrix; the chromophore is located in a relatively inaccessible, hydrophobic domain of the protein and is also highly oriented parallel to the plane of the membrane. Due to the amphipathic nature of the visual pigment, it is thermodynamically unlikely for the protein to rotate end-over-end through the lipid bilayer and such rotational mobility has not been observed. There is evidence that this vectorial orientation of the visual pigment is required for the functional competence of the photo-receptor. Upon absorption of a photon, a charge displacement is immediately induced in the disk membrane without measurable latency, generating a biphasic "fast photovoltage" (FPV) known as the "early receptor potential" or ERP (Brown and Murakami, 1964; cf. Tomita, 1970; Cone and Pak, 1971). The duration of the ERP is on the order of a few milliseconds and is associated with certain intermediates in the bleaching of rhodopsin (Cone, 1967; Hagins and McGaughy, 1967, 1968). This fast photovoltage temporarily precedes the commonly measured photore- sponse of the retina known as the "electroretinogram" or ERG, which is also biphasic but has a latency of about 5 msec and typically has a re- sponse amplitude about $10^6$-fold greater than the ERP (Tomita, 1970, 1972). If one heats freshly-excised, dark-adapted rat eyes (allowing 10 minutes for equilibration at each temperature) and measures the ERP from one eye while monitoring the linear dichroism of the rods in the contralateral retina, it can been shown that the ERP falls to zero between $48^\circ$C and $58^\circ$C with concomitant loss of the linear dichroism (Cone and Brown, 1967). The rhodopsin is still spectroscopically "intact" (i.e. it has not lost its absorbance maximum at 500 nm) under these conditions, but the loss of linear dichroism implies that the
native orientation of the visual pigment has been lost (i.e. it assumes a random orientation in the membrane). It is reasonable to assume that thermal reorganization of the lipid matrix takes place over the temperature range 48–58° C, allowing a relaxation of the vectorial orientation of the photopigment; thus, one may conclude that under physiological conditions the lipid matrix supports and maintains the native orientation of rhodopsin in the membrane. These results also suggest that the lipid environment of rhodopsin protects it from thermal denaturation (thermal bleaching), since the absorbance at 500 nm was not lost even in the absence of linear dichroism.

The tenacious association of lipids with rhodopsin and its hydrophobic nature are suggested by the fact that detergents are required in order to extract the photopigment from the membrane and solubilize it in aqueous solutions. In fact, complete removal of lipids from rhodopsin (i.e. to less than one mole of phospholipid per mole of rhodopsin) can only be achieved by treatment of the ROS membranes with phospholipases, followed by repeated extraction with organic solvents, or by chromatographic purification of the detergent-solubilized membranes (Heller, 1968; Shichi et al., 1969; Shichi, 1971; Borggreven et al., 1971, 1972; Hong and Hubbell, 1972; Osborne et al., 1974; Applebury et al., 1974; Bonting et al., 1977). It was originally thought that rhodopsin was a "lipoprotein" (Krinsky, 1958), i.e. that there was a stoichiometric association of lipids with the visual pigment as a discrete entity or functional complex, and that the presence of lipids was responsible for the insolubility of rhodopsin. However, it has been demonstrated that lipid-free rhodopsin is also insoluble in aqueous solution (in the
absence of detergents) and exhibits the typical absorbance spectrum of
the native visual pigment when solubilized in detergent solutions
(Borggreven et al., 1971, 1972; Hong and Hubbell, 1972, 1973; Applebury
et al., 1974). Thus, phospholipids are not intrinsically essential for
"spectrally intact" rhodopsin. The ability of the bleached photopigment
to recombine with the chromophoric prosthetic group (in the dark) to
reform "native" rhodopsin (i.e. regenerability) appears to be dependent
on the environment of the protein. It has been reported that even par-
tial removal of phospholipids (i.e. a phospholipid:rhodopsin molar ratio
of less than 30) will result in the complete loss of regenerability of
rhodopsin, but that the readdition of phospholipids to the delipidated
protein will restore regenerability (Shichi, 1971; Zorn and Futterman,
1971). However, Bonting et al. (1977) have reported that aqueous sus-
pensions of lipid-free and detergent-free rhodopsin retain up to 52%
of the regenerability of isolated rod outer segment membranes. They also
suggested that mere addition of phospholipids to delipidated rhodopsin
is insufficient to restore regenerability, but that "reconstitution" of
rhodopsin-phospholipid mixed vesicles (i.e. by addition of 100 moles of
phospholipid per mole of rhodopsin in detergent solution, followed by
removal of the detergent by dialysis) restores the level of regenera-
tility to about 92% of that in isolated ROS membranes. Previously, Hong
and Hubbell (1973) reported that removal of detergent from rhodopsin-
detergent solutions in the absence of phospholipids resulted in rapid
denaturation of the photopigment, whereas the presence of phospholipid
during dialysis maintained the molecule "spectrally intact". The
dialyzed rhodopsin-lipid recombinants were found to form lipid bilayers,
as shown by freeze-fracture electron microscopy (Hong and Hubbell, 1972; Chen and Hubbell, 1973), in contrast to micellar suspensions or emulsions which may form merely upon mixing of phospholipids with the delipidated rhodopsin. It is also known that regeneration of rhodopsin may be sustained in solutions of digitonin or alkylglucosides (Hong and Hubbell, 1972, 1973; Applebury et al., 1974; Stubbs et al., 1976), whereas most detergents will not support regeneration of the photobleached pigment (Daemen, 1974; Stubbs et al., 1976; Plante et al., 1977). The results described above suggest that lipids, per se, are not essential for regeneration of the spectrally intact photopigment; however, since digitonin, alkylglucosides and phospholipids are chemically quite distinct from one another but also appear to support the regenerability of rhodopsin under certain conditions with similar effectiveness, they must supply some bulk structural property or particular environment required for regenerability. Bonting et al. (1977) have proposed that it is the phospholipid bilayer that is required under physiological conditions for the thermal stability and functional regenerability of rhodopsin in the rod outer segment.

The thermal stability of rhodopsin (i.e. the resistance of rhodopsin to thermal denaturation, as indicated by the loss of absorbance at 500 nm under nonphotolytic conditions) is also highly sensitive to the micro-environment of the visual pigment (Daemen, 1974; Stubbs et al., 1976). The following order of thermal stability (in order of decreasing stability, based on the loss of absorbance at 500 nm of chromatographically-purified bovine rhodopsin at 42°C) has been reported: un-solubilized disk membranes >> decyl glucoside > digitonin ~ octyl
glucoside > Emulphogene >> N,N-dimethyldodecylamine oxide > cetyltrimethylammonium bromide (Stubbs et al., 1976). At room temperature, rhodopsin is completely unstable in sodium dodecylsulfate (Smith and Pickels, 1941). Similarly, Baker et al. (1977) reported that the thermal stability of bovine rhodopsin decreased in the order sonicated ROS membranes > digitonin >> dodecyl dimethylamine oxide ~ Triton X-100 >> cetyltrimethylammonium bromide. It should be realized that these trends in thermal stability are dependent on the concentration of the detergent and the relative concentrations of lipid, protein and detergent in the mixed micelles formed during solubilization of the membranes (Tanford, 1968; Helenius and Simons, 1975). Bonting et al. (1977) reported that treatment of ROS membranes with phospholipase C (resulting in the hydrolysis of 80–90% of the phospholipids to diglycerides and phosphate esters) or complete delipidation of rhodopsin (and removal of detergent by dialysis) resulted in comparable decreases in thermal stability, whereas reconstitution of rhodopsin-phospholipid vesicles yielded thermal stability which was comparable to that of isolated ROS membranes; again, mere addition of phospholipids to the delipidated rhodopsin did not restore thermal stability, suggesting that a bulk, physical property of phospholipids (i.e. lipid bilayer formation) was critical for maximal stabilization of the visual pigment.

The kinetics of photobleaching of rhodopsin are also quite dependent on the environment of the visual pigment. The transition of metarhodopsin I to metarhodopsin II in detergent solutions proceeds about two orders of magnitude faster than for rhodopsin in isolated disk membranes (Rapp, 1970; Abrahamson and Wiesenfeld, 1972; Applebury et al., 1974;
Stewart et al., 1976). As with thermal stability, the kinetics of this transition are influenced by the type and concentration of detergent used for membrane solubilization (Baker et al., 1977). These studies are complicated by the fact that the rate of metarhodopsin II formation does not appear to be a single exponential process (Abrahamson and Wiesenfeld, 1972) and the units for expressing the rate have not been uniform from lab to lab. It has been suggested that the co-existence of two stable conformations of rhodopsin in thermal equilibrium, differing only by a small increment of free energy, results in the formation of two forms of metarhodopsin I which decay independently to two forms of metarhodopsin II (Stewart et al., 1975). Previously, it was suggested that the differential association of lipids with the photopigment was responsible for the multiplicity of kinetic decay products (Williams et al., 1974). More recently, it has been reported that delipidation of rhodopsin (either by phospholipase C treatment of the membranes or by chromatographic removal of the lipids followed by dialysis) results in the complete loss of the meta I – meta II transition as well as the meta II – meta III transition (Bonting et al., 1977). However, reconstitution of rhodopsin-phospholipid vesicles results in kinetics of photobleaching which are comparable to those of isolated ROS membranes (Applebury et al., 1974; Bonting et al., 1977; O'Brien et al., 1977). It has been reported that the meta I – meta II transition in recombinant membranes requires both membrane fluidity and unsaturation of phospholipid acyl chains, but is independent of the polar "head group" of the phospholipid (O'Brien et al., 1977). Interestingly, Hong and Hubbell (1973) reported that the maintenance of a "regenerable conformation" of
rhodopsin in recombinant vesicles is also independent of the phospholipid head group.

Results of delipidation studies are difficult to interpret, since it is not unambiguous whether the observed effects are due to the absence of lipid or whether they are results of damage incurred by the photopigment itself. However, the results of the studies cited above seem to indicate that the lipids of the photoreceptor membranes support and preserve the native orientation, thermal stability and "regenerable conformation" of rhodopsin, as well as providing a highly fluid environment conducive to the dynamic behavior of the visual pigment.

Important information concerning the lipid requirements of the retina has been gained from experiments involving dietary manipulation of rats. Hands et al. (1965) reported that rats fed a diet deficient in essential fatty acids (i.e. linoleic acid, $C_{18:2 \omega 6}$, and linolenic acid, $C_{18:3 \omega 3}$) exhibited impaired visual acuity in dim light. The experimental rats had only about 7% of the normal complement of docosahexaenoic acid ($C_{22:6 \omega 3}$) in their retinal lipids, as well as having livers which were nearly devoid of vitamin A. Futterman et al. (1971) attempted to reduce the levels of polyunsaturated fatty acids in the ROS membranes of rats by feeding them a diet deficient in essential fatty acids for 280 days. Whereas other body organs (i.e. liver, kidney, spleen and heart) exhibited severe depletion of polyunsaturated acids, only minor decreases in retina polyunsaturates were observed. Since mammals rely on chain elongation of the essential fatty acids for the synthesis of polyunsaturates (i.e. they cannot synthesize these acids de novo), these results were interpreted to indicate that the retina has the
remarkable capability of conserving its pool of polyunsaturated fatty acids when the animal is faced with a precursor-deficient diet. These results were later confirmed by Anderson and Maude (1972). It was apparent from the results of Hands et al (1965) that such dietary manipulation influenced the functional competence of the retina, but it was not clear whether the effect was a direct consequence of the depletion of polyunsaturated acids per se or whether it was due to vitamin A depletion, which is known to cause a condition known as "dietary night blindness" (Fridericia and Holm, 1925). Tansley (1931, 1933) observed that rats which were fed a diet lacking in vitamin A exhibited severe depletion of their visual pigments. Wald studied the carotenoids of vertebrate retinas and found vitamin A present in the crude visual pigments extracted with aqueous digitonin (Wald, 1933, 1935). The persistent research efforts of Wald and collaborators resulted in the elucidation of the relationship between vitamin A metabolism and visual function, for which Wald was awarded the Nobel Prize in 1967 (Wald, 1968).

Further experimental manipulation of the diets of rats have yielded results which implicate the polyunsaturated fatty acids (particularly C_{22:6\omega3}) in the normal physiology of the photoreceptors. Rats which were fed a fat-free diet devoid of essential fatty acids exhibited tenacious conservation of docosahexaenoic acid as well as an attenuated rate of ROS membrane renewal (Landis et al., 1973; Anderson et al., 1974; Dudley et al., 1975). However, more recent experiments failed to confirm the effect of this dietary manipulation on the rate of turnover of ROS membranes (Anderson, 1978). The first-generation offspring of the experimental animals, also maintained on a fat-free diet, had normal
levels of rhodopsin per eye (relative to control littermates fed a normal lab chow diet), but exhibited a dramatic decrease (ca. 50%) in the levels of polyunsaturated fatty acids (especially $\text{C}_{22:6} \omega 3$) in their photoreceptor membranes concomitant with aberrant electroretinograms (Benolken et al., 1973; Anderson et al., 1974; Dudley et al., 1975; Wheeler et al., 1975). The electroretinogram (ERG) is a biphasic electrical response of the retina to photostimulation; the "a-wave" is thought to reflect the electrical processes confined to the photoreceptor membranes, whereas the "b-wave" which follows it is considered to represent the sum of electrical activity of the rest of the neural retina (Tomita, 1970, 1972). In the experimental animals, the amplitude of the "a-wave" was selectively decreased relative to that of the "b-wave", indicating that the photoreceptor outer segments were selectively affected. Furthermore, groups of rats fed the fat-free diet supplemented with $\text{C}_{18:3} \omega 3$ (the precursor of $\text{C}_{22:6} \omega 3$) exhibited considerably higher a-wave amplitudes than littermates fed either the fat-free diet alone or the diet supplemented with $\text{C}_{18:1} \omega 9$ (Wheeler et al., 1975). These results suggest that the $\omega 3$ fatty acids are particularly important to the normal electrophysiology of the photoreceptors. Most recently, it has been observed that when rats which had incurred a selective reduction of the "a-wave" of their ERG (due to lack of one or more essential fatty acids in their diets) were subsequently fed a normal lab chow diet or the "fat-free" lab chow supplemented with the essential fatty acid constituents, their ERG patterns returned to normal (Dr. R. E. Anderson, personal communication). In a subsequent series of experiments, it was reported that weanling rats fed a diet devoid of
either \( \omega 3 \) or \( \omega 6 \) fatty acid precursors during development exhibited a retardation in the appearance of the "b-wave" component of the ERG (Anderson et al., 1977). Since this particular component of the ERG has been correlated with the development of anatomically and functionally intact synaptic connections between the retinal neural cells (Weidman and Kuwabara, 1968), it was proposed that both \( \omega 3 \) and \( \omega 6 \) fatty acids may be salient to the normal post-natal development of the neural retina.

**Rationale for Experimental Investigations**

From the preceding discussion, it should be apparent that the vertebrate retina is a highly organized and highly specialized tissue, maintaining an individuality evident from its unique complement of cell types, its cellular organization and biochemical composition while also exhibiting characteristics common to all tissues, particularly the brain (with which it has a common embryological origin). The retina, sequestered in the globe of the eye, relies on the circulatory system for sustenance while also possessing mechanisms for the recycling of various biochemical constituents and the maintenance of apparently required levels of polyunsaturated fatty acids in its lipids. Rhodopsin is a key element in the complex mosaic of components involved in the visual process, and various physical, chemical and dynamic properties of rhodopsin are affected by the lipid environment which surrounds it in the photoreceptor membranes. The photoreceptor membranes themselves are under strict regulation with regard to their synthesis and turnover, being maintained with uniform dimensions in a highly organized array which is continually renewed throughout life, and various endogenous and environmental factors apparently control this renewal process with
precision.

The composition of vertebrate retinas and their photoreceptor membranes have been studied in some detail. The overall composition of the tissue shows little species variation, as does the phospholipid class composition and the relative distribution of fatty acids in each phospholipid class. The ROS membranes are typified by a high phospholipid content, a very low cholesterol content and an unusually high level of polyunsaturated fatty acids. Rhodopsin is found only in the retina, almost exclusively in the photoreceptor cells (with the exception of minor amounts phagocytized and degraded by the pigment epithelium), and shows little species variation with respect to amino acid composition.

Although several reports have claimed to document the presence and quantities of cholesterol in both whole retinas and ROS membranes, the methods employed for the identification and quantitation of cholesterol were, without exception, insufficient for rigorous characterization of the sterol content of the tissues. The colorimetric assays employed by several investigators (essentially variations of the original Liebermann-Burchard assay for sterols) suffer from the fact that they are not specific for cholesterol alone; a wide variety of sterols react positively with the standard colorimetric reagents commonly used for cholesterol determination. Likewise, standard chromatographic techniques (i.e. TLC, GLC) are incapable of resolving a large number of organic compounds from cholesterol (some of which are not even sterols). Conclusions based merely on gravimetric analyses or differential solubility in organic solvents are even more tenuous. Although the major (if not exclusive) sterol in many mammalian tissues is cholesterol, there are several
examples where cholesterol is a minor constituent of the total sterol content of the tissue and in many tissues a multiplicity of sterols exist (Cook, 1958). The sterol composition of the retina and ROS membranes has never been documented, therefore it is not valid to assume that the component which is extractable with organic solvents, gives a positive reaction with the Liebermann-Burchard reagent (or similar colorimetric reagent), and co-migrates with an authentic standard of cholesterol on TLC (or even certain GLC supports) is necessarily cholesterol. In most instances, the analyses did not even include the criterion of precipitability with digitonin (a general property of many 3β-hydroxy sterols) or survival of the component by purification via the dibromide (a property more specific to cholesterol) (Schoenheimer and Sperry, 1934; Fieser, 1953).

In contrast to the several studies which have examined the synthesis and turnover of phospholipids, fatty acids and proteins in the retina (particularly the components of the ROS membranes), there have been no such studies concerning the biosynthesis or metabolic fate of sterols in the retina. In a study aimed at describing axoplasmic transport of cholesterol in the chick optic system, young chicks (1-5 days old) were injected intravitreally in one eye with labelled compounds, including \(^{3}\text{H}\)-cholesterol and DL-[2-\(^{3}\text{H}\)]-mevalonic acid lactone (the former was dissolved in a small amount of ethanol and diluted in 0.1% sodium stearate solution with sonication, while the latter was "dissolved" in sterile saline) (Rostas, 1975; Rostas et al., 1975; Rostas and Jeffrey, 1977). The uptake and conversion of labelled material was monitored over several days in the injected eye, the optic nerve and tectum as
well as in the contralateral eye, optic nerve and tectum. The researchers claimed that the labelled mevalonic acid lactone was taken up by the experimental retina and converted to several labelled components, one of which had the chromatographic properties of cholesterol on silica gel TLC. They also reported that components which co-migrated with cholesterol and cholesteryl esters on TLC appeared in the contralateral optic tectum over short time intervals (a few hours) as well as several days after injection. They concluded that two phases of axoplasmic transport from the retina via the optic nerve to the contralateral tectum took place: a "fast" phase and a "slow" phase. They found that 75% and 92%, respectively, of the total recovered radioactivity associated with the "fast" and "slow" phases was associated with a component which co-chromatographed on TLC with cholesterol. In contrast, cholesterol injected into the experimental eye was found in the contralateral tectum only after a few days, suggesting that it was transported by the "slow" phase only. These authors concluded that there are two kinetic pools of cholesterol in the retina: pre-existing cholesterol molecules are axonally transported via the optic nerve to the optic tectum (i.e. via the projections of the ganglion cells) exclusively by the "slow" phase, whereas newly-synthesized cholesterol (i.e. from a de novo precursor) is mobilized for "fast" transport selectively. Although there are obvious problems with both the mechanics and interpretation of these experiments, it is evident that the chick retina has the ability to take up both free cholesterol and mevalonic acid lactone (or its free acid) from the vitreal side, and that the de novo precursor may be metabolized to various other components (which may or may not be sterols). It is
also evident that the exchange of certain lipid components between the retina and the central nervous system proper can take place (although "retrograde transport" from the tectum to the retina was not demonstrated).

Cholesterol is a component of all mammalian tissues and most tissues have the ability to synthesize cholesterol de novo. The biochemical steps involved in the conversion of acetate and other common precursors to cholesterol have been elucidated primarily by in vivo and in vitro experimentation with rat liver (Bloch, 1965; Clayton, 1965; Frantz and Schroepfer, 1967; Schroepfer et al., 1972). The enzymology and regulation of cholesterol biosynthesis have been studied in some detail and are areas of active research (Popjak and Cornforth, 1960; Dempsey, 1974; Gaylor, 1974; Siperstein, 1970; Rodwell et al., 1973; Brown and Goldstein, 1976). However, cholesterol metabolism in neural tissues has not been as well-studied and, although there is no evidence to suggest that the individual conversion reactions in the synthesis of sterols are different in neural (as opposed to visceral) tissue, there have been suggestions that the regulation and relative importance of "alternative pathways" may be different, especially when comparing developing versus adult animals (Kabara, 1967, 1973; Paolletti et al., 1969; Davison, 1970; Ramsey and Nicholas, 1972; Bowen et al., 1974). The origin of "cholesterol" in the retina is not known. With the exception of the work of Rostas and collaborators previously mentioned, there have been no reports concerning the de novo biosynthesis of sterols or the convertibility of known precursors to cholesterol by the retina. Since Rostas and co-workers did not sufficiently characterize the labelled
component thought to be cholesterol in their in vivo studies with tritiated mevalonic acid lactone, it is not really known whether or not the retina actually has the ability to form cholesterol from mevalonic acid (a "committed precursor" in the biosynthesis of isoprenoids).

The goals of this dissertation, therefore, were to characterize and quantitate the endogenous sterols of the retina and of purified ROS disk membranes, and to examine the biosynthesis of cholesterol by the retina using known precursors, primarily in in vitro systems. The bovine retina was employed, since bovine eyes are readily available from a local abattoir, the retinas are large and offer a good source of photoreceptor membranes, the biochemical composition has been relatively well-studied, and the procedures have been established for both isolation of purified bovine ROS membranes and in vitro incubation of intact retinas. In vitro incubations of bovine retina homogenates were also performed, using a method of preparation based on that devised for the preparation of the rat liver "10,000 x g supernatant" fraction (Bucher and McGarrah, 1956; Popjak, 1969).
II. MATERIALS AND GENERAL METHODS

Solvents and General Reagents

Unless otherwise stated, all organic solvents and other reagents were of analytical reagent grade and were obtained from Mallinckrodt Chemical Works. Water was deionized and glass-distilled prior to use. Pyridine was distilled over barium oxide and stored in a tightly sealed brown glass bottle over molecular sieves (type 3A, Matheson Coleman and Bell). The composition of solvent mixtures is expressed as the volumetric ratio of the individual components; the composition of other solutions is expressed as the weight:volume percentage of the solute in the given solvent (i.e. grams per 100 ml), unless otherwise specified.

Radiochemical Reagents

The following compounds were purchased from New England Nuclear Corporation: 3RS-[5-^{3}H]-mevalonic acid (dibenzylethylene diamine salt; 5 Ci/mmol, 1.0 mCi/ml in methanol); D(-)-β-[3-{^{14}C}]-hydroxybutyric acid (potassium salt; 18.4 mCi/mmol, 0.1 mCi/ml in ethanol-water, 1:1); [^{3}H]-water (1 Ci/g); [^{3}H]-acetic anhydride (50 mCi/mmol, in benzene); [1,2-{^{3}H}]-cholesterol (50 Ci/mmol, in benzene; "nominally-labelled").

Other radiochemicals were purchased from Amersham Corporation: 3RS-[2-^{3}H]-mevalonic acid lactone (176 mCi/mmol; 1.0 mCi/ml, in benzene); 3RS-[2-{^{14}C}]-mevalonic acid lactone (10.9 mCi/mmol; 250 μCi/ml, in benzene); [2-{^{14}C}]-acetic acid (sodium salt; 55 mCi/mmol); L-[4,5-{^{3}H}]-leucine (58 Ci/mmol, in 2% ethanol).
Sodium 3RS-[5-3H]-mevalonate was prepared from the DBED salt by evaporating a portion of the methanolic stock solution to dryness under a stream of nitrogen and redissolving the residue in 1.0 ml of 5% sodium bicarbonate solution. The solution was extracted three times with 5 ml portions of diethyl ether (extracts discarded); residual ether was removed from the solution under a stream of nitrogen. The mevalonate solution was then added to an appropriate volume of incubation buffer, so as to contain a final activity of 0.01-0.10 mCi/ml. Using the usual volumes of bicarbonate solution and incubation buffer, it was found that the bicarbonate solution raised the pH of the incubation buffer by only 0.07 units.

Sodium or potassium 3RS-[2-3H]-mevalonate was prepared from the lactone by evaporating a portion of the benzene stock solution under nitrogen and redissolving the residue in either 5% sodium bicarbonate solution or in incubation buffer (after the addition of 2-3 drops of 5 M potassium hydroxide solution). In either case, the final volume of the mevalonate solution was chosen so as to obtain a final activity of 0.01-0.10 mCi/ml. Addition of the usual volume of either 5% bicarbonate solution or basic incubation buffer to the incubation medium increased the final pH of the medium by only 0.08 units.

L-[4,5-3H]-Leucine (in 2% ethanol) was diluted with isotonic saline so as to obtain a final stock concentration of 10.0 mCi/ml.

Cofactors

The following compounds, used as cofactors (or coenzymes) in the \textit{in vitro} homogenate incubations, were purchased from Sigma Chemical Company: 8-nicotinamide adenine dinucleotide (NAD; Grade III);
nicotinamide adenine dinucleotide 2'-phosphate (NADP; sodium salt); D-glucose-6-phosphate (G-6-P; monosodium salt); adenosine 5'-triphosphate (ATP; disodium salt). Nicotinamide was a product of the Eastman Organic Chemical Company.

Routinely, a concentrated stock solution was composed in an appropriate incubation buffer (adjusted to pH 7.4 with 5 M potassium hydroxide) such that a specified aliquot of the stock added to the incubation medium would provide the following final concentration of each cofactor: NAD, 1 mM; NADP, 1 mM; G-6-P, 3 mM; ATP, 5 mM.

**Chromatographic Standards**

Cholesterol (Nutritional Biochemical Corporation) was purified via the dibromide, according to the method of Fieser (1953), and recrystallized three times from methanol. 7-Dehydrocholesterol (Sigma Chemical Company) was recrystallized twice from acetone prior to use. Commercial lanosterol ("C.P. grade"; Mann Research Laboratories, Inc.) was found to be a mixture of lanosterol and 24,25-dihydrolanosterol (a 3:2 mole ratio, respectively) by gas chromatography-mass spectrometry; this material was recrystallized three times from methanol and once from acetone prior to use (without appreciable alteration of the mole ratio of the constituent sterols). All other sterols and steryl acetates used in these studies had been synthesized previously in this laboratory.

The following isoprenoids were used as obtained from Aldrich Chemical Company, Inc.: farnesol (approximately 90%; a mixture containing all-trans-, cis, trans- and/or trans, cis- isomers); geraniol; nerol; nerolidol. Squalene, dolichol (approximately 98%; Grade I),
Coenzyme Q₁₀ (ubiquinone 50), dipalmitin, triolein and cholesteryl oleate were purchased from Sigma Chemical Company. Phytol (approximately 60%) was a gift of Professor Ernest Wenkert (Rice University). All-trans geranylgeraniol was a gift of Professor Robert M. Coates (University of Illinois, Urbana). Methyl-Δ²-trans-phytenate (methyl-3,7,11,15-tetramethyl-hexadeca-2-enoate) was obtained from Analabs, Inc. (courtesy of Mr. Richard S. Brinsmade, Jr.). All-trans methyl farnesoate was a generous gift of Professor John Law (University of Chicago). Farnesolic acid (a mixture of all four geometric isomers) was a product of K & K Laboratories. The methyl and ethyl esters of all-trans geranylgeranoic acid and of [2-¹⁴C]-Δ²-cis-geranylgeranoic acid (4.8 mCi/mmol) were supplied most generously by Professor Charles A. West (University of California, Los Angeles).

Fatty acids and fatty acid methyl ester (FAME) standards were purchased from Supelco, Inc.

**Melting Points**

Melting points were recorded in sealed, evacuated glass capillary tubes, using a Thomas Hoover capillary melting point apparatus. Melting points were uncorrected.

**Ultraviolet-Visible Absorption Spectroscopy**

Ultraviolet-visible absorption spectra were recorded on a Cary 118 recording spectrophotometer, using matched quartz cuvettes with a one-centimeter light path.

Enzyme assays requiring visible absorbance measurements were performed with a Varian Techtron Model 635 dual-beam recording spectro-
photometer, using optical glass cuvettes with a one-centimeter light path.

Other visible absorbance measurements were performed with a Coleman Junior spectrophotometer, using optical glass culture tubes (10 x 75 mm; Kimble Glass Company).

**Infrared Spectroscopy**

Infrared (IR) spectra were recorded on a Beckman IR-9 infrared spectrophotometer. Samples were prepared as potassium bromide pellets, at a concentration of 1% (w:w). In some instances (i.e. where a sample was a liquid at room temperature), the sample was sandwiched (neat) between two potassium bromide disks.

**Nuclear Magnetic Resonance Spectroscopy**

Nuclear magnetic resonance (NMR) spectra were recorded on either a Perkin-Elmer R-12 (60 MHz) or a Varian EM-390 (90 MHz) nuclear magnetic resonance spectrometer. Samples were dissolved in deuterated chloroform, containing tetramethylsilane as an internal standard.

**Low-Resolution Mass Spectrometry**

Mass spectral analyses were performed on an LKB 9000S mass spectrometer. Solid samples were introduced by direct inlet, using an accelerating voltage of 3.5 KeV, an ion source temperature of 60°C and a filament current of 60 μA. Alternatively, liquid samples (or samples prepared as liquid solutions) were analyzed by coupled gas chromatography-mass spectrometry (GC-MS), using commercially-prepared supports of Gas-Chrom Q (100/120 mesh) coated with either 3% OV-17 or 3% OV-1 (Applied Science Laboratories, Inc.), packed in silanized glass
columns (6 ft. x 3 mm, i.d.). The column temperature was varied as a function of the type of sample being analyzed (routine range, 130–270°C); the injection port temperature was routinely 50–100°C higher than the column temperature.

Only ions of intensity greater than one per cent of the base peak were plotted in the spectra.

**Measurement of Radioactivity**

Radioactivity was measured in a Beckman LS-250 liquid scintillation system, using interchangeable iso-sets (external standard mode), in most instances. Alternatively, a Packard Tri-Carb Model 2650 liquid scintillation system was employed.

Labelled material dissolved in organic solvents was counted in a solution containing 0.4% PPO (2,5-diphenyl-1,3-oxazole; Beckman Instruments, Inc.) and 0.005% POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene; Packard Instrument Company), dissolved in toluene (scintillation grade; Beckman Instruments, Inc.). This solution was used routinely with the Beckman scintillation system. Tritium-labelled material was counted at 45–55% efficiency; $^{14}$C-labelled samples were counted at 90–95% efficiency. Alternatively, a counting solution suitable for both aqueous and non-aqueous samples was employed (using the Packard scintillation spectrometer). This solution was composed of Beckman Bio-Solv-3 (a solubilizing agent; Beckman Instruments, Inc.) saturated sodium citrate solution and toluene (containing Omnifluor, a commercially-prepared, pre-measured fluor, 15 g per gallon of toluene; Beckman Instruments, Inc.), in the volumetric ratio of 80:7:400, respectively. Using this type of counting solution, tritium-labelled
material was counted at 30-40% efficiency, while $^{14}$C-labelled material was counted at 75-85% efficiency.

Regardless of the counting solution or scintillation instrument used, a constant volume (10 ml per vial) of counting solution was employed. Throughout the text, the terms "radioactivity" and "activity" will be used interchangeably when referring to the assay or presence of radioactively-labelled compounds or products.

**Thin-Layer Chromatography (TLC)**

Unless otherwise stated, thin-layer chromatography was performed on commercially prepared glass plates (length, 20 cm) coated with Silica Gel G (0.25 mm thickness; Analtech, Inc.). In some instances, these plates were argentated by spraying with a 5% silver nitrate solution (in 50% methanol) until saturated. The plates were allowed to air dry in darkness for one hour and were then activated by heating in an oven (110-120°C) for one hour. The argentated plates were stored in darkness in a sealed desiccator until ready for use.

Compounds which had been chromatographed on such plates were visualized either by exposure to iodine vapors or by heating in an oven (110-120°C, 10-20 minutes) after spraying with molybdic acid reagent. The molybdic acid reagent was prepared by a modification of the method of Clark (1964): ammonium molybdate (20 g) was mixed with concentrated sulfuric acid (25 ml), warmed on a steam bath to dissolve, and diluted to 400 ml with water. Alternatively, plates were sprayed with a 50% sulfuric acid solution and heated in an oven for 0.5-2 hours (especially when visualizing saturated compounds or when using argentated plates).
Thin-Layer Radiochromatographic Analysis

Radioactive samples were chromatographed routinely on thin-layer plates, as described above. Appropriate chromatographic standards were applied to both the sample lane (as internal standards) and an adjacent lane. After developing in the chosen solvent system, plates were air dried and the standards were visualized by exposure to iodine vapors. The sample lane was divided horizontally into evenly-spaced sections (usually 0.5 or 1.0 cm each), then scraped with a razor blade into an appropriate scintillation counting solution in vials and the radioactivity measured as previously described. The position of each standard was marked on the plate for further comparison with the radioactivity distribution.

Gas-Liquid Chromatography (GLC)

Gas-liquid chromatographic analyses were performed on either a Hewlett-Packard Model 402 or a Model 5730A gas chromatograph, using silanized glass columns (6 ft. x 2 mm, i.d.) and helium as the carrier gas (approximately 66 ml/min. for the former unit and 60 ml/min. for the latter unit, at 40 p.s.i.). Both chromatographs utilized flame ionization detectors. The injection port and detector were maintained routinely at 50-100°C above the column temperature.

Chromatographic columns were packed with the following commercially-prepared phases (Applied Science Laboratories, Inc.): Gas-Chrom Q (100/200 mesh), coated with either 1% or 3% OV-1 (methyl silicone), 1% or 3% OV-17 (50% methyl silicone, 50% phenyl silicone), or 1% QF-1 (trifluoropropyl silicone); Gas-Chrom P (80/100 mesh), coated
with 12% DEGS (diethylene glycol succinate, a polyester; alternatively called HI-EFF 1 BP).

Gas-Liquid Radiochromatographic Analysis (Radio-GLC)

The Hewlett-Packard Model 402 gas chromatograph was equipped with a splitter attachment at the column outlet to facilitate the collection of effluent gas. The column effluent was collected by condensation in tapered glass tubes (20 cm x 2 mm, o.d.), at timed intervals (usually one minute). The condensed vapors were eluted several times with scintillation fluid into vials and the radioactivity was assayed as previously described. Recovery of the injected radioactivity varied from 45-99% (the lower range of recoveries was usually due to mechanical obstruction of the splitter attachment).

Appropriate chromatographic standards were chromatographed before and after collection of the radioactive effluent, and the mass tracings were compared with the time course of elution of the radioactive components. The minor delay routinely observed between the initial elution of radioactivity and the time course of the mass tracing was compensated for by initiating the collection time-interval 20 seconds after injecting the radioactive sample into the chromatograph.

Colorimetric Assay of Sterols and Steryl Acetates

Sterols and steryl acetates were quantitated by the Liebermann-Burchard colorimetric assay, as modified by Abell et al. (1952). A mixture of acetic anhydride and concentrated sulfuric acid (20:1) was cooled on ice in a sealed glass flask for nine minutes. At the end of this time, glacial acetic acid (10 parts, by volume) was added, and
the reagent was allowed to warm to room temperature. An aliquot of
this reagent (1.8 ml) was added to the dry sterol or steryl acetate
sample in glass culture tubes (10 x 75 mm), and the absorbance was
read at 620 nm (for cholesterol and other C27 sterols or steryl ace-
tates) with a Coleman Junior spectrophotometer. The following
developing times were allowed for free sterols and steryl acetates:

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>DEVELOPING TIME (min.)</th>
<th>O.D. _620/\text{mg}^{**}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta^5 )</td>
<td>30.0*</td>
<td>1.31</td>
</tr>
<tr>
<td>( \Delta^7 )</td>
<td>1.5*</td>
<td>6.33</td>
</tr>
<tr>
<td>( \Delta^8(14) )</td>
<td>13.0*</td>
<td>1.55</td>
</tr>
<tr>
<td>( \Delta^8(9) )</td>
<td>4.5</td>
<td>---</td>
</tr>
<tr>
<td>Dienes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta^7,14 )</td>
<td>1.25*</td>
<td>6.2</td>
</tr>
<tr>
<td>( \Delta^8,14 )</td>
<td>7.0*</td>
<td>5.0</td>
</tr>
<tr>
<td>( \Delta^5,7 )</td>
<td>1.5</td>
<td>5.0</td>
</tr>
<tr>
<td>( \Delta^5,24 )</td>
<td>25.0</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*Lutsky (1971).

**Values determined for sterylacetates.

Lanosterol and dihydrolanosterol were allowed to develop for
20 minutes prior to reading the absorbance at 460 nm. The developing
time was the same for sterols and steryl acetates having equivalent
double bond positions; however, the O.D./mg values were always slight-
ly lower for steryl acetates than for the corresponding free sterols.
Silicic Acid-Super Cel Column Chromatography

Silicic acid-Super Cel columns were used for the separation of relatively nonpolar components from the more polar constituents of the nonsaponifiable lipid (NSL) extracts of tissues or homogenate preparations, essentially as described by Clayton et al. (1963).

Silicic acid (A.R. grade, 100 mesh; Mallinckrodt Chemical Works) and Hy-Flo Super Cel (Johns-Manville Corp.) were mixed thoroughly in ratios of 2:1 or 1:1 (w:w) in an appropriate solvent (i.e. benzene, toluene, or a hexane-benzene mixture), forming a slurry. The slurry was poured into a glass column (100 x 1 cm or 50 x 1 cm) and packed under nitrogen pressure (5 p.s.i.).

Samples were applied in a small volume of the first eluting solvent (1-2 ml). Fractions were collected in glass test tubes using an automatic fraction collector (either an LKB Radi-Rac, type 3403B, or an ISCO Model 273 fraction collector). In most instances, fractions were evaporated to dryness under nitrogen and redissolved in a measured volume of solvent (i.e. 2.0 ml of benzene). Aliquots were then assayed for radioactivity and/or carrier mass.

Silica Gel G-Super Cel-Silver Nitrate Column Chromatography

Columns of Silica Gel G-Super Cel-silver nitrate were prepared essentially as described by Lutsky (1971). Silica Gel G (20 g; type 60, containing calcium sulfate binder; Brinkmann Instruments, Inc.) was mixed with Hy-Flo Super Cel (20 g) in a one-litre flask. To this dry mixture, a solution of silver nitrate (8 g, in 150 ml of water) was added, and the resulting slurry was shaken vigorously to insure complete and homogeneous mixing. The slurry was transferred to a two-
litre lyophilization flask, frozen solid in a Dry Ice-acetone bath, and lyophilized (30-100 microns pressure) for 48 hours. During lyophilization, the flask was protected from exposure to light by wrapping it with aluminum foil. The resulting buff-colored material was slurried in an appropriate solvent (i.e. benzene, toluene or a hexane-benzene mixture), poured into glass columns (50 x 1 cm) and packed under nitrogen pressure, as described above. Samples were applied to the columns, eluted with appropriate solvents, and fractions collected and assayed as previously described.

Such chromatographic columns were used for the separation of monoene and diene sterols or steryl acetates, and for the resolution of individual diene sterols or steryl acetates from a mixture of dienes.

Alumina-Super Cel-Silver Nitrate Column Chromatography

Monoene isomers of steryl acetates were separated on columns of alumina-Super Cel-silver nitrate by a modification of the method of Paliokas et al. (1968), essentially as described by Lutsky (1971).

Neutral alumina (30 g; type AG-7, without binder; Bio-Rad Laboratories) was mixed thoroughly with Hy-Flo Super Cel (15 g) in a one-litre flask. A silver nitrate solution (9 g, in 75 ml of water) was added to the dry mixture, and the resulting slurry was mixed well. The slurry was transferred to a two-litre lyophilization flask, frozen solid in a Dry Ice-acetone bath, and lyophilized for 48 hours, as described previously. The resulting white powder was slurried in an appropriate solvent (i.e. hexane-benzene or hexane-toluene, 85:15), and chromatographic columns (100 x 1 cm) were prepared as described
above. The steryl acetate samples were applied to the columns, eluted with appropriate solvent systems, and fractions were collected and assayed for radioactivity and carrier mass as previously described.

**Acetylation of Nonsaponifiable Lipids**

Sterols and isoprenoid alcohols were acetylated by the method of Johnston and Bloch (1957). A mixture of dry pyridine and acetic anhydride (1:1) was added to the dry sample (i.e. 2 ml per mg, for sterols) in a glass vial. The reaction vessel was purged with nitrogen, sealed tightly and allowed to stand at room temperature for 24 hours; samples which resisted acetylation under these conditions were either allowed to incubate further (24–48 hours) at room temperature or for several hours at 40°C. At the end of the allowed reaction time, the contents of the vial were diluted with two volumes of ice water and the solution was extracted three times with either hexane or petroleum ether (two volumes each). The combined organic extracts were washed once with water (0.5 volume), once with a saturated sodium bicarbonate solution (0.5 volume), once with a 5% tartaric acid solution (0.5 volume), and twice more with water (0.5 volume each). The organic extract was then dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure or evaporated to dryness under nitrogen and redissolved in a measured volume of an appropriate solvent.

Sterols were labelled with $[^{3}\text{H}]$-acetic anhydride as follows. The sterol sample (1–10 mg) was dissolved in 4.0 ml of dry pyridine in a glass vial. The original stock of $[^{3}\text{H}]$-acetic anhydride (in about 0.05 ml of benzene) was diluted with 0.10–0.20 ml of benzene (dried over molecular sieves prior to use) and 0.10 ml of unlabelled carrier
acetic anhydride. An aliquot of the diluted stock (ca. 0.10 ml) was added to the sterol sample, and the acetylation and work-up was carried out as described above.

**Preparation of Methyl Esters**

Fatty acids and isoprenoid acids were derivatized with freshly-generated diazomethane to form the corresponding methyl esters, as follows. A small amount (i.e. 50-100 mg) of N-nitrosomethylurea (Lot 99148; K & K Laboratories) was hydrolyzed with 5 M potassium hydroxide (5-10 ml) in a 50 ml erlenmeyer flask fitted with a two-hole rubber stopper and two U-shaped glass tubes. One of the tubes served as an inlet through which nitrogen was bubbled, while the other served as an effluent port for the diazomethane. The free end of the exit tube was submerged in a test tube containing a solution of diethyl ether-methanol (9:1, dried over molecular sieves); the solution was chilled on a Dry Ice-acetone bath. Diazomethane was bubbled through the chilled solution under nitrogen pressure until a deep yellow color persisted.

The sample to be derivatized was dissolved in diethyl ether-methanol, 9:1 (usually about 1-2 ml) in a small vial; an excess of the chilled diazomethane solution (i.e. 2-5 volumes) was then added, and the vial was sealed and allowed to stand at room temperature (usually 1-4 hours). After the appropriate time, the solvent was removed from the sample under a stream of nitrogen and the derivatized sample was redissolved in an appropriate volume of hexane.

Routinely, methyl esters were purified by chromatography on a small Unisil column (activated silicic acid, 100-200 mesh; Clarkson Chemical Company, Inc.). A hexane slurry of Unisil (ca. 4 g) was
poured into a glass column (21 x 1 cm) and allowed to settle under gravity. The derivatized sample was applied to the column in a small volume of hexane (i.e. 1-2 ml), and eluted with hexane-diethyl ether, 95:5. Fractions were collected automatically in glass tubes, as previously described; methyl esters usually eluted within the first twelve fractions. The elution of radioactive samples was monitored by assaying aliquots of the fractions, as described previously; unlabelled samples were assayed by thin-layer chromatography, in comparison with an appropriate authentic standard.

Fractions containing the methyl esters were pooled, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in a measured volume of hexane.

**Digitonin Precipitation of Sterols**

Sterols were precipitated with digitonin according to the method of Sperry and Webb (1950). Samples (usually containing 5-10 mg of sterol) were dissolved in a solution of ethanol-acetone, 1:1 (10 ml) in conical glass centrifuge tubes. Digitonin (Sigma Chemical Company) was dissolved in 50% aqueous ethanol (5 mg/ml) and 5 ml of the solution were added to each sample, along with 3-5 drops of 10% acetic acid. The samples were sealed, mixed well and left overnight in a dark cabinet at room temperature (12-20 hours). The resulting precipitate was collected by centrifugation in a desk-top centrifuge (20-30 minutes at 1500 xg; Clay Adams clinical centrifuge). The supernatant was removed by aspiration, and the pellet was resuspended and washed once with anhydrous ether; the pellet was collected each time by centrifugation, as described above. Residual ether in the final pellet was removed under
a stream of nitrogen and the pellet was dried \textit{in vacuo} over phosphorous pentoxide.

The digitonide complex was split by the addition of dry pyridine (0.5 ml) to each sample, followed by heating the samples in sealed tubes for 20–30 minutes in a boiling water bath. After the allotted time, anhydrous ether (5.0 ml) was added rapidly to each sample and the samples were mixed vigorously (by Vortex mixer) and then centrifuged as described above. The supernatants (containing the liberated sterols) were removed carefully by aspiration and saved; the digitonin pellets were washed twice more with ether (5.0 ml portions), and the combined supernatants for each sample were evaporated to dryness under nitrogen and redissolved in a measured volume of benzene. Aliquots were assayed for radioactivity and for sterol mass as usual; a comparison of the specific activity of a sample (dpm/mg sterol) before and after digitonin precipitation revealed the percentage of the original sample which was digitonin-precipitable.

The efficiency of precipitation and recovery of cholesterol varied from 85–95%, whereas that of lanosterol and dihydrolanosterol averaged only 35–45%.

\textbf{Dibromide Purification of Cholesterol}

Radioactive cholesterol recovered from incubations was purified by a modification of the micro-dibromide procedure of Frantz \textit{et al.} (1959). The radioactive sample was diluted with unlabelled carrier cholesterol (ca. 50 mg) and dissolved in a measured volume of benzene. Aliquots were removed for assays of radioactivity and sterol mass. The sample was evaporated to dryness under nitrogen and the residue re-
dissolved in anhydrous ether (2 ml) in a Corex centrifuge tube, chilled on ice. Bromine was added to this solution, dropwise, until a reddish-brown color persisted; the solution was maintained on ice for 1-2 hours. At the end of this time, a solution of chilled glacial acetic acid-methanol (95:5, 1 ml) was added, the precipitated cholesteryl dibromide was collected by centrifugation (20 minutes at 2000 rpm, Sorvall SS-34 rotor; 0–4°C) and the pellet was washed three times with glacial acetic acid-methanol (95:5; 2 ml) and saved.

The resulting cholesteryl dibromide was dissolved in anhydrous ether (5 ml) and zinc dust (25 mg) was added, with continuous agitation on a Vortex mixer for 15 minutes. Water was added dropwise to dissolve the zinc bromide, and the ether layer was washed once with 0.7 N hydrochloric acid (2 ml), twice with water (2 ml), once with 8% sodium hydroxide (2 ml) and three times more with water (2 ml portions), until the ether layer was neutral to litmus. The ether layer was evaporated and the residue was recrystallized from methanol (usually twice). The resulting cholesterol was redissolved in a measured volume of benzene, and aliquots were removed again for assays of radioactivity and sterol mass.

Routinely, recovery of sterol ranged from 40-65%.

**Saponification Procedures**

The saponification of biological samples and pure compounds was performed by the addition of 15% ethanolic potassium hydroxide, followed by heating for 3-4 hours under nitrogen atmosphere either on a steam bath, a water bath (75–80°C), or a heating mantle (80°C).

The potassium hydroxide solution was prepared fresh for each use.
by dissolving 15 grams of potassium hydroxide in 100 ml of 95% ethanol.

Lyophilized samples were hydrolyzed at reflux directly in the 15% ethanolic potassium hydroxide solution (0.5 ml per mg, for membrane samples; 1.0 ml per mg, for lyophilized retinas). After cooling to room temperature, the samples were diluted with an equal volume of water and extracted four times with petroleum ether or hexane (one volume each). The combined extracts were washed once with 0.9% saline (0.25 volumes), and three times with water (0.25 volumes each); the extracts were dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure, transferred to tared vials, evaporated to dryness under nitrogen and desiccated under vacuum over phosphorous pentoxide. The nonsaponifiable residues were then weighed and redissolved in a measured volume of benzene, prior to further analyses.

Small-scale in vitro homogenate incubations were terminated, in general, by the addition of 1 ml of an ethanolic cholesterol solution (4 mg per ml, in absolute ethanol) plus 3 ml of 15% ethanolic potassium hydroxide. After saponification as described above, the hydrolyzates were diluted with one volume of water (usually 6 ml) and extracted three times with 15 ml portions of hexane or petroleum ether. The combined organic extracts were washed once with 10 ml of water, dried over anhydrous sodium sulfate, evaporated to dryness under nitrogen and redissolved in a measured volume of benzene. Aliquots of the nonsaponifiable lipid extract were then taken for various analyses (i.e. colorimetric assay, chromatographic analyses, digitonin precipitation, etc.).

Large-scale in vitro homogenate incubations were terminated by the addition of one volume of 15% ethanolic potassium hydroxide,
followed by the usual saponification procedure. The hydrolyzates were
diluted with an equal volume of water, allowed to cool, and extracted
three times with hexane or petroleum ether (one volume each). The com-
bined extracts were washed once with water (0.25 volume), dried over
anhydrous magnesium sulfate, filtered, concentrated under reduced
pressure, transferred to vials, evaporated to dryness under nitrogen
and redissolved in a measured volume of benzene, prior to further
analyses.

**Acidification and Extraction of Saponifiable Lipids**

After saponification and extraction of the nonsaponifiable lipid
material, the aqueous ethanolic phase which remained (including the
water washes of the combined hexane or petroleum ether extracts) was
stored in a freezer (-20°C). The frozen solution was allowed to thaw
(to approximately 4°C); the mixture was then acidified by dropwise
addition of concentrated hydrochloric acid, with continuous rapid
stirring, until the pH was about 1-2. The acidified mixture was ex-
tracted three times with dichloromethane (two volumes); the combined
extracts were washed once with water (one volume), then dried over an-
hydrous magnesium sulfate, filtered, concentrated under reduced
pressure, transferred to a vial, evaporated to dryness under nitrogen
and redissolved in a measured volume of an appropriate solvent.

**Extraction of Total Lipids**

Two similar techniques were employed routinely for the extraction
of "total lipids" from biological tissues and incubation mixtures.

A modification of the method of Folch et al. (1957) was used on
lyophilized tissues. The tissue was homogenized in chloroform-methanol (2:1) in a tight-fitting, Ten Broeck ground-glass tissue grinder (1 ml per 8 mg of membranes; 1 ml per 3 mg of dried retinas). The extract was transferred to a separatory funnel; the homogenization sleeve was rinsed with 0.2 volume of 0.9% saline and the eluant was transferred to the funnel. After vigorous shaking, the phases were allowed to settle (at 4°C) and the lower phase was removed and saved. The upper (aqueous) phase was extracted twice more with a volume of chloroform equivalent to that which was previously removed during the initial extraction. The combined chloroform phases were washed once with water (0.2 volume) and dried over anhydrous magnesium sulfate.

Alternatively, extraction of incubation mixtures was performed by a modification of the procedure of Bligh and Dyer (1959). The incubation mixture was diluted with an equal volume of methanol and transferred to a separatory funnel. Chloroform and methanol were added to the mixture so as to form a monophase of chloroform-methanol-water with the volumetric ratio of 60:45:11. Water was added (dropwise) with the mixture separated into two distinct phases and the lower (chloroform) phase was removed and saved. The upper phase was extracted again with a volume of chloroform equivalent to that which was previously removed. The combined extracts were washed once with water (0.2 volume) and dried over anhydrous magnesium sulfate.

The extracts were concentrated under reduced pressure, transferred to vials, evaporated to dryness under nitrogen and redissolved in a measured volume of chloroform.
Hydrogenation Procedures

Hydrogenation of sterols was carried out in benzene solution, using a Parr hydrogenator, over freshly-prepared Raney nickel (K & K Laboratories; prepared by the method of Moringo, 1955). Routinely, the hydrogenation was performed at room temperature for 24 hours with hydrogen pressure of 40-48 p.s.i. After the allotted time, catalyst was removed by vacuum filtration through a bed of Hy-Flo Super Cel in a fritted funnel. The solvent was then removed under reduced pressure and the sterol was recrystallized or purified further by chromatographic techniques.

Other lipids were hydrogenated by bubbling hydrogen through the sample, dissolved in hexane-ethanol, 2:1, for 15-30 minutes (room temperature, one atmosphere) over platinum oxide catalyst (J. Bishop & Co. Platinum Works). The procedure was performed in 15 ml-capacity conical glass centrifuge tubes. Catalyst was removed by centrifugation (10 minutes at 2400 rpm, Clay Adams clinical centrifuge); the supernatant was withdrawn by aspiration and saved, while the catalyst was washed twice with fresh solvent (5-10 ml each). The combined supernatants were evaporated under nitrogen and redissolved in a measured volume of an appropriate solvent.

Preparation of Buffers and Incubation Media

Tris-acetate buffer (1.0 M, pH 7.4) was prepared by dissolving Trizma base (tris-(hydroxymethyl)-aminomethane, 12.11 g; Sigma Chemical Co.) in water (90 ml) in a 100 ml volumetric flask. The pH was adjusted to 7.4 with glacial acetic acid, dropwise, and the volume was then adjusted to 100 ml with water. A portion of this stock was diluted
100-fold with water to make 0.01 M Tris-acetate, pH 7.4, buffer. By an analogous procedure, 0.1 M Tris-acetate, pH 8.0 was made (using 1.211 g of Trizma base in a final volume of 100 ml, adjusted to pH 8.0 with glacial acetic acid).

Potassium phosphate buffer (1.0 M, pH 7.4) was prepared by dissolving anhydrous potassium dihydrogen phosphate (\(\text{KH}_2\text{PO}_4\), 136.09 g) in water (900 ml), adjusting the pH to 7.4 with 5 N potassium hydroxide and then adjusting the volume to one liter with water.

Sodium potassium phosphate buffer (67 mM, pH 7.0) was made by mixing solutions of potassium dihydrogen phosphate (\(\text{KH}_2\text{PO}_4\), 9.073 g in one litre of water) and disodium hydrogen phosphate (\(\text{Na}_2\text{HPO}_4\), 11.87 g in one liter of water) in the volumetric ratio of 413:587.

Phosphate-buffered saline (PBS) was prepared by dissolving 8 g of sodium chloride per 100 ml of 67 mM sodium potassium phosphate buffer, pH 7.0.

Sodium phosphate buffer (0.1 M, pH 7.0) was prepared by dissolving monosodium dihydrogen phosphate (\(\text{NaH}_2\text{PO}_4\), 0.39 g) and disodium hydrogen phosphate (\(\text{Na}_2\text{HPO}_4\), 1.89 g) in 99 ml of water, adjusting the pH to 7.4 by dropwise addition of 5 N sodium hydroxide.

Potassium phosphate incubation buffer (0.10 M, pH 7.4), used for in vitro homogenate incubations, was prepared as follows. Solutions of dibasic potassium phosphate (\(\text{K}_2\text{HPO}_4\), 0.20 M, 400 ml) and monobasic potassium phosphate (\(\text{KH}_2\text{PO}_4\), 0.20 M, 100 ml) were mixed in a one-litre volumetric flask. Nicotinamide (3.66 g; Eastman Organic Chemical Co.) and magnesium chloride hexahydrate (\(\text{MgCl}_2\cdot6\text{H}_2\text{O}\), 1.00 g) were added and the volume was increased to 950 ml by the addition of water. The pH
of the solution was adjusted to 7.4 with 5 N potassium hydroxide and the final volume was adjusted to one liter with water. The final concentrations of nicotinamide and magnesium chloride were 30 mM and 5 mM respectively. In some instances, this same phosphate buffer was prepared without nicotinamide and magnesium chloride, and will be referred to as simply 0.10 M potassium phosphate buffer (pH 7.4).

In vitro incubations of intact bovine retinas were carried out in three different media: RPMI-1640 (a synthetic, defined tissue culture medium developed at Roswell Park Memorial Institute; a product of Grand Island Biological Co.); Krebs-Ringer Bicarbonate (KR-B); Krebs-Ringer Phosphate (KR-P).

The RPMI-1640 medium is a bicarbonate-buffered system containing the following complex assortment of constituents (concentrations expressed as mg per liter): inorganic salts (Ca(NO₃)₂·4H₂O, 100.0; KCl, 400.0; NaCl, 6000.0; MgSO₄·7H₂O, 100.0; NaHCO₃, 2000.0; Na₂HPO₄·7H₂O, 1512.0); amino acids (L-arginine (free base), 200.0; L-asparagine, 50.0; L-aspartic acid, 20.0; L-cystine, 50.0; L-glutamic acid, 20.0; L-glutamine, 300.0; glycine, 10.0; L-histidine (free base), 15.0; L-hydroxyproline, 20.0; L-isoleucine (allo-free), 50.0; L-leucine (methionine-free), 50.0; L-lysine HCl, 40.0; L-methionine, 15.0; L-phenylalanine, 15.0; L-proline (hydroxyproline-free), 20.0; L-serine, 30.0; L-threonine (allo-free), 20.0; L-tryptophane, 5.0; L-tyrosine, 20.0; L-valine, 20.0); vitamins (biotin, 0.2; D-calcium pantothenate, 0.25; choline chloride, 3.0; folic acid, 1.0; i-inositol, 35.0; nicotinamide, 1.0; para-aminobenzoic acid, 1.0; pyridoxine HCl, 1.0; riboflavin, 0.2; thiamine HCl, 1.0; vitamin B₁₂, 0.005); glucose 2000.0; reduced gluta-
thione, 1.0; phenol red, 5.0.

The Krebs-Ringer Bicarbonate medium contained the following constituents (concentrations expressed in millimolar units): NaCl, 120.0; KCl, 5.1; CaCl$_2$·2H$_2$O, 2.75; MgSO$_4$, 1.25; NaHCO$_3$, 25.0; KH$_2$PO$_4$, 1.25; glucose, 10.0.

The Krebs-Ringer Phosphate medium had the following composition (concentrations expressed in millimolar units): NaCl, 120.0; KCl, 5.1; CaCl$_2$·2H$_2$O, 2.75; MgSO$_4$, 1.25; KH$_2$PO$_4$, 1.25; glucose 10.0; sodium phosphate buffer, pH 7.4 (final concentration, 10.0 mM).

All three media were supplemented with penicillin-G (potassium salt, 8 µg/ml; Sigma Chemical Co.) and streptomycin sulfate (12 µg/ml; Sigma Chemical Co.). Media were autoclaved and stored at 4°C prior to use. About 30 minutes before the initiation of incubations, the media were removed from the refrigerator, aerated with oxygen-carbon dioxide (95:5, v:v) and allowed to warm to room temperature.

Protein Assays

In cases where large amounts of samples were available, protein concentrations were determined by a modification of the Biuret assay (Goo, 1953). Aliquots of each sample (in duplicate, at two different volumes) were adjusted to 1 ml with an appropriate buffer; Biuret reagent (4 ml) was added to each, the solutions were mixed well and the absorbance at 550 nm was read after thirty minutes of developing time (room temperature; in 10 x 75 mm optical glass culture tubes), using a Coleman Junior spectrophotometer. A blank of water (1 ml) plus Biuret reagent (4 ml) was used to "zero" the instrument; a buffer blank was also included in each assay series. Protein concentrations were deter-
mined from the absorbance readings by comparison with a reference curve, using serial dilutions of a bovine serum albumin solution (BSA; Sigma Chemical Co.); the linear range of the assay was 0.5 - 7.5 mg of protein.

Biuret reagent was prepared by dissolving cupric sulfate pentahydrate (1.5 g) and sodium potassium tartrate (6.0 g) in water (500 ml), in a one-liter volumetric flask. A freshly-prepared solution of 10% sodium hydroxide (300 ml) was added to the mixture, with continuous stirring; the reagent was diluted to one liter with water.

Alternatively, small amounts of protein (i.e. 10 - 100 μg) were assayed by a modification of the method Lowry et al. (1951), essentially as described by Legget-Bailey (1967). The following stock solutions were made: (A) 2% sodium carbonate, in 0.1 M sodium hydroxide; (B) 0.5% cupric sulfate pentahydrate, in 1% sodium citrate; (C) 1 ml of solution B plus 50 ml of solution A; (D) Folin reagent (2 N phenol; Sigma Chemical Co.), diluted 1:1 with water. Aliquots of each sample (0.20 ml each) were added to optical glass culture tubes (12 x 75 mm); solution C (1.0 ml) was added to each tube, and the samples were mixed by vortexing and incubated at room temperature for 10 minutes. At the end of this time, solution D (0.10 ml) was rapidly added to each sample with vigorous mixing and the samples were incubated an additional 30 minutes. Each sample was then diluted with water (1.0 ml), and the absorbance at 650 nm was read on a Coleman Junior spectrophotometer.

A calibration curve was obtained, using serial dilutions of a BSA stock solution. Exact concentrations of each BSA standard were determined by the absorbance at 280 nm, using a value of 0.66 for a
1.0 mg/ml solution of BSA as a reference.

**Subcellular Fractionation of Tissues**

Fresh bovine eyes were obtained from a local abattoir (Blue Ribbon Meat Packing Co.) and stored on ice for less than one hour prior to processing. All procedures were performed under normal room illumination at 0-4°C. An incision was made in the sclera along the circumference of the eye with a razor blade, just below the ora serrata; the cornea, lens and vitreous were extruded and the eyecup was inverted to expose the retina. Retinas were gently teased away from the retinal pigmented epithelium (RPE) and detached at the optic nerve with a small surgical scissors. After gentle swirling in ice-chilled incubation buffer (0.10 M potassium phosphate, pH 7.4, containing 30 mM nicotinamide and 5 mM MgCl₂·6H₂O) to remove adhering RPE and vitreal debris, the retinas were transferred to an ice-chilled glass homogenization sleeve containing incubation buffer (2 ml per retina) and homogenized for 90 seconds with a loose-fitting, motor-driven teflon pestle (660 rpm, 15-20 passes). The resulting homogenate was distributed into 50 ml capacity polyethylene centrifuge tubes and centrifuged for 5 minutes at 500 x g (Sorvall SS-34 rotor; Sorvall RC-2B centrifuge; 0-4°C). The supernatants were carefully decanted into another set of 50 ml capacity centrifuge tubes and then centrifuged again for 30 minutes at 10,000 xg. The resulting supernatants were pooled and stored in an ice-chilled flask until ready for use; this supernatant preparation will be referred to as the retina \( S_{10} \) homogenate throughout the text. This procedure was based on the method originally described by Bucher and McGarrah (1956) for the preparation of rat liver \( S_{10} \) homogenates, with the
modifications developed by Raulston (1979) concerning the specifics of
the homogenization procedure (i.e. duration of homogenization and
pestle speed for optimal incorporation of mevalonate into digitonin-
precipitable material in incubations of rat liver $S_{10}$ homogenates).

By a strictly analogous procedure, $S_{10}$ homogenates were prepared
from rat livers. Male Sprague-Dawley rats (120-150 g each) were
maintained on an ad lib cholesterol-free lab chow diet (United States
Biochemical Corp.) at least two weeks prior to sacrifice by decapita-
tion. The livers were removed intact, rinsed briefly with ice-chilled
0.9% saline and blotted dry with Kimwipes; after weighing (9-13 g
each), the livers were minced into small pieces (ca. 0.5 cm sections)
with a razor blade and homogenized in ice-chilled incubation buffer
(2.5 ml per gram wet weight) as described above. The liver $S_{10}$ homo-
genate was obtained by centrifugation per the above protocol; the super-
natant from the 500 x g centrifugation was withdrawn with a Pasteur
pipet, carefully excluding the copious white lipid film at the air/
buffer interface, and then centrifuged at 10,000 x g as described above.

Whereas the rat liver $S_{10}$ homogenates routinely had an opaque, red-
dish-tan appearance, the bovine retina $S_{10}$ homogenates were essentially
colorless and translucent (and did not form an appreciable film at the
air/buffer interface). These preparations were presumed to be free of
gross cell debris, nuclei and mitochondria. Typically, 10 retinas
would provide about 20-25 ml of $S_{10}$ homogenate, while about 18-20 ml
would be obtained per rat liver.
FIGURE II-1 - Protocol used for the preparation of an $S_{10}$ homogenate of bovine retinas. All procedures are carried out under normal room illumination at 0-4°C.
III. CHEMICAL SYNTHESIS

Synthesis of Cholesta-8,14-dien-3β-ol

The synthesis of cholesta-8,14-dien-3β-ol was performed by a modification of the method of Fieser and Ourisson (1953). Cholesta-5,7-dien-3β-ol (20 g; United States Biochemical Company) was dissolved in absolute ethanol-benzene, 5:1 (600 ml) and heated at reflux for four hours in the presence of concentrated hydrochloric acid (50 ml). The mixture was allowed to cool to room temperature and then washed once with 0.9% sodium chloride (500 ml) and twice with water (500 ml each). The organic phase was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The solid residue was re-crystallized twice from methanol and once from acetone-water, yielding 12.8 g of white needles (m.p. 111-113°C; literature: 116-117°C, Fieser and Ourisson, 1953; 119-120°C, Adams et al., 1951; 113.5-114.5°C, Lutsy and Schroepfer, 1970).

Analysis of the product by TLC (silica gel G) in two different solvent systems (chloroform; chloroform-ethyl acetate, 65:35) revealed a single component, even on overloaded plates. However, GLC analysis (3% OV-17; column temperature, 260°C; carrier flow rate, 66 ml/min.) revealed at least three components having the following relative retention times (relative to cholestane; retention time, 5.95 min.): 2.30 (ca. 4%), 2.48 (ca. 9%), and 2.72 (ca. 87%). Analysis of the following authentic sterols gave the following relative retention times under the same conditions: cholest-8,14-dien-3β-ol, 2.72; cholest-7,14-dien-3β-ol, 2.76; cholest-5,7-dien-3β-ol, 2.99. The starting material ex-
hibited a major peak (ca. 96%) with a relative retention time of 2.99, and a minor peak (ca. 4%) with a relative retention time of about 2.49.

In an attempt to eliminate the minor contaminants (thought to be due to incomplete isomerization of the 7-dehydrocholesterol), the product was exposed again to acid isomerization conditions, this time by refluxing for four hours in methanol-benzene, 8:1 (450 ml) in the presence of hydrochloric acid (50 ml). The mixture was washed once with 200 ml of water, and the aqueous phase was re-extracted with petroleum ether (four times, 400 ml each). The combined organic phases (benzene and petroleum ether layers) were washed with water (three times, 200 ml each) and dried over anhydrous sodium sulfate. After filtration, the solvent was removed under reduced pressure and the residue was dissolved in hot methanol (100 ml, heated on a steam bath) containing several grams of decolorizing carbon. The mixture was filtered and the carbon residue was washed with hot methanol (three times, 25 ml each). The combined eluants were evaporated to dryness under reduced pressure and recrystallized twice from methanol and once from acetone. Dense clear prisms were obtained (5.57 g; m.p. 113-114°C).

Thin-layer chromatographic analyses using three different solvent systems (benzene; chloroform; chloroform-ethyl acetate, 65:35) showed only a single component, even when grossly overloaded. Analysis of the product by GLC (same conditions as previously employed) exhibited one major component (ca. 97%), preceded by a minor constituent (ca. 2-3%).

The ultraviolet absorption spectrum (scanned from 200-350 nm) of the product (dissolved in absolute ethanol) exhibited an absorption maximum at 250 nm with a molar extinction coefficient of 17,600
The infrared spectrum exhibited a broad band centered around 3360 cm\(^{-1}\) (hydroxyl group), a weak band at 3055 cm\(^{-1}\) (olefinic C-15 carbon-hydrogen stretch), another weak band at 1632 cm\(^{-1}\) (olefinic carbon-carbon stretch) and two moderate, sharp bands at 1030 and 1052 cm\(^{-1}\) (carbon-oxygen stretch of the 3-\(\alpha\)-hydroxyl group).

The NMR spectrum of the product showed a normal methylene envelope (centered around 1.2 ppm), a broad multiplet at 3.6 ppm (the 3\(\alpha\)-proton) and a partially-resolved triplet at 5.3 ppm (C-15 olefinic proton). The integrated areas of the peaks at 3.6 ppm and 5.3 ppm gave a ratio of 1.0, demonstrating that only one kind of olefinic proton was present (i.e. contamination of the product with either the \(\Delta^7,14\) diene or \(\Delta^5,7\) diene sterol was not detected).

Analysis of the product by mass spectrometry revealed the expected molecular ion at m/e 384 (base peak), as well as the following prominent fragments: m/e 369 (88%; M-C\(_3\)H\(_7\)), 351 (45%; M-C\(_3\)H\(_2\)O), 271 (17%; M-C\(_8\)H\(_{17}\)), and 253 (12%; M-C\(_8\)H\(_{17}\)H\(_2\)O).

The above data are consistent with the known structure of cholesta-8,14-dien-3\(\beta\)-ol. The product was approximately 97% pure according to the physical properties listed above.

**Synthesis of Cholesta-8,14-dien-3-one**

Cholesta-8,14-dien-3\(\beta\)-ol was converted to the corresponding 3-ketone by the method of Corey and Suggs (1975). The sterol (1.00 g, dissolved in 10 ml of dry dichloromethane) was added to a solution of pyridinium chlorochromate (1.68 g dissolved in 10 ml of dry dichloro-
methane; Aldrich Chemical Co.) and allowed to react at room temperature for 1.5 hours with continuous stirring. About 100 ml of diethyl ether was added and the mixture was filtered through a bed of anhydrous magnesium sulfate; the tarry brown residue remaining in the flask was extracted with diethyl ether (three times, 50 ml each), filtered, and the combined eluants were evaporated to dryness under reduced pressure and redissolved in 10 ml of benzene.

The benzene-soluble material was purified by chromatography on a silica gel column (60-200 mesh, Baker Reagents; 58 x 2 cm, packed in benzene), eluting with benzene-ether, 95:5 (21.5 ml per 8-minute fraction). Fractions were assayed for mass by TLC (silica gel G; chloroform), and fractions 5-16 (corresponding to the ketone) were pooled, evaporated to dryness under reduced pressure and recrystallized once from methanol and once from acetone, yielding translucent cubes (0.24 g, m.p. 134.0-135.5°C; literature: 133°C, Canonica et al., 1968; 137-138°C, Lutsky and Shcroepfer, 1970).

Analysis of the product by TLC in chloroform ($R_f = 0.40$) and chloroform-ethyl acetate, 65:35 ($R_f = 0.70$) showed only a single band, which migrated considerably ahead of the starting material.

Gas-liquid chromatography (3% OV-17; column temperature, 260°C; carrier flow rate, 66 ml/min.) revealed one major component (ca. 93%; relative retention time, 2.93) followed by a minor component (ca. 7%; relative retention time, 3.62) (relative to cholestane; retention time, 5.16 minutes).

The infrared spectrum showed the loss of the previously observed hydroxyl band at 3360 cm$^{-1}$ and the presence of a strong, sharp band at
1710 cm$^{-1}$ (carbonyl stretch, six-membered saturated ring). Also noted was the loss of the bands previously observed at 1030 and 1052 cm$^{-1}$.

The NMR spectrum showed a complete loss of the resonance signal at 3.6 ppm, but retention of the C-15 olefinic proton signal at 5.3 ppm. Again, no other olefinic protons were detected.

The mass spectrum of the product exhibited a molecular ion at m/e 382 (base peak), consistent with the known molecular weight of the steryl ketone. Other prominent fragments were observed at m/e 367 (61%; M-CH$_3$), 269 (38%; M-C$_8$H$_{17}$) and 255 (31%; M-CH$_3$-C$_8$H$_{17}$).

The above data are consistent with the known structure of cholesta-8,14-dien-3-one.

Synthesis of [2,4-$^3$H]-Cholesta-8,14-dien-3-ol

The steryl ketone was labelled with tritium by base-catalyzed enolization in the presence of [$^3$H]-water. All glassware was dried in an oven overnight (120°C) and allowed to cool in vacuo in a desiccator. Dioxane (spectrograde) was freshly-distilled over lithium aluminum hydride (Matheson Coleman and Bell) and stored over molecular sieves (type 3A, Matheson Coleman and Bell). The steryl ketone was stored in vacuo over phosphorous pentoxide overnight.

Cholesta-8,14-dien-3-one (101.60 mg) was dissolved in dry dioxane (4 ml), followed by the addition of sodium methoxide (50 mg; Aldrich Chemical Co.). Approximately 0.04 ml of [$^3$H]-water was added to the sample and the reaction vessel was sealed, vortexed and allowed to stand for five days at room temperature, with intermittent mixing.

The reaction was terminated by the addition of 10 ml of a 5% sodium bicarbonate solution and the mixture was extracted with diethyl
ether (three times, 15 ml each). The combined extracts were washed once with water (10 ml), dried over anhydrous magnesium sulfate, filtered and the solvent volume was reduced to approximately 28-30 ml under a stream of nitrogen. The organic and aqueous phases were assayed for total radioactivity, as previously described (organic phase, ca. $3.8 \times 10^9$ dpm; aqueous phase, $6.6 \times 10^9$ dpm).

The product in the organic phase was reduced by the addition of lithium aluminum hydride (400 mg, in small portions), allowing the reduction to proceed with intermittent vortexing for 30 minutes at room temperature. An additional 100 mg of reducing agent was then added and the mixture was allowed to stand overnight at room temperature.

The excess hydride reagent was destroyed by the careful addition of small amounts of ice followed by 10 ml of 2.4 N HCl and then drop-wise addition of concentrated HCl (to dissolve the aluminum hydroxide colloid which formed). The ether layer was removed by aspiration to another flask; the aqueous phase was extracted with fresh diethyl ether (three times, 20 ml each). The combined organic phases were dried over anhydrous sodium sulfate, filtered, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and then desiccated in vacuo over phosphorous pentoxide. The product was re-dissolved in 10.0 ml of benzene and assayed for radioactivity (1.44 mCi; $3.17 \times 10^9$ dpm).

Aliquots of the product (ca. 1 μl) were applied to silica gel G thin-layer plates along with authentic cholesta-8,14-dien-3β-ol and developed in two different solvent systems (chloroform; benzene-ether,
Sections (one cm each) were scraped into vials and assayed for radioactivity, as usual; mass was visualized by charring the plates, after spraying with molybdic acid reagent. Approximately 77% of the radioactivity co-migrated with the authentic sterol standard, while about 8% migrated just ahead of the standard (in the region expected for the 3α-epimer). The remainder of the radioactivity trailed to the origin of the plate.

The remainder of the sample (ca. 3.17 x 10⁹ dpm) was applied to a silica gel column (60-200 mesh, chloroform slurry; 58 x 2 cm) and fractions (11 ml per 4 minutes) were eluted with chloroform-acetone, 98.5:1.5, and assayed for radioactivity as previously described. After fraction 80, the column was stripped with 300 ml of chloroform-acetone, 9:1. The elution profile indicated that resolution of the 3α- and 3β-epimers was not effected; therefore, the fractions were pooled, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in benzene. The sample was applied to a Unisil column (100-200 mesh, in benzene slurry; Clarkson Chemical Co.; 58 x 2 cm) and eluted with benzene-ether, 9:1 (fractions, 11 ml per 5 minutes). The fractions were assayed for radioactivity as usual and those corresponding to the 3β- epimer (fractions 28-80) were pooled, evaporated to dryness under reduced pressure and recrystallized once from methanol. White spindles (42.65 mg) were obtained (m.p. 112.5-114.5°C); the sample was redissolved in 10.0 ml of benzene and assayed in triplicate for radioactivity (1.66 x 10⁹ dpm; 3.89 x 10⁷ dpm per mg).

Aliquots of the sample were analyzed by thin-layer chromatography
FIGURE III-1

Radio-TLC of chemically-synthesized [2,4-³H]-cholesta-8,14-dien-3β-ol. Chromatographic systems: (A) Silica Gel G, developed once with chloroform; (B) Silica Gel G-4% silver nitrate, developed once with chloroform and then once with benzene-diethyl ether petroleum ether, 7:3:2. Chromatographic standards: (A) Δ⁸,¹⁴-cholestadien-3β-ol; (B) Δ⁸,¹⁴-cholestadien-3β-ol (solid) and Δ⁸(⁹)-cholesten-3β-ol (open).

FIGURE III-2

Radio-GLC of chemically-synthesized [2,4-³H]-cholesta-8,14-dien-3β-ol. Chromatographic system: 3% OV-17, 260°C isothermal; carrier flow rate, 66 ml/min. The mass peak is due to unlabelled authentic cholesta-8,14-dien-3β-ol.
on two different systems: silica gel G (chloroform) and silica gel G-4% silver nitrate (developed once with chloroform and a second time with benzene-ether-petroleum ether, 7:3:2). An authentic standard of cholesta-8,14-dien-3β-ol was applied to each plate. The plates were sectioned and assayed for radioactivity as usual; the sterol standard was visualized by charring after spraying the plates with a solution of sulfuric acid diluted 1:1 with 50% methanol. The radiochromatograms are shown in Figure III-1. At least 99% of the radioactivity co-migrated with the sterol standard on the silica gel G plate, while about 96% co-migrated with the standard on the silver-impregnated plate.

Radio-GLC analysis of the product on a 3% OV-17 column (column temperature, 260°C/ carrier flow rate, 66 ml/min.) exhibited a single, homogeneous peak of radioactivity (Figure III-2) which exactly co-migrated with the co-injected authentic standard of cholesta-8,14-dien-3β-ol (retention time, 22.20 min.). Recovery of applied activity was 83% under these conditions.

According to the above data, the labelled sterol radiopurity was at least 99%.

**Synthesis of Lanost-8-en-3β-ol (24,25-Dihydrolanosterol)**

Commercial lanosterol ("C.P. grade"; Mann Research Laboratories, Inc.) was found to be a mixture of lanosterol and 24,25-dihydrolanosterol (3:2 molar ratio) by GLC-mass spectroscopy analysis. The sterol mixture (4.0 g) was reduced by hydrogenation over freshly-prepared Raney nickel catalyst (15 g) in about 800 ml of benzene, as previously described (see Materials and General Methods). After filtration,
solvent was removed under reduced pressure and the residue was recrystalized twice from methanol, yielding white plates (3.54 g, m.p. 146.0-146.5°C; literature: 149-150°C, Wieland et al., 1937; 145°C, Lacoume and Levisalles, 1964; 138-139.5°C, Kandutsch and Russell, 1959).

Analysis by TLC on silica gel G plates in two different solvent systems showed only a single component (chloroform, \( R_F = 0.19 \); chloroform-ethyl acetate, 65:35, \( R_F = 0.62 \)). Chromatography on silica gel G-4% silver nitrate plates also exhibited a single band (chloroform-ethyl acetate, 65:35, \( R_F = 0.53 \)). In all three cases, the observed component co-migrated with an authentic standard of lanost-8-en-3α-ol.

Gas-liquid chromatographic analysis of the product (3% OV-17; column temperature, 260°C; carrier flow rate, 66 ml/min.) exhibited a single component (full-scale, symmetrical peak); co-injection of the product with the starting material showed a selective enhancement of the dihydrolanosterol peak. Under the conditions employed, baseline separation of lanosterol and dihydrolanosterol was achieved (absolute retention times: dihydrolanosterol, 18.50 min.; lanosterol, 22.20 min.).

The infrared spectrum exhibited a broad band around 3450 cm\(^{-1}\) (hydroxyl group), as well as several other prominent bands: 2950, 2870 and 2815 cm\(^{-1}\) (aliphatic carbon-hydrogen stretch), and 1462, 1372 and 1066 cm\(^{-1}\) (assignment ambiguous). No bands corresponding to olefinic carbon-hydrogen stretching frequencies were detected, but a very weak and broadened band around 1635 cm\(^{-1}\) was noted.

The NMR spectrum exhibited the usual methylene envelope (centered around 1.25 ppm) in addition to a multiplet at 3.25 ppm (the 3α-proton). No olefinic proton signals were detected.
Analysis of the product by mass spectrometry revealed a molecular ion at m/e 428 (26%), consistent with the known molecular weight of the desired compound. Other prominent fragments were observed at m/e 413 (base peak; M-CH₃) and 395 (50%; M-CH₃-H₂O).

The above data are consistent with the known structure of lanost-8-en-3β-ol (24,25-dihydrolanosterol).

**Synthesis of Lanost-8-en-3-one**

Lanost-8-en-3β-ol (500 mg, 1.17 mmole, dissolved in 10 ml of dry dichloromethane) was oxidized to the corresponding 3-ketone with pyridinium chlorochromate (663 mg, 3.08 mmole, dissolved in 10 ml of dry dichloromethane), as previously described. The work-up and extraction of the reaction mixture was performed exactly as described previously. The reaction products (dissolved in 10 ml of benzene) were purified by silica gel column chromatography (60-200 mesh, in benzene slurry; 58 x 2 cm column), eluting with benzene (14 ml per 4-minute fraction). Fractions were assayed for mass by TLC (silica gel G, chloroform), and the fractions corresponding to the steryl ketone (fractions 5-16) were pooled, evaporated to dryness under reduced pressure and the residue was recrystallized once from methanol, yielding white plates (469.6 mg, m.p. 120-120.5°C; literature: 115.5-116.5°C, Wieland et al., 1937; 119-120°C, Lacoume and Levisalles, 1964). Based on TLC of the reaction mixture, the reaction proceeded to approximately 98% completion, with little or no side products.

A single component was observed upon analysis by thin-layer silica gel G chromatography (chloroform, Rₖ = 0.55; chloroform-ethyl acetate, 65:35, Rₖ = 0.77) and silica gel G-4% silver nitrate chromatography.
(chloroform-ethyl acetate, 65:35, $R_f = 0.66$). Gas-liquid column chromatography (3% OV-17; column temperature, 260°C; carrier flow rate, 66 ml/min.) also revealed a single component (relative retention time, 3.70; relative to cholestane, retention time 6.19 min.).

The infrared spectrum showed the expected loss of the hydroxyl band at 3450 cm$^{-1}$ and the concomitant appearance of a strong band at 1708 cm$^{-1}$, due to the carbonyl function (saturated, six-membered ring).

The NMR spectrum exhibited the loss of the 3α-proton signal and a new multiplet was observed at 2.45 ppm (the C-4 and C-2 protons, coupled to the 3-keto function).

Analysis of the product by mass spectrometry revealed a molecular ion at m/e 426 (19%) and a base peak at m/e 411 (M-CH$_3$). Other fragments were observed at m/e 271 (11%), 257 (20%) and 244 (18%).

The data are consistent with the known structure of lanost-8-en-3-one.

**Synthesis of [2-3H]-Lanost-8-en-3-one**

Base-catalyzed enolization of the steryl ketone in the presence of tritiated water and subsequent work-up of the reaction mixture were carried out exactly as described previously for the labeling of the C$_{27}$ steryl ketone, using 101.25 mg of lanost-8-en-3-one. The organic extract of the reaction mixture contained a total radioactivity of approximately $1.69 \times 10^9$ dpm (0.77 mCi).

Analysis of the reaction products by TLC (silica gel G) in two different solvent systems (chloroform; benzene-diethyl ether, 7:3) revealed that about 90% of the radioactivity corresponded to the migration of authentic 24,25-dihydrolanosterol, while about 6% exhibited the
expected migration of the 3α-hydroxy epimeric sterol.

The products were purified by column chromatography on silica gel (60-200 mesh, in chloroform slurry; 58 x 2 cm column), eluting with a solvent system of chloroform-acetone (98.5:1.5). Fractions (10.5 ml per four minutes) were collected and assayed for radioactivity, as usual. As observed previously, the elution profile did not exhibit resolution of the epimeric sterols. Therefore, the column was stripped with 300 ml of chloroform-acetone, 9:1 (after fraction 80) and the total eluted material from the column was pooled, evaporated under reduced pressure and redissolved in 5 ml of benzene. The entire sample (ca. 1.68 x 10^9 dpm) was applied to a Unisil column (100-200 mesh, in benzene slurry; 58 x 2 cm) and eluted with benzene-diethyl ether, 95:5. Fractions (10 ml per five minutes) were collected and assayed for radioactivity, as usual, and the fractions corresponding to the 3β-epimer (fractions 21-35) were pooled, evaporated to dryness under reduced pressure and recrystallized from methanol once. White needles were obtained (82.0 mg, m.p. 146-147°C). The product was dissolved in 10.0 ml of benzene and triplicate aliquots were taken for measurement of radioactivity (1.54 x 10^9 dpm; specific activity, 1.88 x 10^7 dpm per mg).

The product co-migrated with authentic lanost-8-en-3β-ol on two different TLC systems (silica gel G, in chloroform; silica gel G-4% silver nitrate, developed once in chloroform and a second time in benzene-diethyl ether-petroleum ether, 7:3:2), with an apparent radiopurity in excess of 99% (Figure III-3).

Radio-GLC of the product (3% OV-17; column temperature, 260°C; flow rate, 66 ml/min.) revealed a single, homogeneous peak of radio-
FIGURE III-3
Radio-TLC of chemically synthesized [2-$^3$H]-lanost-8-en-3β-ol. Chromatographic systems: (A) Silica Gel G, developed once with chloroform; (B) Silica Gel G-4% silver nitrate, developed once with chloroform and then once with benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards: (A) lanost-8-en-3β-ol; (B) lanosterol (open) and lanost-8-en-3β-ol (solid).

FIGURE III-4
Radio-GLC of chemically synthesized [2-$^3$H]-lanost-8-en-3β-ol. Chromatographic system: 3% OV-17, 260°C isothermal; carrier flow rate, 66 ml/min. The first mass peak is due to lanost-8-en-3β-ol; the second mass peak is due to lanosterol.
A

RADIOACTIVITY (DPM x 10^{-4})

B

MIGRATION (cm)
FIGURE III-5

Outline of the procedures used for the chemical synthesis of [2,4-$^3$H]-cholesta-8,14-dien-3β-ol (Ia.) and [2-$^3$H]-lanost-8-en-3β-ol (IIa.). 7-dehydrocholesterol (Ia.); cholesta-8-14-dien-3β-ol (Ib.); cholesta-8,14-dien-3-one (Ic.); lanosterol (IIa.); lanost-8-en-3β-ol (IIb.); lanost-8-en-3-one (IIc.).
\[
R_1 = \begin{array}{c}
\text{CH}_3 \\
\text{CH}_2 \\
\text{CH}_2 \\
\text{CH}_3
\end{array}
\]

\[
R_2 = \begin{array}{c}
\text{CH}_3 \\
\text{CH}_2 \\
\text{CH}_2 \\
\text{CH} = \text{CH}
\end{array}
\]

**Reactions:**
1. \(\text{HCl} / \Delta\)
2. Pyridinium Chlorochromate
3. \(\text{LiAlH}_4\)
4. \(\text{H}_2O / \text{NaOCH}_3\)

**Steps:**
- **Ia:** Hydrolysis
- **Ib:** Reduction
- **Ic:** Oxidation
- **Id:** Reduction
- **IIa:** Reduction
- **IIb:** Oxidation
- **IIc:** Reduction
- **IId:** Reduction
activity (Figure III-4) which exactly co-migrated with the co-injected standard of lanost-8-en-3β-ol (retention time, 29.45 min.). Recovery of applied radioactivity was approximately 86%.

The above data suggests that the desired sterol was obtained with a radiopurity in excess of 99%. A schematic outline of the procedures used for the synthesis of the tritiated sterols is given in Figure III-5.

**Synthesis of Methyl-3,7,11-trimethyldodecanoate**

Farnesolic acid (ca. 150 mg; K & K Laboratories) was esterified with excess freshly-generated diazomethane (three hours at room temperature), as previously described. The solvent was removed under a stream of nitrogen (forming an amber yellow oil), the residue was re-dissolved in 2 ml of hexane and purified by Unisil column chromatography (4 g, in hexane slurry; 21 x 1 cm column). Fractions (3 ml per 4 minutes) were eluted with hexane-diethyl ether, 95:5, and analyzed by TLC for mass (silica gel G; chloroform). The fractions which corresponded to an authentic standard of all-trans methyl farnesoate (fractions 5-10) were pooled, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and re-dissolved in about 4 ml of hexane.

Analysis of a portion of the sample by GLC (12% DEGS; column temperature, 135°C; carrier flow rate, 66 ml/min.) showed four major peaks having the following relative retention times (relative mole percent in parentheses): 0.638 (15.9%), 0.753 (24.5%), 0.912 (25.2%) and 1.000 (34.4%). The component which eluted last (retention time, 31.32 min.) was found to co-migrate with an authentic standard of all-trans
methyl farnesoate under the same chromatographic conditions. GLC/MS analysis of the sample revealed four components, each having an apparent molecular ion at m/e 250 (3% OV-1; column temperature, 130°C). The chromatographic and mass spectroscopic properties of the four components are consistent with the expected behavior and known structure of the four geometric isomers of methyl farnesoate. In analogy to the known chromatographic behavior of the isomers of farnesol (Bates et al., 1963), it is assumed that the fastest-eluting component is all-cis methyl farnesoate while the two components of intermediate migration represent the cis, trans- and trans, cis- isomers of methyl farnesoate (order of elution not known).

Approximately half of the methyl farnesoate sample was transferred to a 15 ml conical glass centrifuge tube, evaporated under nitrogen, redissolved in about 5 ml of ethanol-hexane, 2:1, and hydrogenated over about 6 mg of platinum oxide (30 minutes, at room temperature and pressure) as previously described. After removal of the catalyst by sedimentation, the sample was transferred to another vial, evaporated under nitrogen (forming a colorless oil) and redissolved in 5 ml of hexane.

Aliquots of the sample were compared before and after hydrogenation by thin-layer chromatography on two different systems (silica gel G and silica gel G-5% silver nitrate), each developed once with hexane-ether-glacial acetic acid, 94:4:2 (Figure III-6). On both TLC systems, a single band was observed for the hydrogenated sample, which migrated considerably ahead of the unhydrogenated material on the argentated plate.
FIGURE III-6

Thin-layer chromatograms of methyl farnesoate (1) and methyl-3,7,11-trimethyldodecanoate (2), chromatographed one plates of Silica Gel G (A) and Silica Gel G-5% silver nitrate (B). Each plate was developed once with hexane-diethyl ether-glacial acetic acid, 94:4:2, and charred after spraying with molybdic acid reagent. 0, origin; SF, solvent front.
Analysis of the hydrogenated sample by gas-liquid column chromatography (3% OV-17; column temperature, 130°C; carrier flow rate, 66 ml/min.) revealed a single symmetrical peak (> 98% of the observed mass). An aliquot of the hydrogenated sample was co-injected with authentic standards of methyl palmitate and methyl stearate (3% OV-17; column temperature, 160°C; carrier flow rate, 66 ml/min.) and the following relative retention times were recorded (relative to methyl stearate; retention time, 27.68 min.): 0.102 (methyl-3,7,11-trimethyl-dodecanoate), 0.379 (methyl palmitate) and 1.000 (methyl stearate). A plot of $\log_{10}$ of the retention time versus carbon number revealed that the hydrogenated isoprenoid methyl ester retention time was actually less than that calculated for a C$_{14}$ saturated n-fatty acid (calculated relative retention time, 0.147, relative to methyl stearate).

GLC/MS analysis of the hydrogenated sample (3% OV-1; column temperature, 130°C) revealed a single component with an apparent molecular ion at m/e 256, consistent with the known molecular weight of methyl-3, 7,11-trimethyl-dodecanoate.

A portion of the hydrogenated sample was evaporated under nitrogen and about 1.5 µl of the clear oil was applied between two potassium bromide discs. The infrared spectrum of the oil exhibited the following major bands: 1015 cm$^{-1}$ (medium), 1172 cm$^{-1}$ (strong), 1370 and 1385 cm$^{-1}$ (strong, doublet), 1440 and 1448 cm$^{-1}$ (strong, doublet), 1749 cm$^{-1}$ (strong) and 2936 cm$^{-1}$ (strong). The spectral properties were consistent with those expected for an aliphatic ester containing an isopropyl group (Bellamy, 1958). The above data are consistent
with the known structure of methyl-3,7,11-trimethylododecanoate (methyl perhydrofarnesoate).

**Synthesis of Dihydrophytol**

Phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol; ca. 0.85 g) was dissolved in 50 ml of ethyl acetate and hydrogenated in a Parr apparatus over 100 mg of 5% palladium on carbon (lot #022/96-2; International Chemical Corp.) as previously described (20 hours at 40 p.s.i. hydrogen pressure). Catalyst was removed by filtration (Whatman #2 paper) and the eluant was concentrated to about 25 ml under reduced pressure.

Portions of the hydrogenated sample and the starting material were compared chromatographically on two different TLC systems (silica gel G; silica gel G-5% silver nitrate), both developed once with benzene-ether-petroleum ether, 7:3:2. It was apparent that the starting material was only about 60% pure, containing minor less polar constituents (ca. 4%) as well as a very polar or polymerized component near the origin (ca. 36%). The hydrogenated material also showed a multiplicity of components, with the major band representing about 60% of the total mass and migrating ahead of the major phytol constituent on the argentated plate.

Analysis of the phytol starting material by GLC (3% OV-17; column temperature, 160°C; carrier flow rate, 66 ml/min.) revealed only a single peak (ca. 98% of the observed mass); thus, the other components observed by TLC must have been either larger and/or more polar than the major component, and did not elute from the column under the conditions specified. The hydrogenated sample showed one major component (ca. 95%; retention time, 17.12 min.) and one minor component (ca. 5%;
retention time, 3.56 min.) under the same chromatographic conditions. GLC/MS of the hydrogenated sample (3% OV-1; column temperature, 150°C) indicated that the largest ion fragment of the minor component was at m/e 282, whereas the major component exhibited its largest ion fragment at m/e 280. Co-injection of aliquots of the phytol sample and the hydrogenated sample on the 3% OV-17 column (under the conditions cited above) revealed three components having the following relative retention times (relative to the major component of phytol; retention time, 21.73 min.): 0.163 (minor hydrogenated component); 0.790 (major hydrogenated component); 1.000 (major phytol component).

It is likely that the minor hydrogenated species represented an allylic elimination product formed during hydrogenation (i.e. loss of water from phytol, MW 296, would yield a product of MW 282), whereas the major hydrogenated species (expected MW 298) represented dihydrophytol which became dehydrated during the mass spectral analysis.

It was decided to use the hydrogenated material at the given level of purity for the subsequent steps of the overall synthesis of methyl phytanate.

Synthesis of Methyl Phytanate (Methyl-3,7,11,15-tetramethylhexadecanoate)

The remainder of the hydrogenated phytol sample was dissolved in 50 ml of glacial acetic acid. Chromium trioxide (0.57 g) was dissolved in 5 ml of 90% acetic acid and added to the hydrogenated sample. The oxidation was allowed to continue at room temperature for 18 hours. Water (200 ml) was then added and the reaction mixture was further acidified by dropwise addition of concentrated HCl (to pH ~ 2). The mixture was extracted with petroleum ether (three times, 200 ml each) and the
combined extracts were washed with water (twice, 100 ml each), dried
over anhydrous magnesium sulfate, filtered and concentrated under
reduced pressure to about 100 ml final volume.

An aliquot of the sample was analyzed by TLC (silica gel G; de-
veloped once with hexane-ether-glacial acetic acid, 80:20:1) in compari-
son with an authentic standard of palmitic acid. Mass was visualized
by spraying the plate with 50% sulfuric acid (in 50% methanol) and
charring for 3 hours at 120°C. About 60-65% of the observed mass co-
migrated with the authentic fatty acid standard, while the remaining
35-40% was observed to migrate almost to the solvent front.

The sample was transferred to a 125 ml screw-top erlenmeyer flask
and the solvent was removed under a stream of nitrogen (forming a vis-
cous green oil). The residue was redissolved in 25 ml of diethyl
ether-methanol, 9:1, and esterified with 50 ml of a saturated solution
of diazomethane in diethyl ether-methanol, 9:1 (chilled on a dry ice-
acetone bath); the flask was lightly sealed and esterification was
allowed to proceed overnight at room temperature (ca. 9 hours).

The solvent was removed under reduced pressure and the residue was
redissolved in 100 ml of hexane. Aliquots were analyzed by TLC with
three different solvent systems (hexane; hexane-diethyl ether-glacial
acetic acid, 80:20:1; hexane-diethyl ether-glacial acetic acid, 94:4:2).
Although there was no starting material apparent in the sample, at
least two other products were observed in addition to the major compoent
(ca. 65%, assumed to be the desired methyl ester).

The sample was concentrated under reduced pressure to about 10 ml
and applied to a Unisil column (hexane slurry; 46 x 3 cm column);
fractions 1-49 were eluted with 2% diethyl ether in hexane (5.8 ml/min.), fractions 50-84 were eluted with 10% diethyl ether in hexane and fractions 85-130 were eluted with 50% diethyl ether in hexane. Fractions were assayed for mass by TLC (silica gel G; hexane-diethyl ether-acetic acid, 94:4:2) as usual. A very nonpolar component (assumed to be a hydrocarbon) eluted in fractions 15-20; no other components were observed between fractions 21 and 84. The remainder of the mass eluted with the hexane-ether, 1:1, solvent system without apparent resolution. Fractions 85-108 were pooled, concentrated under reduced pressure, transferred to a vial and concentrated further under a stream of nitrogen (forming a viscous pale yellow oil).

The sample was redissolved in 2 ml of hexane-ether, 98:2, and then applied to a silica gel medium pressure column (particle mesh, 0.032-0.063 mm; 100 x 1.5 cm column, equilibrated with hexane-ether, 98:2). Fractions were eluted with hexane-ether, 98:2 (flow rate, 4.8 ml/min.; fraction volume, 19.2 ml) and assayed for mass as before. Fractions 11-16, corresponding to the major product, were pooled, concentrated under reduced pressure, transferred to a tared vial, and the solvent was removed under a stream of nitrogen. The sample was dried in vacuo over phosphorous pentoxide for several hours and then weighed. The final yield was 502 mg of product; assuming only 60% purity of the initial phytol starting material, this represents essentially quantitative conversion and recovery of the desired product.

Analysis of the product by TLC with two different solvent systems showed only a single component (hexane-ether, 98:2, R_f = 0.253; hexane-ether-glacial acetic acid, 94:4:2, R_f = 0.445), even when grossly over-
GLC analysis of the product (3% OV-17; column temperature, 160°C; carrier flow rate, 66 ml/min.) showed one major product (ca. 96% of the observed mass) having a retention time, relative to methyl stearate, of 0.766 (methyl stearate retention time, 28.98 min.) (Figure III-7).

The infrared spectrum of the product exhibited the following major bands (cm$^{-1}$): 1014 (medium), 1171 (strong), 1370 and 1381 (strong, doublet), 1748 (strong) and 2935 (strong). The spectrum was consistent with that of a branched-chain aliphatic ester (Bellamy, 1958).

The NMR spectrum of the compound showed a complex methylene envelope centered at 1.2 ppm, a complex multiplet centered at 2.18 ppm (protons adjacent to the carbonyl carbon) and a strong singlet at 3.63 ppm (methyl protons adjacent to oxygen). No signals downfield from 3.63 ppm were detected, confirming that the compound was aliphatic.

GLC/MS analysis (3% OV-1; column temperature, 130°C) showed a single component having an apparent molecular ion at m/e 326 (60%), consistent with the known molecular weight of methyl phytanate. Other fragments were observed at m/e 101 (base peak; CH$_3$CHCH$_2$COOCH$^+$), 111 (82%; M-C$_{13}$H$_{27}$-CH$_3$OH), 139 (62%; M-C$_{11}$H$_{23}$-CH$_3$OH), 143 (79%; M-C$_{13}$H$_{27}$), 191 (91%; M-C$_{11}$H$_{23}$) and 311 (20%; M-CH$_3$). The methyl ester functionality was further confirmed by major rearrangement peaks at m/e 74 (87%; M-CH$_2$COOCH$_3$+H) and 102 (66%; CH$_3$CHCH$_2$COOH$_3$+H), while the terminal isopropyl group was confirmed by the presence of a peak at m/e 283 (14%).

Other fragments which are characteristic of the structure of methyl phytanate were observed at m/e 87 (28%), 157 (5%), 209 (19%), 213 (28%), 227 (2%), 241 (17%), 279 (6%) and 295 (9%).
FIGURE III-7
Gas-liquid chromatogram of chemically-synthesized methyl phytanate (methyl-3,7,11-15-tetramethylhexadecanoate) (A) co-injected with an authentic standard of methyl stearate (B). Chromatographic system: 3% OV-17, 160°C isothermal; carrier flow rate, 66 ml/min.

FIGURE III-8
Outline of the procedures used for the chemical synthesis of methyl phytanate (IV). Phytol (I.); dihydroyphytol (II.); phytanic acid (III.).
I. (MW 296)\[\text{Pd/C, H}_2\] \rightarrow II. (MW 298) \rightarrow \text{III. (MW 312)} \rightarrow \text{IV. (MW 326)}
The physical, chromatographic and spectral properties of the product are consistent with those published for methyl phytanate (Sonneveld et al., 1962; Lough, 1964; Hansen et al., 1965). A summary outline of the procedures used for the chemical synthesis of methyl phytanate is given in Figure III-8.
IV. STEROL COMPOSITION OF BOVINE RETINAS AND ROD

OUTER SEGMENT MEMBRANES

INTRODUCTION

The vertebrate rod cell has been the object of extensive investigations directed at elucidating the functional relationships between ultrastructure, biochemical composition and molecular architecture and their importance to the phototransduction process (Daemen, 1973; Ebrey and Honig, 1975; Montal and Korenbrok, 1976; Ostroy, 1977). Despite the acknowledged importance of sterols as structural components in cell membranes and their apparent role as modulators of physical properties which regulate the physiology of cells at the membrane level (Oldfield and Chapman, 1972; Nes, 1974; Papahadjopoulos, 1974; Demel and De Kruijff, 1976; Green, 1977), there have been no reports of systematic studies concerning the types, quantities and metabolism of sterols in either retinas or rod outer segments.

Previous determinations of the cholesterol content of bovine and frog retinas and/or rod outer segment membranes (see Chapter I.) relied on either thin-layer chromatography, colorimetric assay (variations of the Liebermann-Burchard method), gas-liquid column chromatography, or a combination of these methods. These methods of identification and quantitation of cholesterol are only valid if it has been demonstrated that appreciable amounts of sterols other than cholesterol are not present in the tissue. The chromatographic properties of most C_{27} monohydroxy sterols on conventional TLC plates and certain GLC supports are very similar. In addition, most sterols react with the standard colorimetric
reagents used for sterol determination; the intensity, color and kinetics of color development depend on the number and positional distribution of the double bonds in the sterol molecule (and the number and position of "extra" methyl groups, in the case of C_{28}-C_{30} sterols).

It is well known that cholesterol, although the major sterol in most mammalian tissues, is accompanied by "companion" sterols in many tissues, especially in neural tissues (Fieser, 1953, 1954; cf. Cook, 1958). The relative amounts of these companion sterols are a function of the type of tissue as well as the species and age of the animal. For instance, guinea pig intestine contains appreciable amounts of both 7-dehydrocholesterol and lathosterol (cholest-7-en-3β-ol) (Glover et al., 1952; Glover and Green, 1957). Lathosterol is a major sterol in rat skin (Miller and Baumann, 1954), a tissue having a common embryological origin with the retina. In the developing rat brain, desmosterol is the predominant sterol (Fish et al., 1962; Kritchevsky and Holmes, 1962; Paoletti et al., 1965; Smith et al., 1967); the functional and anatomical maturation of the brain is accompanied by a decline in the desmosterol content and a concomitant increase in the level of brain cholesterol (i.e. during myelination). Cholestanol is present in small amounts (i.e. 1-3 percent of fresh tissue weight) in many tissues (Fieser, 1953; Gould, 1958; Werbin et al., 1962); this sterol, however, does not react with the "sterol-specific" colorimetric reagents routinely used for sterol determination.

In view of the lack of information concerning the sterol composition of vertebrate retinas and ROS membranes as well as the known presence of "companion" sterols in other neural tissues, previous
determinations of the cholesterol content of retinas and ROS membranes are suspect. The present study was undertaken in order to elucidate the types and quantities of sterols present in bovine retinas and rod outer segment membranes.

Materials and Methods

Trizma base, ethylenediamine tetraacetate (EDTA; disodium salt), sodium dodecylsulfate (SDS), dithiothreitol (DTT), 2-mercaptoethanol, phenazine methosulfate (PMS), 2,6-dichlorophenolindophenol (DCIP), TPNH (tetrasodium salt, Type X), cytochrome c (Type VI, horse heart) and Ficoll (a nonionic, synthetic polymer of sucrose; approximate MW 400,000) were obtained from Sigma Chemical Co.

N,N'-Methylenedisacrylamide (BIS), N,N,N',N'-tetramethylmethacryl-
diamine (TEMED), both products of Eastman Organic Chemical Co., and acrylamide (Bio-Rad Laboratories) were used without further purification.

Sucrose (enzyme grade, "ultra-pure") was obtained from Schwarz-Mann Co.

Coomassie brilliant blue (R-250; Mann Research Laboratories, Inc.) and pyronin Y (Fischer Scientific Co.) were used as purchased.

Ammonyx LO (a 30% aqueous solution) was obtained as a gift from the Onyx Chemical Co. (Jersey City, N.J.). Tween-80 (polyethoxyethylene (20) sorbitan monooleate) and Tween-20 (polyethoxyethylene sorbitan monolaurate) were obtained from J. T. Baker Chemical Co. and Sigma Chemical Co., respectively.

11-cis Retinaldehyde was a generous gift of Hoffman-La Roche (Nutley, N.J.).
Organic solvents, inorganic salts and all other reagents were used as purchased from Mallinckrodt Chemical Works, unless otherwise stated. Petroleum ether (b.p. 35-60°C) was used without prior redistillation. The term "ether" (used alone) will refer to diethyl ether throughout the text.

Homogenization buffer was made by dissolving sucrose (170 g) in a buffer containing 0.01 M Tris-acetate, pH 7.4, 65 mM NaCl, 0.15 mM CaCl$_2$ • 2H$_2$O and 0.10 mM EDTA, adjusting the mixture to a final weight of 500.0 grams with buffer (final sucrose concentration, 34%, w:w).

Sucrose solutions used for density gradient centrifugation were prepared according to the following protocol:

<table>
<thead>
<tr>
<th>42% (w:w) SUCROSE (g)</th>
<th>BUFFER * (ml)</th>
<th>0.1 M MgCl$_2$ (ml)</th>
<th>FINAL WT. ** (g)</th>
<th>DENSITY (mg/ml)</th>
<th>SUCROSE (Wt. %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>136.8</td>
<td>0.2</td>
<td>0.2</td>
<td>222.0</td>
<td>1.11</td>
<td>26 - 27</td>
</tr>
<tr>
<td>162.8</td>
<td>0.2</td>
<td>0.2</td>
<td>226.0</td>
<td>1.13</td>
<td>30 - 31</td>
</tr>
<tr>
<td>186.0</td>
<td>0.2</td>
<td>0.2</td>
<td>230.0</td>
<td>1.15</td>
<td>34 - 35</td>
</tr>
<tr>
<td>214.0</td>
<td>0.2</td>
<td>0.2</td>
<td>234.0</td>
<td>1.17</td>
<td>38 - 39</td>
</tr>
</tbody>
</table>

* 1.0 M Tris-acetate, pH 7.4

**Adjusted to final weight with water

Sucrose densities were determined by measuring the refractive index of the solutions with a Bausch and Lomb refractometer.

A 1.0 M solution of neutralized hydroxylamine was prepared by dissolving hydroxylamine hydrochloride (694.9 mg) in 67 mM sodium potassium phosphate buffer, pH 7.0 (8.0 ml), adjusting the pH to 7.0 with 5 M sodium hydroxide, and then adjusting the final volume to 10.0 ml with
buffer.

3% Ammonyx LO buffer was made by mixing 10.0 ml of neutralized hydroxylamine solution (1.0 M, pH 7.0) with 10.0 ml of Ammonyx LO and adjusting the final volume to 100.0 ml with 67 mM sodium potassium phosphate buffer (pH 7.0).

Concentrated SDS buffer was prepared with the following components: 20% aqueous SDS (8.0 ml), 0.4 M DTT (4.0 ml), 2-mercaptoethanol (0.5 ml), sucrose (2.0 g), pyronin Y (0.004 mg) and 0.1 M Tris-acetate buffer, pH 8.0 (4.0 ml). The final volume was adjusted to 20.0 ml with water.

Procedures used for the saponification, extraction and acetylation of lipids, TLC, GLC, column chromatography, colorimetric assay of sterols and steryl acetates, and protein determination were as described previously (see Materials and General Methods).

Preparation of Rod Outer Segments (ROS)

Bovine rod outer segments (ROS) were prepared by a modification of the method of Papernagter and Dreyer (1974) as outlined in Figure IV-1. All procedures were carried out under dim red light at 0-4°C. Prior to use, all solutions were boiled (2 minutes), aerated with argon (15 minutes) and allowed to equilibrate to 4°C in a cold room. In addition to purging with argon, all buffers were supplemented with CaCl₂·2H₂O (0.15 mM) and EDTA (0.10 mM), to attenuate transition metal-catalyzed autoxidation of lipids (Farnsworth and Dratz, 1976).

Freshly-enucleated bovine eyes (Blue Ribbon Meat Packing Co.) were stored on ice in a light-tight container, about 2-4 hours prior to removal of retinas. An incision was made in the sclera along the circumference of the eye (just below the ora serrata) with a razor blade; the
cornea, lens and vitreous were extruded and the retina was exposed by
inverting the eyecup. The retina was peeled away from the retinal pig-
mented epithelium (RPE) and detached at the optic nerve with small surgi-
cal scissors. Retinas were deposited into homogenization buffer (one
ml per retina), chilled on ice, in a 500 ml screw-top erlenmeyer flask.

The following procedure was used routinely for groups of 100-200
retinas. The ROS were detached from retinas by vigorous manual shaking
for two minutes in homogenization buffer, and distributed into 50 ml
polyethylene centrifuge tubes. The homogenate was centrifuged for 10
minutes at 5000 rpm (Sorvall SS-34 rotor; Sorvall RC-2B centrifuge); the
supernatants were pooled, and the pellets were rehomogenized in fresh
homogenization buffer (20 ml per pellet), centrifuged again, and the
resulting supernatants were pooled with those from the initial centrifu-
gation. The combined supernatants were diluted three-fold (1:2) with a
buffer containing 0.01 M Tris-acetate, pH 7.4, 0.15 mM CaCl₂·2H₂O and
0.10 mM EDTA; the diluted supernatants were then distributed into 250 ml
centrifuge bottles and centrifuged for 20 minutes at 10,000 rpm (Sorvall
GSA rotor).

The pellets (containing crude ROS) were combined and homogenized in
50-60 ml of a 1.17 g/ml sucrose solution with a tight-fitting ground-
glass Duall tissue grinder (15 passes, by hand). The homogenate was dis-
tributed evenly into six 35 ml polycarbonate centrifuge tubes (10-12 ml
per tube); each sample was aspirated repeatedly through a #20 hypodermic
needle with a 10 ml disposable syringe (10 times each). Decreasingly
dense sucrose solutions were then individually layered into each tube
with another 10 ml syringe (#12 hypodermic needle), in the following
order: 1.15 g/ml sucrose (5 ml), 1.13 g/ml sucrose (10-11 ml), and 1.11 g/ml sucrose (6-8 ml). The discontinuous sucrose gradients were then centrifuged for one hour at 25,000 rpm (SW 27 rotor, L3-50 ultracentrifuge; Beckman Instruments, Inc.), and the reddish material which banded at the 1.11/1.13 g/ml interface (purified ROS) was collected by aspiration with a Pasteur pipet.

The ROS material was washed twice with 40 ml of 67 mM sodium potassium phosphate buffer, pH 7.0 (30 minutes at 18,000 rpm, Sorvall S-34 rotor) and then resuspended in a measured volume (20-50 ml) of either 0.25 M sucrose buffer (containing 0.01 M potassium phosphate buffer, pH 7.4, 0.15 mM CaCl₂·2H₂O and 0.10 mM EDTA) or 67 mM sodium potassium phosphate buffer. Aliquots were removed for electron microscopy, SDS polyacrylamide gel electrophoresis, spectroscopic assays, protein determination and enzymatic analyses. The remainder of the material was subjected to one of the following procedures: (1) storage under argon in a light-tight container (~20°C); (2) washing three times with distilled water (30 minutes at 25,000 rpm, Beckman SW 27 rotor) and lyophilizing (24-48 hours at 30-60 microns pressure); (3) further processing to obtain individual disc membranes.

Typically, the yield of ROS membranes was about one mg per retina (by dry weight).

**Preparation of ROS Disc Membrane Vesicles**

ROS disc membrane vesicles were isolated from purified bovine ROS by the method of Smith *et al.* (1975). All procedures were carried out at 0-4°C under dim red light or in total darkness. All solutions were purged with argon prior to use.
Purified ROS (suspended in buffer) were pelleted after diluting three-fold with water (30 minutes at 18,000 rpm, Sorvall SS-34 rotor). The pellet was resuspended in 40 ml of water and homogenized with a loose-fitting teflon-on-glass homogenizer (10 passes, by hand). The osmotically-shocked ROS membranes were sedimented again, resuspended in 30-34 ml of water and evenly distributed into two 35 ml polycarbonate centrifuge tubes. An equal volume of a 10% Ficoll solution was added to each tube, the mixture was stirred with a glass rod, and the membrane suspensions were incubated for 10-12 hours at 4°C in total darkness under argon atmosphere.

At the end of the incubation time, the suspensions were centrifuged (2 hours at 25,000 rpm, Beckman SW 27 rotor) and the material which floated to the air/Ficoll interface was collected with a spatula and pipet. This material was washed twice in 40 ml of 67 mM sodium potassium phosphate buffer, pH 7.0 (30 minutes at 20,000 rpm, Sorvall SS-34 rotor), resuspended in a measured volume of the same buffer (usually 20-50 ml) and aliquots were taken for electron microscopy, gel electrophoresis and various assays. The remaining material was then sedimented and washed three times with water (30 minutes at 25,000 rpm, Beckman SW 27 rotor) and lyophilized (24-48 hours at 30-60 microns pressure).

Preparation of Whole Retinas for Analysis

Retinas were dissected from freshly-enucleated bovine eyes (stored on ice, 2-4 hours) under normal room illumination. The retinas were rinsed gently in chilled 0.9% saline (to remove adhering RPE and vitreal debris), then rinsed and homogenized in water (10 ml per retina) and lyophilized (24-48 hours at 30-60 microns pressure).
Electron Microscopy

Aliquots of ROS and disc vesicle preparations were sedimented in polyethylene tubes (17 ml capacity, Sorvall SS-34 rotor; 30 minutes at 20,000 rpm) and resuspended in 10 ml of fixative (2% formaldehyde, 2% gluteraldehyde, in 0.087 M potassium phosphate buffer, pH 7.4). Samples were incubated at 4°C for 10-12 hours in total darkness; the material was then centrifuged again, washed twice with 15 ml portions of 0.087 M potassium phosphate buffer and post-fixed in 10 ml of 1% aqueous osmium tetroxide (one hour at 4°C, in total darkness). The samples were then collected by centrifugation as before, washed twice with 15 ml portions of water and dehydrated in a graded ethanol series (15 minutes in 15 ml of 50% ethanol, 15 minutes in 15 ml of 75% ethanol, 15 minutes in 15 ml of 95% ethanol, and three five-minute washes (15 ml each) in absolute ethanol) at 4°C.

The dehydrated samples were transferred to glass vials and incubated in propylene oxide (two 10 ml washes, 20 minutes each), followed by an overnight incubation (10-12 hours) in about 5 ml of a 1:1 (v:v) mixture of propylene oxide and Araldite 502 embedding medium, at room temperature (continuously mixed by gentle rotation). Portions of each sample were embedded in 100% Araldite 502, cured for 48 hours at 60°C, and silver-grey sections were collected with an OMU Reichert ultramicrotome.

Sections were mounted on copper grids (200 mesh), stained with ethanolic uranyl acetate, counter-stained with lead citrate, and viewed with an AEI 801 electron microscope (accelerating voltage, 80 KeV).

Spectral Assays and Regeneration of Rhodopsin with 11-cis Retinaldehyde

Aliquots of ROS and disc vesicle suspensions (0.20 ml, in 67 mM...
sodium potassium phosphate buffer, pH 7.0) were dispersed in 0.80 ml of
3% Ammonyx LO buffer, and the absorption spectra were recorded from 650-
250 nm with a Cary 118 recording spectrophotometer, in dim red light; a
blank containing 0.20 ml of buffer and 0.80 ml of 3% Ammonyx LO solution
was used as a reference. After recording the absorption spectra, the
samples were bleached for five minutes under normal room illumination
and the spectra were recorded again.

Rhodopsin content was quantitated by the method of Raubach et al.
(1974a); correction for bleached photopigment was made by in situ incu-
bation of membranes with 11-cis retinaldehyde, following the regenera-
tion of rhodopsin spectrophotometrically. Aliquots of ROS or disc
vesicle suspensions (0.20 ml, having O.D.₄₉₅ values of 2.5-4.0) were
distributed into three glass tubes (at 4°C, under dim red light). One
sample served as a control (no additions); a second sample was treated
with one μl of ethanolic 11-cis retinaldehyde solution (the "native +
11-cis" sample); the third sample was bleached for five minutes under
normal room illumination and then treated with one μl of 11-cis retinal-
dehyde solution under dim red light (the "bleached + 11-cis" sample).
The three samples were flushed with argon, sealed, vortexed for 5
seconds and then incubated in total darkness at 37°C for 1.25 hours.
The reaction was then terminated by immersing the tubes in an ice-water
bath, followed by vortexing for 5 seconds. Duplicate aliquots (0.10 ml
each) were each dissolved in 0.90 ml of 3% Ammonyx LO buffer and the
absorption spectra were scanned from 650-250 nm; the samples were
bleached in their cuvettes and the absorption spectra were re-scanned.
The spectral region below 400 nm was obscured in samples containing
exogenous retinaldehyde; therefore, the absorbance at 280 nm of the control sample was used for calculations. The spectral ratio $\frac{O.D._{280}}{\Delta O.D._{498}}$ was obtained for each sample, where $\Delta O.D._{498}$ represents the difference in absorbance at 498 nm before and after bleaching. Rhodopsin concentrations were determined by dividing the $O.D._{498}$ value of the "bleached + 11-cis" sample by an average molar extinction coefficient of 41,800 (Wald and Brown, 1953; Matthews et al., 1963; Shichi et al., 1969; Shichi, 1970; Daemen et al., 1972; Bridges, 1971; Rotmans et al., 1972). Quantitation of rhodopsin on a weight basis was calculated using an average value of 37,500 for the molecular weight of bovine rhodopsin (Cavanaugh and Wald, 1969; Daemen et al., 1972; Heitzmann, 1972; Papermaster and Dreyer, 1974; Kossi et al., 1977).

The ethanolic retinaldehyde solution was prepared by dissolving a few crystals of 11-cis retinaldehyde in 0.20 ml of absolute ethanol at room temperature under dim red light. One μl of the solution was added to a cuvette containing 1.0 ml of absolute ethanol and the absorption spectrum was scanned from 500-230 nm (versus an ethanol blank). The following spectral characteristics were observed: $O.D._{380} = 1.68$; $O.D._{250} = 1.34$. Assuming a molar extinction coefficient of 27,000 for 11-cis retinal in ethanol at 380 nm (Wald and Brown, 1953), the concentration of the stock solution of 11-cis retinaldehyde was approximately 62 mM.

**SDS-Polyacrylamide Gel Electrophoresis**

Membrane preparations were analyzed by polyacrylamide gel electrophoresis in the presence of SDS, per the method of Fairbanks et al. (1971). Aliquots of membrane suspensions were diluted in 1:1 (v:v) with
concentrated SDS buffer (see Materials and Methods), such that the final protein concentration was less than one mg/ml. The final concentration of the SDS buffer constituents was as follows: SDS, 8%; EDTA, 2 mM; DTT, 80 mM; 2-mercaptoethanol, 5%; sucrose, 10%; pyronin Y, 10 μg/ml; Tris-acetate, pH 8.0, 20 mM. Samples were solubilized at room temperature for two hours.

Aliquots of each sample (containing 10-40 μg of protein in 25-100 μl) were applied to 7.5% polyacrylamide gels (9 cm x 4 mm), containing 1% SDS; electrophoresis was carried out at 1 mA/gel for the first 15-20 minutes, then 4 mA/gel for the duration of the run (about 3-4 hours). At the end of the run, gels were removed from their tubes, stained with coomassie brilliant blue and then destained as described by Fairbanks et al. (1971). The destained gels were scanned at 550 nm with a Gilford Model 214 recording spectrophotometer, equipped with a linear transport attachment.

Molecular weight calibration was performed using the following standards: β-galactosidase (130 kilodaltons; Sigma), BSA (68 kilodaltons; Sigma), hen ovalbumin (43 kilodaltons; Boehringer-Mannheim), rabbit muscle aldolase (40 kilodaltons; Boehringer-Mannheim), pepsin (35 kilodaltons; Sigma), chymotrypsinogen A (25 kilodaltons; Boehringer-Mannheim), and horse heart cytochrome c (12.5 kilodaltons; Boehringer-Mannheim). Each standard, dissolved in 67 mM sodium potassium phosphate buffer, pH 7.0 (2 mg/ml), was diluted 1:1 with the concentrated SDS buffer and boiled for two minutes in a hot water bath; 10 μl of each detergent-solubilized standard was applied to a polyacrylamide gel and electrophoresed simultaneously with the membrane samples. Plots of the
relative mobility ($R_f$) versus log molecular weight were linear in the range of 12.5-68 kilodaltons; anomalous deviation from linearity was observed for $\beta$-galactosidase consistently, thus, calibration of molecular weights in the range above that of the BSA standard was considered unreliable.

Enzymatic Analyses of ROS and Disc Vesicle Preparations

Mitochondrial and microsomal "marker enzymes" were assayed in an attempt to quantitate the extent of subcellular contamination of the membrane preparations. Succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) and cytochrome oxidase (cytochrome c:o$_2$ oxidoreductase, EC1.9.3.1) were assayed as mitochondrial marker enzymes, while TPNH-cytochrome c reductase (TPNH:cytochrome c oxidoreductase, EC 1.6.2.3) was assayed as the microsomal enzyme marker.

Succinate dehydrogenase was assayed essentially as described by King (1967), with the modifications suggested by Singer et al. (1973). In a 3 ml capacity optical glass cuvette, the following assay cocktail was prepared (path length, one cm): 0.20 M potassium phosphate buffer, pH 7.4 (0.50 ml), 0.20 M potassium succinate (0.40 ml), 1% aqueous BSA (0.20 ml), 9 mM phenazine methosulfate (PMS, 0.40 ml) and 45 mM KCN (50 l). Aliquots of membrane suspensions were added to each cuvette (ca. 30-200 $\mu$g protein); the volume was adjusted to 1.93 ml with water and the samples were incubated at room temperature for five minutes in total darkness (to allow "substrate activation" of the enzyme; Singer et al., 1973). The cuvette was then placed in the spectrophotometer (Varian Techtron Model 635 recording spectrophotometer) and the reactions were initiated by rapid addition of 2,6-dichlorophenolindophenol (DCIP,
1.5 mM, 0.07 ml). The loss of absorbance at 600 nm was monitored as a function of time, corresponding to the reduction of DCIP (molar extinction coefficient, 20,500). Bovine heart submitochondrial particles (SMP's, 12.41 mg protein/ml in 0.25 M sucrose buffer; a gift of Dr. Gerald T. Babcock) were diluted ten-fold and 25 μl aliquots were used as the "enzymatic control" (0-1.0 O.D.₆₀₀ units, full scale; chart speed, 2 min./inch). ROS membranes (2.0 mg protein/ml, in 0.25 M sucrose buffer) were assayed undiluted (0.10 and 0.20 ml each; 0-1.00 O.D.₆₀₀ units, full scale; chart speed, 1 min./inch). Phosphate buffer was substituted for the membrane suspensions as the "nonenzymatic control".

Under the assay conditions employed, a rate of 0.001 ΔO.D.₆₀₀/min. could be detected. The nonenzymatic control (buffer) gave rates of 0.020-0.027 ΔO.D.₆₀₀/min.; the nonenzymatic rate was subtracted from the rates observed for the membrane suspensions and the activity was then calculated from the expression,

\[
\text{Activity} = \frac{(O.D.\text{corr}./\text{min.}) (F)}{(20.5)}
\]

where F is the dilution factor (taking into account aliquot volume, stock dilution and total assay volume) and 20.5 is the millimolar extinction coefficient. One unit of activity is defined as 1.0 μmole of substrate converted per minute per ml of reaction volume. The specific activity is then calculated by dividing the activity value by the protein concentration (mg/ml) of the undiluted membrane suspension stock.

Cytochrome oxidase activity was determined by the method of Smith and Conrad (1956). In a 3 ml capacity optical glass cuvette (one cm
light path), the following constituents were added: 3 mM cytochrome c (Type VI, 0.025 ml, freshly reduced with sodium dithionite) and an appropriate volume (i.e. 2.875-2.950 ml) of 40 mM potassium phosphate buffer, pH 6.65, containing 0.5% Tween-80. The absorbance at 550 nm was monitored for several minutes (0-0.5 O.D.\textsubscript{550} units, full scale; chart speed, 1 min./inch) to assess the autoxidation rate (ca. 0.002 ΔO.D.\textsubscript{550}/min., at room temperature). The enzymatic reaction was then initiated by the addition of an aliquot of membrane suspension (0.025 ml for SMP’s; 0.025 or 0.100 ml for ROS), bringing the final assay volume to 3.000 ml; the loss of absorbance at 550 nm was monitored as a function of time.

An aliquot of 45 mM KCN (0.050 ml) was substituted for membrane suspensions as a control to assess cyanide-insensitive oxidation of cytochrome c; it was found that the nonenzymatic reaction (autoxidation) was insensitive to cyanide (i.e. the Tween-80 detergent promotes autoxidation).

For the SMP samples, a first-order rate constant (k) could be calculated from the equation

\[ k = \frac{0.693}{t_{1/2}} \]

where \( t_{1/2} \) is the half-time for the reaction and \( k \) is the rate constant (in units of \( \text{min}^{-1} \)). Multiplying this value by the dilution factor and dividing by the protein concentration of the undiluted SMP preparation (mg/ml), one can calculate a specific activity value (in units of \( \text{min}^{-1} \text{mg protein}^{-1} \)). A value of 119 \( \text{min}^{-1} \text{mg}^{-1} \) was obtained for the SMP sample used as the enzymatic control. However, the oxidation rate catalyzed by aliquots of ROS suspension did not appear to be first-order; rather, the loss of absorbance at 550 nm appeared linear over several
minutes of assay time. Therefore, the activity of the ROS sample was derived from the slope of the chart tracing (ΔO.D. 550/min.), multiplying the rate by the dilution factor and dividing by the protein concentration of the undiluted membrane suspension, yielding a specific activity value in units of ΔO.D. 550 min. -1 mg -1. For comparison, an "apparent initial rate" was calculated for the SMP oxidation of cytochrome c by taking the slope of the chart tracing at the initial phase of the reaction, multiplying this rate by the dilution factor and dividing by the stock protein concentration. The initial rates were corrected for nonenzymatic oxidation as previously described. Under the conditions employed, a rate of 0.0005 ΔO.D. 550 /min. could be detected.

TPNH-cytochrome c reductase activity was assayed by a modification of the method of Masters et al. (1967). In a one ml capacity optical glass cuvette (one cm light path), 0.90 ml of potassium phosphate buffer, pH 7.4 (10 mM, containing 0.25 M sucrose and 0.04 mM oxidized cytochrome c) was adjusted to 1.00 ml with aliquots of membrane suspension and 45 mM KCN (i.e. 0.05 ml of membrane suspension and 0.05 ml of KCN, etc.). The enzymatic reaction was initiated by the addition of 0.01 ml of 10 mM TPNH (dissolved in 10 mM potassium phosphate buffer). The increase in absorbance at 550 nm was monitored as a function of time (0-0.5 O.D. 550 units, full scale). A partially-purified preparation of calf liver microsomal TPNH-cytochrome c reductase (flavoprotein complex II; a gift of Dr. Scott D. Power) was used as the "enzymatic control" (chart speed, 0.167 min/inch; 0.010 ml, 6.26 mg protein/ml). The ROS enzymatic activity was assayed at two different levels of aliquots (0.10 and 0.05 ml each; chart speed, 1 minute/inch). The nonenzymatic control rate was
obtained by monitoring the absorbance in the absence of added TPNH and also by substituting phosphate buffer for the membrane suspensions. One unit of activity is defined as a change of 1.0 O.D. 550 unit at 25°C; this corresponds to 0.0476 μmole of cytochrome c reduced per minute per ml of reaction mixture. Under the conditions employed, a rate of 0.0005 ΔO.D. 550 units per minute could be detected; the nonenzymatic control was found to have a rate of 0.013-0.015 ΔO.D. 550/min., while the enzymatic control exhibited a rate of 1.16 ΔO.D. 550/min. Porcine liver microsomes were reported to have an activity of 0.48 units/mg protein (i.e. 0.0229 μmole per min. per mg protein) (Masters et al., 1967). Assuming that bovine retina microsomes possess a similar enzymatic activity, I could detect a microsomal contamination representing ≥0.5% of the total protein of the ROS membrane suspension, i.e. using 0.20 mg of ROS protein, I could have detected an activity corresponding to 0.00012 μmole per min. per mg.

These enzymatic assays offer a level of sensitivity considerably greater than that afforded by the use of alternative methods for determining the purity and homogeneity of the ROS preparations, with regard to mitochondrial and microsomal contamination.

RESULTS

Preparation of Rod Outer Segments and Disc Vesicles

The procedures used to isolate rod outer segments from bovine retinas are outlined in Figure IV-1. Inspection of the discontinuous sucrose gradient after centrifugation revealed a reddish band of material at both the 1.11/1.13 g/ml interface and the 1.13/1.15 g/ml interface.
FIGURE IV-1

Protocol for the isolation of purified, relatively intact rod outer segments (ROS) and disk membrane vesicles. All procedures were carried out at 0-4°C under dim red light or complete darkness.
RETINAS

Homogenize (34% sucrose buffer)
Centrifuge (10 min. @5000 rpm)

PELLET

(repeat)

PELLET

SUPERNATANT

Dilute 3-fold
Centrifuge
(20 min. @10,000 rpm)

PELLET
(Crude ROS)

SUPERNATANT

Homogenize (1.17 g/ml sucrose)
Discontinuous Sucrose Gradient
Centrifugation (1 hr. @25,000 rpm)

Sucrose (Wt. %) | Density (g/ml)
--- | ---
26 - 27 | 1.11
30 - 31 | 1.13
34 - 35 | 1.15
38 - 39 | 1.17

Purified ROS
Contaminated ROS
Other Membranes & Debris
Heavy Subcellular Debris

Ficoll Floatation
(2 hr. @25,000 rpm)

Collect & Wash Purified ROS
Hypotonic Shock

Purified ROS Disk Vesicles

5% Ficoll

Membrane Pellet
Depending on the individual preparation, the upper band constituted between 30% and 60% of the total material banding at the two interfaces. The lower band of material could be harvested, rehomogenized and aspirated in 1.17 g/ml sucrose solution, and re-run on a second discontinuous density gradient to obtain another crop of material which banded at the 1.11/1.13 g/ml interface. Papernstal and Dreyer (1974) determined that the material which banded at the 1.11/1.13 g/ml interface represented purified, mostly intact rod outer segments, whereas the material from the 1.13/1.15 g/ml interface represented ROS contaminated with particulate debris (i.e. mitochondria, synaptosomes, etc.). Overloading the gradients (by using more than 200 retinas per preparation), agitation of the sucrose gradients (either mechanically or thermally), or deviating from the prescribed sucrose concentrations resulted in a diffuse distribution of membrane material throughout the gradient instead of distinct bands of material.

The 1.15/1.17 g/ml interface contained a band of off-white membranous material, most likely due to synaptosomes and microsomal and mitochondrial debris (Papernstal and Dreyer, 1974); a dark pellet was always observed at the bottom of each tube (pigment granules and other heavy subcellular debris).

After osmotic shock and floatation of the purified ROS on a cushion of 5% Ficoll, a thick carpet of reddish membranes formed at the air/Ficoll interface, representing at least 95% of the total membrane material in each tube. A slight amount of reddish material was observed also as a pellet at the bottom of each tube. It has been suggested by other investigators that the floated material represents intact indivi-
dual ROS disc membranes which survived the osmotic shock, whereas the pellet is composed of membranous material which is either lamellar and unsealed or vesicular and enclosing material more dense than the 5% Ficoll solution (Smith, et al., 1975).

Typically, 1.0-1.2 mg of lyophilized membranes were obtained per retina; the yield of disc vesicles, of course, was always slightly less than that obtained for isolated rod outer segments.

Assessment of Purity of ROS and Disc Vesicle Preparations

Several criteria for assessing the purity of rod outer segment preparations generally have been recognized by the vision research community. The criteria may be divided into five types of evidence; buoyant density (i.e. behavior on sucrose density gradients), morphology (general anatomy and ultrastructure), spectrophotometric properties, behavior on SDS-polyacrylamide gel electrophoresis, and enzymatic activity.

It has been known for some time that rod outer segment membranes will float in sucrose solutions having a concentration of 0.88-1.00 M (corresponding density, approximately 1.11-1.13 g/ml), even when exposed to centrifugation (Wald and Brown, 1951-1952; Collins, et al., 1952; Matthews et al., 1963; McConnell, 1965; Shichi et al., 1969; Ebrey, 1971; Borrgreven et al., 1970; Papermaster and Dreyer, 1974; Raubach et al., 1974a; Hemminki, 1974). More recently, it has been demonstrated that the individual, intact ROS disc membranes (free-floating, not enclosed by a plasma membrane) are buoyant in 5% Ficoll solution (Smith et al., 1975). Since the material collected from the discontinuous sucrose density gradient banded at the 1.11/1.13 g/ml interface, and the membranes subsequently processed on a cushion of 5%
Ficoll floated to the air/Ficoll interface, the buoyant density criterion was satisfied. This behavior implied that the membranes were not appreciably contaminated with other heavier subcellular debris.

The vertebrate rod outer segment isolated free from the retina should retain the general morphology and ultrastructure of ROS observed in the intact retina, i.e. an ordered stack of closely-opposed, disc-shaped membranes enclosed by a limiting plasma membrane, containing no other discernable subcellular organelles (Sjostrand, 1953; Moody and Robertson, 1960; De Robertis and Lasansky, 1961). The isolated discs, however, should appear like unilamellar membrane vesicles in suspension, without any apparent order or restriction by an enclosing membrane (Smith et al., 1975).

Electron micrographs of crude ROS preparations (obtained prior to discontinuous sucrose density centrifugation) exhibited apparently intact as well as fragmented ROS, with a substantial amount of attendant subcellular debris (mitochondria, pigment granules, vesiculated membranes, etc.), as shown in Figure IV-2A. After centrifugation on a discontinuous sucrose gradient, the material harvested from the 1.11/1.13 g/ml interface exhibited the characteristic morphology of intact ROS, with no apparent subcellular contamination (Figure IV-2B). After osmotic shock and floatation on a 5% Ficoll solution, only unilamellar vesicles were observed in electron micrographs of the material harvested from the air/Ficoll interface (Figure IV-3A); closer examination of this material revealed the appearance of swollen disc-shaped membranes having the characteristic "hair-pin loop" ultrastructure of ROS disc membranes (Figure IV-3B).
FIGURE IV-2
Electron micrographs of crude (A) and purified (B) rod outer segments (ROS). The material was fixed in potassium phosphate buffer (0.087 M, pH 7.4) containing 2% gluteraldehyde and 2% formaldehyde, followed by post-fixation in 1% aqueous OsO₄ and dehydration in a graded ethanol series. After embedding in Araldite, thin sections (silver-grey) were stained with lead citrate-uranyl acetate. Note the presence of numerous mitochondria (M), pigment granules (PG) and other subcellular debris in the crude ROS preparation. Magnification, 8,000 X.

FIGURE IV-3
Electron micrographs of the purified disk membranes prepared by hypotonic lysis and Ficoll floatation of purified ROS. A, low magnification (9,250 X); B, higher magnification (231,250 X). Note that even swollen disks retain the pinched "hair-pin loops" routinely observed in electron micrographs of intact ROS (arrows).
When dispersed in an appropriate detergent solution, ROS and disc vesicles isolated from dark-adapted retinas should exhibit the characteristic absorption spectrum of rhodopsin (Wald, 1968), i.e. absorbance maxima near 280 nm and 500 nm; the absorbance near 500 nm should be lost upon exposure to room illumination ("bleaching"), with a subsequent increase in the absorbance near 367 nm (in the presence of hydroxylamine), due to the formation of all-trans retinaldehydeoxime. In addition, the spectral ratio of absorbance maxima near 280 nm and 500 nm, respectively, \((0.\text{D}_{280}/\Delta 0.\text{D}_{500})\) for dark-adapted, detergent-solubilized ROS membranes should be within the range 2.0-2.5 (De Grijp et al., 1972; Papermaster and Dreyer, 1974; Hemminki, 1974; Raubach et al., 1974). The lower the value of the spectral ratio, the higher the purity is of the ROS membrane preparation.

The ROS membranes isolated from the discontinuous sucrose gradients in this experiment were dissolved in a 3% Ammonyx LO buffer (containing 67 mM sodium potassium phosphate buffer, pH 7.0, and neutralized 0.1 M hydroxylamine), and exhibited the characteristic absorption spectrum of rhodopsin (Figure IV-4). The spectral ratio of the "native" ROS preparations ranged from 2.77-3.14 (average, 3.01); the spectral ratios were somewhat high, due to the fact that the retinas used for the preparation of the ROS membranes were not obtained from dark-adapted animals and, therefore, a portion of the rhodopsin (ca. 30-40%) had been bleached. Upon regeneration of the ROS membranes with 11-cis retinaldehyde, however, the spectral ratios of the ROS preparations decreased markedly to 2.26-2.44 (average, 2.35). These values are comparable to those obtained for the more pure ROS preparations described in the literature.
FIGURE IV-4
Absorption spectrum of bovine ROS membranes solubilized in 67 mM phosphate buffer, pH 7.0, containing 3% Ammonyx-LO and 0.10 M hydroxylamine. Solid line (-----), "native" ROS; dashed line (----), "bleached" ROS.

FIGURE IV-5
Absorption spectrum of ROS disk vesicle membranes solubilized in 67 mM phosphate buffer, pH 7.0, containing 3% Ammonyx-LO and 0.1 M hydroxylamine. A, "bleached" membranes; B, "native" membranes; C, "native" membranes after incubation for 1.25 hours in the dark with 11-cis retinaldehyde; D, "bleached" membranes after incubation for 1.25 hours in the dark with 11-cis retinaldehyde. Rhodopsin was approximately 86% regenerable; the optimal O.D.280/O.D.500 ratio was 2.07.
TABLE IV-1

Spectral Ratios Obtained for Purified ROS
Membranes and Chromatographically Purified
Rhodopsin from Bovine Retinas

<table>
<thead>
<tr>
<th>REFERENCE</th>
<th>ROS MEMBRANES</th>
<th>RHODOPSIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mc Connell (1965)</td>
<td>6.3 - 7.7&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;g&lt;/sup&gt;</td>
<td>---</td>
</tr>
<tr>
<td>Heller (1968)</td>
<td>---</td>
<td>1.55 - 1.68&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>Shichi et al. (1969)</td>
<td>3.7&lt;sup&gt;b&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.75&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ebrey (1971)</td>
<td>3.0 - 3.5&lt;sup&gt;b&lt;/sup&gt;,&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.65 - 1.80&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>De Grip et al. (1972)</td>
<td>3.3 - 3.8&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>2.0 - 2.5&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
<td>---</td>
</tr>
<tr>
<td>Raubach et al. (1974a)</td>
<td>1.9 - 2.4&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;h&lt;/sup&gt;</td>
<td>---</td>
</tr>
<tr>
<td>Papermaster and Dreyer (1974)</td>
<td>2.2 - 2.4&lt;sup&gt;b&lt;/sup&gt;,&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 - 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hemminki (1974, 1975)</td>
<td>2.19 - 2.37&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;g&lt;/sup&gt;</td>
<td>---</td>
</tr>
<tr>
<td>This study</td>
<td>2.77 - 3.14&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;d&lt;/sup&gt;</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>2.26 - 2.44&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;d&lt;/sup&gt;</td>
<td>---</td>
</tr>
</tbody>
</table>

a. From fresh eyes, stored on ice in darkness.
b. From dark-adapted, frozen retinas (Hormel Company, Austin, Minn.)
c. After regeneration with ll-cis retinaldehyde.
d. In Ammonyx-L0.
e. In Cetyltrimethylammonium Bromide (CTAB).
f. In Emulphogene BC270.
g. In Deoxycholate.
h. Corrected for light scattering.
(see Table IV-1). In contrast, material obtained from the 1.13/1.15 g/ml interface yielded "native" spectral ratios in the range 4.61-5.48, indicating contamination of the ROS with extraneous proteins (i.e. from heavier subcellular debris, as expected from its density gradient behavior).

The disc vesicle preparations always exhibited lower spectral ratios than the corresponding ROS preparations; the regenerated ROS membranes yielded spectral ratios in the range 2.53-2.71 (average 2.62), whereas the disc membranes incubated with 11-cis retinaldehyde gave values of 2.04-2.09 (average, 2.07) (see Figure IV-5). For both the ROS and disc vesicle preparations, exposure to room illumination caused almost a complete loss of the absorbance near 500 nm.

Using an average extinction coefficient of 41,800 for the absorbance near 500 nm and an average apparent molecular weight of 37,500 for rhodopsin, the membrane preparations contained an average of 31 ± 3 mg of rhodopsin per 100 mg of lyophilized membranes.

It has been suggested that the spectral ratio of the absorbance minima near 250 nm to the maxima near 280 nm may provide an indication of the extent of lipid peroxidation in membrane preparations, where a value of 0.5 or less indicates essentially negligible peroxidation (Farnsworth and Dratz, 1976). It was noted that the ROS preparations exhibited 0.Δ.D.\textsubscript{250}/\Delta.D.\textsubscript{280} ratios of 0.53-0.57, whereas the disc vesicle material gave spectral ratios of 0.45-0.48. These data, coupled with the fact that the 0.Δ.D.\textsubscript{280}/\Delta.D.\textsubscript{500} ratios of disc vesicles were always lower than those of ROS preparations, seem to indicate that extraneous proteins (including bleached or "damaged" rhodopsin) and lipid peroxidation
products may be selectively removed from ROS by osmotic shock and floatation on a 5% Ficoll solution (i.e. the Ficoll floatation procedure results in a further purification of the ROS membranes).

The banding pattern of purified, washed ROS membranes after solubilization, electrophoresis on SDS-polyacrylamide gels and staining with appropriate protein-specific dyes, should be highly simplified, relative to that of other biological membranes. There should be only one major protein component (opsin), having an apparent molecular weight of 30,000-39,000 (depending on the type of gel system employed); other protein components should be minor, each not exceeding about one mole per cent of the major component (Papermaster and Dreyer, 1974). Several investigators have observed the appearance of other components on such gels, having apparent molecular weights which are integral multiples of the major component; these have been interpreted as aggregates of opsin and are resistant to even high concentrations of SDS and reducing agents (cf. Papermaster and Dreyer, 1974). In addition, a very large component (ca. 240,000 MW) has been observed routinely in bovine ROS preparations, and has been identified as an intrinsic membrane protein located in the marginal incisures of the outer segment (Papermaster and Dreyer, 1974; Krebs and Kuhn, 1977; Papermaster et al., 1976, 1978).

Analysis by SDS-polyacrylamide electrophoresis of ROS preparations (Fairbanks et al., 1971) revealed a very simple banding pattern (Figure IV-6), with the major component having an apparent molecular weight of about 34,000 (band I, presumed to be opsin). Invariably, higher molecular weight species were observed (bands II and III) which had apparent molecular weights consistent with those expected of dimers and trimers.
FIGURE IV-6

Densitometer scan of an SDS-polyacrylamide gel of SDS-solubilized ROS membranes (24 μg total protein). Electrophoresis and staining of the gel with Coomassie blue were carried out as described by Fairbanks et al. (1971). The major band (I) exhibits the electrophoretic properties of opsin; two of the minor bands exhibit the electrophoretic properties consistent with the dimer (II) and trimer (III) aggregates of opsin. In addition, a "high molecular weight component" (IV) is observed near the origin of the gel. D, pyronin-Y dye. The original gel is shown above the scan.

FIGURE IV-7

Densitometer scan of an SDS-polyacrylamide gel of SDS-solubilized ROS disk membrane vesicles (19 μg total protein). Electrophoresis and staining of the gel with Coomassie blue were carried out as described by Fairbanks et al. (1971). The major band has the characteristic electrophoretic mobility of opsin; no aggregates of opsin are apparent. The "high molecular weight component" is seen as a minor band near the origin of the gel. D, pyronin-Y dye. The original gel is shown above the scan.
of opsin. In addition, a very high molecular weight component was routinely observed to migrate near the origin of the gels (band IV, \( \sim 200,000 \text{ MW} \)). The peak area ratios of bands II and III relative to band I could be altered by lowering the total protein concentration (\( \lesssim \text{one mg/ml} \)), varying the SDS concentration, or increasing the temperature and duration of membrane solubilization; the peak area ratio of band IV relative to band I, however, was not changed by such procedures. These data suggest that bands II and III represent aggregates of the major component, whereas band IV is an intrinsic constituent of the ROS membranes. In no case were components observed which had molecular weights smaller than the 34,000 dalton species, suggesting that proteolysis was negligible during the preparation of the ROS membranes.

When solubilized disc vesicle preparations were electrophoresed on polyacrylamide gels in the presence of SDS, only the opsin monomer and the very high molecular weight component were observed (Figure IV-7). The apparent molecular weight of the opsin monomer varied from 30,000 to 35,000 daltons (brackets, Figure IV-8) for different membrane preparations. The variation in the observed apparent molecular weight of opsin, as well as the deviation from the commonly accepted molecular weight range for bovine opsin (36-40 kilodaltons), may be due to the fact that opsin is a glycoprotein and an intrinsic membrane protein, whereas the proteins used for molecular weight calibration were aqueous-soluble and (with the exception of ovalbumin) not glycoproteins. Using the polyacrylamide gel system of Fairbanks et al. (1971), other investigators have observed an apparent molecular weight of 30-35 kilodaltons for bovine opsin, also (Papernost and Dreyer, 1974).
FIGURE IV-8

Calibration plot of protein molecular weight standards used for SDS-polyacrylamide gel electrophoresis. The range of opsin's relative migration is bounded by brackets [] . The following soluble protein standards were employed: β-galactosidase (BG), bovine serum albumin (BSA), hen ovalbumin (OVN), rabbit muscle aldolase (ALD), pepsin (PEP), chymotrypsinogen A (CHY) and horse heart cytochrome c (C).
Since the outer segment of the vertebrate rod cell is physically separated from the rest of the cell, purified ROS membranes should be devoid of the enzymatic activity commonly associated with other subcellular organelles, such as mitochondria, endoplasmic reticulum and nuclei (Mc Connell, 1965; Hemminki, 1974; Zimmerman et al., 1976). It has been reported that all-trans retinol dehydrogenase (Lion et al., 1975) and a light-stimulated, ATP-dependent cyclic nucleotide phosphodiesterase (Miki et al., 1973) are endogenous constituents of ROS membranes and may be considered marker enzymes of that organelle; however, other enzymatic activities classically ascribed to plasma membranes (i.e. 5'-nucleotidase, Na\(^+\)/K\(^+\)-dependent ATPase, etc.) may not be useful as ROS marker enzymes, due to their widespread occurrence (cf. Zimmerman et al., 1975, 1976).

Enzymatic analyses were performed on ROS preparations (in duplicate, with at least two different protein concentrations per assay), assaying for the presence of mitochondrial and microsomal enzymes. The activities were compared with those of bovine heart submitochondrial particles (SMP's) and a crude microsomal TPNH-cytochrome c reductase preparation (FP-II). The results are shown in Table IV-2. After subtracting the nonenzymatic rates from the rates obtained for the ROS samples, no apparent activity was detected for either succinate dehydrogenase or TPNH-cytochrome c reductase activity. Assuming the given level of detectability for these two enzymatic activities under the conditions employed, the contamination of ROS membranes with the given mitochondrial or microsomal activities was less than 0.5%. Although the ROS suspensions exhibited an apparent rate for the oxidation of cytochrome c, the
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PROTEIN (µg/assay)</th>
<th>SUCCINATE DEHYDROGENASE $^2$ (µmole-min.$^{-1}$-mg$^1$)</th>
<th>CYTOCHROME OXIDASE $^3$ (ΔO.D. $^{550}_{550}$-min.$^{-1}$-mg$^{-1}$)</th>
<th>TPNH-CYTOCHROME C REDUCTASE $^4$ (µmole-min.$^{-1}$-mg$^{-1}$)</th>
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<tbody>
<tr>
<td>SMP$^5$</td>
<td>31</td>
<td>0.450</td>
<td>29.492</td>
<td>---</td>
</tr>
<tr>
<td>FP-II$^6$</td>
<td>63</td>
<td>---</td>
<td>---</td>
<td>0.891</td>
</tr>
<tr>
<td>ROS</td>
<td>50</td>
<td>---</td>
<td>0.120$^7$</td>
<td>---</td>
</tr>
<tr>
<td>100</td>
<td>(n.d.$^8$)</td>
<td>---</td>
<td>(n.d.)</td>
<td>(n.d.)</td>
</tr>
<tr>
<td>200</td>
<td>(n.d.)</td>
<td>0.138$^7$</td>
<td>(n.d.)</td>
<td></td>
</tr>
</tbody>
</table>

1. Average of duplicate assays, corrected for nonenzymatic activity.
2. Assayed by the method of King (1967), with modifications according to Singer et al. (1973).
3. Assayed by the method of Smith and Conrad (1956).
4. Assayed by modification of the method of Masters et al. (1967).
5. Bovine heart submitochondrial particles.
6. Crude TPNH-cytochrome c reductase from calf liver microsomes.
7. Cyanide-insensitive activity.
8. n.d. = below level of detectability (see text).
rate appeared linear (i.e. not first-order) and was also insensitive to potassium cyanide. These characteristics suggest that the oxidation of cytochrome c by ROS membranes was not due to mitochondrial cytochrome oxidase, but was due to some other endogenous oxidant in the ROS (perhaps acting in concert with the Tween-80 detergent). In any case, the ROS oxidation rate was less than 0.5% of the rate exhibited by the SMP suspension. This further supports the conclusion that mitochondrial contamination of the ROS membranes was less than 0.5%.

I have found that repeated freezing and thawing and/or repeated washing and resuspending of SMPs in buffer can decrease the activity of succinate dehydrogenase and cytochrome oxidase by as much as 50% of the untreated control values. Care was taken with the ROS, SMP and FP-II samples to freeze/thaw them only once prior to assay (i.e. several aliquots of each sample were stored individually at -20°C; the samples were then allowed to thaw slowly on ice). In addition, the samples were suspended in phosphate buffer containing 0.25 M sucrose (a cryoprotective agent and mild antioxidant) and Ca-EDTA (to stabilize the membranes and inhibit metal-catalized autoxidation of lipids). The activity of the SMP succinate dehydrogenase was found to be much more labile than that of the cytochrome oxidase.

It was observed that replacement of Tween-80 with Tween-20 in the cytochrome oxidase assay resulted in a marked reduction of the rate of autoxidation of cytochrome c. Unfortunately, this fact was not realized until after the assays were performed on the ROS membrane sample for which the complete analyses of sterol composition will be presented in this chapter.
ANALYSIS OF ROS DISC MEMBRANE NONSAPONIFIABLE LIPIDS

Preliminary Analyses

Disc membrane vesicles obtained from 125 fresh bovine retinas were resuspended in a measured volume of 67 mM sodium potassium phosphate buffer, pH 7.0, and aliquots were removed for protein determination. The remainder of the membrane suspension was centrifuged (30 minutes at 20,000 rpm, Sorvall SS-34 rotor) and the resulting pellet was washed three times with 45 ml portions of distilled water (30 minutes at 25,000 rpm, Beckman SW 27 rotor) and lyophilized (48 hours at 30-60 microns pressure, in a foil-wrapped flask). After weighing, the lyophilized membranes were saponified in 50 ml of 15% ethanolic potassium hydroxide and the nonsaponifiable lipids were extracted, washed, evaporated to dryness in a tared vial and weighed, as previously described (see Materials and General Methods). The residue was redissolved in benzene (10.0 ml) and duplicate aliquots (0.40 ml each) were withdrawn, evaporated to dryness under nitrogen in glass tubes and assayed colorimetrically for cholesterol, as previously described.

The colorimetric assay of the nonsaponifiable material revealed a sequence of color changes upon addition of the modified Liebermann-Burchard reagent which were not apparent for the purified cholesterol standard. Immediately after addition of the reagent, the samples turned dark blue, then faded to a pale green followed gradually by a persisting blue-green color, over a period of 30 minutes. In contrast, the authentic cholesterol standard only exhibited a gradual color development to
changes observed for the membrane extract. This behavior suggested that the nonsaponifiable extract contained other "colorimetrically active" components (of undetermined origin) in addition to cholesterol.

This preparation of membranes yielded 120 mg of lyophilized material from 125 retinas, of which 54.3 mg was due to protein (45.3% of the membrane dry weight). The apparent cholesterol content was approximately 2.1 mg, or about 1.7% of the membrane dry weight; the reliability of this value was questionable, considering the colorimetric behavior of the sample.

**Preliminary Chromatographic Analyses of the Nonsaponifiable Lipids**

Analysis of the nonsaponifiable lipids of the disc membrane preparation by TLC (silica gel G), using four different solvent systems, revealed that the major component (ca. 95% of the observed mass) comigrated with an authentic standard of cholesterol. At least two more polar components were observed, in addition to trace components at the origin and near the solvent front (Figure IV-9).

Chromatographic analysis of the extract by GLC on a 3% OV-17 column (column temperature, 250°C; flow rate, 66 ml/min.) showed a major component which co-migrated with the authentic cholesterol standard (Figure IV-10). Other minor components eluted prior to an internal standard of cholestane; these components of unknown identity did not exhibit the chromatographic behavior of sterols.

**Acetylation and Column Chromatography of Nonsaponifiable Lipids**

Approximately half of the nonsaponifiable lipid sample (ca. 1.04 mg of sterol) was acetylated with tritiated acetic anhydride, as described
FIGURE IV-9
Silica Gel G thin-layer chromatograms of the nonsaponifiable lipids from bovine ROS disk membrane vesicles (1) and an authentic standard of cholesterol (2). Solvent systems: A, benzene-diethyl ether, 9:1; B, chloroform; C, chloroform-ethyl acetate, 65:35; D, petroleum ether-diethyl ether, 9:1. 0, origin; SF, solvent front.

FIGURE IV-10
Gas-liquid chromatograms of the nonsaponifiable lipids from bovine ROS disk membrane vesicles (A), co-injected with authentic standards of either cholestane (B) or cholesterol (C). Chromatographic system: 3% OV-17, 250°C isothermal; carrier flow rate, 60 ml/min. Under these conditions, squalene co-chromatographs with cholestane.
previously (see Materials and General Methods). A sample of authentic cholesterol (7.9 mg) was acetylated simultaneously under identical conditions. The extent of acetylation was monitored by radio-TLC. After the usual work-up, authentic carrier cholesteryl acetate (11.2 mg) was added to the acetylated nonsaponifiable lipid sample. Both acetylated samples were evaporated to dryness under nitrogen and redissolved in 10.0 ml of benzene; aliquots of each (ca. 10 μl) were applied to silica gel G plates and chromatographed, along with authentic standards of cholesterol and cholesteryl acetate, with benzene-diethyl ether, 9:1. Plates were sectioned (one cm each) and assayed for radioactivity, as usual, and the chromatographic standards were visualized by charring after treatment with molybdic acid reagent.

The radiochromatogram of the acetylated disc vesicle nonsaponifiable extract (Figure IV-11) exhibited one major band of radioactivity (ca. 73% of the total activity recovered from the plate), which co-migrated with the cholesteryl acetate standard. The remaining activity was diffusely distributed over the plate. A similar radiochromatogram was obtained for the acetylated cholesterol standard, with about 76% of the total radioactivity co-migrating with the cholesteryl acetate standard.

The acetylated nonsaponifiable lipids sample (3.99 x 10^6 cpm; 7.83 x 10^6 dpm) and the acetylated cholesterol sample (2.01 x 10^7 cpm; 3.94 x 10^7 dpm) were applied to silicic acid-Super Gel chromatographic columns (100 x 1 cm), and eluted with hexane-benzene, 84:16. Fractions were collected and assayed for radioactivity and (colorimetrically) for cholesteryl acetate, as previously described. The elution profile for the nonsaponifiable lipid sample is shown in Figure IV-12. About 97.5%
FIGURE IV-11

FIGURE IV-12
Silicic acid-Super Cel column chromatography of the acetylated nonsaponifiable lipids from bovine ROS disk membrane vesicles. Solvent system: hexane-benzene, 84:16. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), cholesteryl acetate, determined colorimetrically.
of the recovered activity co-eluted with the carrier cholesteryl acetate; the major peak was preceded by two minor components (ca. 2-3% of the total radioactivity) of unknown identity. Overall recovery of radioactivity was approximately 83%. An almost identical radiochromatogram was obtained for the cholesteryl acetate sample (including the minor components of radioactivity which preceded the major peak).

Fractions corresponding to the major peak of radioactivity (99-200, representing the C27 monohydroxy steryl acetates) were pooled, evaporated to dryness under nitrogen and redissolved in 10.0 ml of benzene. The pooled fractions of the acetylated cholesterol standard were assayed, in triplicate, for radioactivity and colorimetrically for cholesteryl acetate. The specific activity of the acetylated cholesterol standard was determined to be $3.26 \times 10^6$ cpm/mg cholesteryl acetate ($6.52 \times 10^6$ dpm/mg).

The C27 steryl acetate sample obtained from the nonsaponifiable lipid extract was divided into two approximately equal portions; one sample was assayed for total radioactivity ($9.59 \times 10^5$ cpm; $1.94 \times 10^6$ dpm) and approximately 5 mg each of the following steryl acetate chromatographic standards were added: cholesteryl acetate, choleseta-8,14-dien-38-y1 acetate, and choleseta-5,7-dien-38-y1 acetate. The sample was evaporated to dryness under nitrogen and redissolved in about 0.5 ml of hexane-benzene, 7:3; the other half of the sample was stored (-20°C).

The sample containing the steryl acetate standards was applied to a silica gel G-Super Cel-silver nitrate column (50 x 1 cm, in hexane-benzene, 7:3) and eluted with hexane-benzene, 7:3 (net activity applied, $9.46 \times 10^5$ cpm; $1.91 \times 10^6$ dpm). Fractions were collected and assayed.
for radioactivity and carrier steryl acetate mass as previously specified. The radiochromatogram is shown in Figure IV-13. Approximately 98% of the recovered activity co-eluted with the monoene standard (cholesteryl acetate); a small amount of residual activity (less than 2%) trailed just after the major peak of radioactivity. No detectable radioactivity eluted with the diene steryl acetate standards; however, this particular column failed to resolve the diene standards for reasons yet unknown (therefore, only the elution of the 7-dehydrocholesterol acetate standard is shown). The recovery of activity from this column was about 97%.

The fractions corresponding to monoene steryl acetates (13-29) were pooled as before, transferred to a vial, evaporated under nitrogen to dryness and redissolved in 5.0 ml of benzene. The sample was assayed for radioactivity (8.25 x 10^5 cpm; 1.67 x 10^6 dpm) and the following carrier steryl acetate standards were added: cholest-8(14)-en-3β-yl acetate (6 mg) and cholest-7-en-3β-yl acetate (5 mg). The sample was evaporated to dryness under nitrogen and redissolved in about 0.5 ml of hexane-benzene, 9:1. The sample was chromatographed on an alumina-Super Cel-silver nitrate column (100 x 1 cm) and eluted with hexane-benzene, 9:1 (net applied activity, 8.18 x 10^5 cpm; 1.65 x 10^6 dpm). Fractions were collected and assayed as usual for radioactivity and carrier mass. The results are shown in Figure IV-14. Approximately 98.5% of the eluted radioactivity corresponded to cholesteryl acetate; the remainder of the activity eluted just prior to the cholest-8(14)en-3β-yl acetate standard and was resolved into three components. The most prominent of the three minor peaks exhibited the chromatographic behavior expected for cholest-
FIGURE IV-13

Silica Gel G-Super Cel-silver nitrate column chromatography of the acetylated C_{27} sterols (fractions 99-200) obtained by silicic acid-Super Cel column chromatography of the acetylated nonsaponifiable lipids from bovine ROS disk membrane vesicles (see Figure IV-12). Open circles (o — o), radioactivity; closed triangles (▲ — ▲), steryl acetates, determined colorimetrically. The first colorimetric peak is due to cholesteryl acetate; the second colorimetric peak is due to 3β-acetoxy-cholesta-5,7-diene. Solvent system: hexane-benzene, 70:30.

FIGURE IV-14

Alumina-Super Cel-silver nitrate column chromatography of the C_{27} monoeny steryl acetates (fractions 13-29) from the previous Silica Gel G-Super Cel-silver nitrate column (see Figure IV-13). Open circles (o — o), radioactivity; closed triangles (▲ — ▲), steryl acetates, determined colorimetrically. The first colorimetric peak is due to 3β-acetoxy-cholesta-8(14)-ene; the second colorimetric peak is due to 3β-acetoxy-cholesta-7-ene; the third colorimetric peak is due to 3β-acetoxy-cholesta-5-ene (cholesteryl acetate). Solvent system: hexane-benzene, 9:1.
anyl acetate (Schroepfer, et al., 1972). The recovery of radioactivity from this column was approximately 95%.

The fractions which co-eluted with the authentic cholesteryl acetate standard (180-386) were pooled and evaporated to dryness under nitrogen. Carrier cholesteryl acetate (50 mg) was added and the sample was repeatedly recrystallized from methanol (twice) and acetone-water (three times). The specific activity (dpm/mg cholesteryl acetate) was measured before and after each recrystallization (triplicate aliquots each for radioactivity and colorimetric assays). As a control, the sample of authentic cholesterol which had been acetylated with tritiated acetic anhydride and purified by silicic acid-Super Cel column chromatography was diluted with about 50 mg of carrier cholesteryl acetate and recrystallized simultaneously in the identical manner. The results are shown in Table IV-3.

The specific activity of the material pooled from fractions 180-386 of the alumina-Super Cel-silver nitrate column exhibited considerable variation upon successive recrystallizations, but no consistent trend toward either increased or decreased specific activity was observed. The authentic labelled cholesteryl acetate standard, however, showed less variability and co-crystallized to essentially constant specific activity. In view of the chromatographic properties of the acetylated sterol sample obtained from the disc membrane preparation, it is unlikely that the variation represents the presence of a component or components other than cholesteryl acetate; rather, the variability is most probably due to a lack of technical expertise in manipulation of the sample, in addition to normal experimental error.
TABLE IV-3

Co-Crystallization of Authentic Cholesteryl Acetate with Pooled Fractions 180-386 from the Alumina-Super Cel-Silver Nitrate Column and with an Authentic Standard of Tritiated Cholesteryl Acetate

<table>
<thead>
<tr>
<th>RECRYSTALLIZATION</th>
<th>FRACTIONS 180-386</th>
<th>STANDARD</th>
</tr>
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<tbody>
<tr>
<td>Initial</td>
<td>3.86 ± 0.35</td>
<td>60.6 ± 6.8</td>
</tr>
<tr>
<td>Once from Methanol</td>
<td>3.15 ± 0.62</td>
<td>63.5 ± 2.4</td>
</tr>
<tr>
<td>Twice from Methanol</td>
<td>3.26 ± 0.53</td>
<td>62.2 ± 4.3</td>
</tr>
<tr>
<td>Once from Acetone-Water</td>
<td>2.77 ± 0.23</td>
<td>67.5 ± 1.1</td>
</tr>
<tr>
<td>Twice from Acetone-Water</td>
<td>3.29 ± 0.21</td>
<td>58.6 ± 1.8</td>
</tr>
<tr>
<td>Three times from Acetone-Water</td>
<td>2.66 ± 0.24</td>
<td>57.0 ± 2.8</td>
</tr>
</tbody>
</table>

S.D. = Standard Deviation (n = 3)

The remaining half of the sample pooled from the silicic acid-Super Cel column (1.14 x 10^6 cpm; 3.63 x 10^6 dpm) was chromatographed on a silica gel G-Super Cel-Silver nitrate column, along with about 5 mg each of added Δ^5, Δ^8,14 and Δ^5,7 steryl acetate standards, exactly as described for the previous sample. The elution profile was essentially identical to that previously described; approximately 98.3% of the recovered radioactivity co-eluted with the cholesteryl acetate standard, while no detectable radioactivity eluted with the diene steryl acetate standards. Again, the dienes failed to resolve from one another on this column (the same chromatographic packing material was used as in the
FIGURE IV-15

Alumina-Super Cel-silver nitrate column chromatography of the C_{27} monoene steryl acetates (fractions 10-32) from a second Silica Gel G-Super Cel-silver nitrate column (not shown). Open circles (o --- o), radioactivity. The following steryl acetates were used as chromatographic standards: 3β-acetoxy-cholest-8(14)-ene (▲ — ▲), 3β-acetoxy-cholest-7-ene (■ — ■) and cholesteryl acetate (● — ●). Solvent system: hexane-benzene, 9:1.
previous analysis). Overall recovery of radioactivity was about 96%.

The material which co-migrated with the monoene steryl acetate standard (fractions 10-32; $8.75 \times 10^5$ cpm, $2.83 \times 10^6$ dpm) was chromatographed on an alumina-Super Cel-silver nitrate column, exactly as previously described, along with about 5 mg each of additional monoene steryl acetate standards (i.e. $\Delta^5,\Delta^8(14)$ and $\Delta^7$). In this case, a fresh batch of column packing material was prepared and used after 30 hours of lyophilization. The elution profile is shown in Figure IV-15.

Unlike the previous such elution profile, the steryl acetates were eluted much sooner (using a freshly prepared batch of the same solvent system of hexane-benzene, 9:1) with incomplete resolution of the $\Delta^8(14)$ and $\Delta^7$ standards. Approximately 98.6% of the recovered radioactivity co-eluted with the authentic cholesteryl acetate standard; the remaining 1.4% of the activity eluted as a single peak (as opposed to the three components previously observed) just under the $\Delta^8(14)$ steryl acetate peak (although the peak fractions of radioactivity and carrier mass were not identical). The recovery of applied radioactivity from this column was greater than 99%.

The fractions which co-migrated with the cholesteryl acetate standard (fractions 30-50) were pooled as usual, diluted with about 50 mg of $\Delta^5$ steryl acetate carrier mass and repeatedly recrystallized from methanol and acetone-water, as previously described. The results of the co-crystallization are given in Table IV-4. The pooled material was found to co-crystallize to constant specific activity with the authentic cholesteryl acetate carrier, within experimental error.
### TABLE IV-4

Co-Crystallization of Authentic Cholesteryl Acetate with Pooled Fractions 30-50 from the Second Alumina-Super Cel-Silver Nitrate Column

<table>
<thead>
<tr>
<th>RECRYSTALLIZATION</th>
<th>SPECIFIC ACTIVITY (dpm-mg(^{-1}) ± S.D.) (\times 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>3.56 ± 0.07</td>
</tr>
<tr>
<td>Once from Methanol</td>
<td>3.58 ± 0.33</td>
</tr>
<tr>
<td>Twice from Methanol</td>
<td>3.48 ± 0.13</td>
</tr>
<tr>
<td>Once from Acetone-Water</td>
<td>3.76 ± 0.32</td>
</tr>
<tr>
<td>Twice from Acetone-Water</td>
<td>3.43 ± 0.40</td>
</tr>
<tr>
<td>Three times from Acetone-Water</td>
<td>3.55 ± 0.45</td>
</tr>
</tbody>
</table>

S.D. = Standard Deviation (n = 3).

The cholesterol content of the original nonsaponifiable lipid extract may be calculated, taking into account the percentage of radioactivity which co-migrated with the authentic cholesteryl acetate standard, the overall recovery of radioactivity from each of the chromatographic columns and the specific activity value derived from the sample of authentic cholesterol acetylated with tritiated acetic anhydride. The results of both isotopic and colorimetric determinations of cholesterol content of the disc vesicle membrane preparation are compared in Table IV-5. Quantitation of cholesterol was comparable using both techniques; cholesterol represented at least 98% of the total sterol composition of the photoreceptor membranes, and approximately 1.76% of the membrane dry weight.
TABLE IV-5

Cholesterol Content of Bovine Photoreceptor Disc Membranes

<table>
<thead>
<tr>
<th>CHOLESTEROL (mg)</th>
<th>% DRY WEIGHT</th>
<th>% NON-PROTEIN RESIDUE</th>
<th>% TOTAL STEROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.04, 2.16¹</td>
<td>1.70, 1.80</td>
<td>3.11, 3.29</td>
<td>---</td>
</tr>
<tr>
<td>2.13²</td>
<td>1.77</td>
<td>3.24</td>
<td>&gt; 98</td>
</tr>
</tbody>
</table>

¹ Determined colorimetrically (duplicate assays).
² Determined isotopically.

Two additional preparations of disc vesicle membranes of comparable purity (according to the previously described procedures and criteria) were analyzed for sterol content (both colorimetrically and isotopically) in an analogous manner as described above. One of the lyophilized membrane preparations was extracted by a modification of the method of Folch et al. (1957) (as described in Materials and General Methods) prior to saponification and extraction of the nonsaponifiable lipids. The cumulative results obtained for all three membrane preparations are given in Table IV-6. The combined data indicate that the photoreceptor disc membranes of bovine retinas are composed of nearly equal amounts of protein and lipid (on a weight per cent basis); approximately 90% of the non-protein residue was composed of material which was soluble in chloroform-methanol, 2:1 (most likely lipids). Cholesterol represents about 1.67% of the membrane dry weight, or about 3.24% of the non-protein residue dry weight. Using the known molecular weight of cholesterol (386), an average extinction coefficient of 41,800 and an average molecular weight
TABLE IV-6
Composition of Bovine Photoreceptor Membranes

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>% PROTEIN</th>
<th>% NON-PROTEIN(^2) RESIDUE</th>
<th>% DRY WEIGHT</th>
<th>% NON-PROTEIN RESIDUE</th>
<th>CHOLESTEROL:RHODOPSIN (^3) MOLE RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>45.3</td>
<td>54.7</td>
<td>1.77 (1.75)</td>
<td>3.24 (3.20)</td>
<td>5.37 (5.28)</td>
</tr>
<tr>
<td>B</td>
<td>47.4</td>
<td>52.6</td>
<td>1.87 (1.55)</td>
<td>3.56 (2.95)</td>
<td>4.43 (3.68)</td>
</tr>
<tr>
<td>C</td>
<td>51.9</td>
<td>48.1(^2)</td>
<td>1.47 (1.65)</td>
<td>3.06 (3.43)</td>
<td>4.46 (5.00)</td>
</tr>
<tr>
<td>AVE. ± S.D.</td>
<td>48.2 ± 3.4</td>
<td>51.8 ± 3.4</td>
<td>1.68 ± 0.15</td>
<td>3.24 ± 0.23</td>
<td>4.70 ± 0.64</td>
</tr>
</tbody>
</table>

1 Values obtained by isotopic analysis, with the average of colorimetrically determined values in parentheses.

2 Chloroform-methanol soluble material, extracted by a modification of the method of Folch et al. (1957).

3 Rhodopsin determined spectrophotometrically, using an average extinction coefficient of 41,800 (498 nm) and an average molecular weight of 37,500 daltons.

S.D., standard deviation.
weight of 37,500 for bovine rhodopsin, the mole ratio of cholesterol: 
rhodopsin in the disc membranes was calculated to be 4.70 (i.e. approxi-
mately five moles of cholesterol per mole of rhodopsin).

**ANALYSIS OF BOVINE RETINA NONSAPONIFIABLE LIPIDS**

**Preliminary Analyses**

Two groups of bovine retinas (A and B, 10 each) were rinsed, lyophi-
lized, weighed, saponified, and the nonsaponifiable lipids were extracted 
as previously described (see Materials and General Methods). The extracts 
were washed, dried and filtered, evaporated to dryness under nitrogen in 
tared vials and the weighed residues were redissolved in benzene (10.0 
ml each). Duplicate aliquots (0.10 and 0.20 ml each) were assayed colori-
metrically for cholesterol. Unlike the ROS membrane samples, however, no 
"fast color reactions" were observed during the course of the color 
development. These analyses gave the following data: retina sample A, 
579.45 mg dry weight, 2.05% cholesterol (by weight); retina sample B, 
636.85 mg dry weight, 2.03% cholesterol (by weight). For reasons previ-
ously discussed, the values obtained for cholesterol content are only 
tentative and must be confirmed by more rigorous methodology.

Thin-layer chromatographic analysis of the retina nonsaponifiable 
material (Figure IV-16) revealed essentially the same pattern as observed 
previously for the photoreceptor membrane lipids. Greater than 96% of 
the observed mass co-migrated with an authentic standard of cholesterol 
on three different solvent systems. At least two minor components were 
apparently more polar than cholesterol, while a trace component migrated 
neat the solvent front.
FIGURE IV-16
Silica Gel G thin-layer chromatograms of the nonsaponifiable lipids obtained from whole bovine retinas (1) and an authentic standard of cholesterol (2). Solvent systems: A, benzene; B, chloroform; C, chloroform-ethyl acetate, 65:35. O, origin; SF, solvent front.

FIGURE IV-17
Gas-liquid chromatogram of the nonsaponifiable lipids obtained from whole bovine retinas before (A) and after (B) co-injection with an authentic standard of cholesterol. Chromatographic system: 3% OV-17, 250°C isothermal; carrier flow rate, 60 ml/min.
Analysis of the nonsaponifiable lipid extract confirmed the TLC results (Figure IV-17). Using a 3% OV-17 column (column temperature, 250°C; carrier flow rate, 60 ml/min.), the major peak of the lipid extract co-migrated with an authentic standard of cholesterol upon co-injection of the two samples. Other components were observed also, trailing from the solvent front; these components did not exhibit the behavior expected of sterols.

The remainder of one of the retina extracts (sample A) was evaporated to dryness under nitrogen and desiccated under vacuum over phosphorous pentoxide. The other retina sample was purged with nitrogen and stored (-20°C).

Acetylation and Column Chromatography of Retina Nonsaponifiable Lipids

The desiccated nonsaponifiable residue was acetylated, as previously described, with tritiated acetic anhydride. An authentic sample of cholesterol (ca. 10 mg) was also acetylated in an identical manner. Both samples were worked up in the usual fashion and the acetylated samples were redissolved in 10.0 ml of benzene; both samples were assayed for radioactivity, as usual.

Duplicate aliquots (ca. 10 µl each) of each sample were applied to silica gel G plates, along with standards of cholesterol and cholesteryl acetate; plates were developed in benzene-diethyl ether, 9:1. One lane of each plate was divided into one-cm sections and scraped into vials for scintillation counting; the other sample lane and the standard lane were sprayed with molybdic acid reagent and charred to visualize mass.

The radiochromatogram of the acetylated retina lipids is shown in Figure IV-18. About 71% of the total radioactivity co-migrated with the
FIGURE IV-18

FIGURE IV-19
Silicic acid-Super Cel column chromatography of the acetylated nonsaponifiable lipids from whole bovine retinas. Open circles (○ — ○), radioactivity; closed triangles (▲ — ▲), cholesteryl acetate, determined colorimetrically. Solvent system: hexane-benzene, 84:16.
cholesteryl acetate standard; the remainder of the radioactivity was distributed among more polar components. Essentially all of the visible mass also co-migrated with the cholesteryl acetate standard; no mass was observed in the region of the cholesterol standard. An almost identical radiochromatogram was observed for the authentic acetylated cholesterol sample. Since essentially the same profiles were obtained for both the retina lipids and the purified authentic cholesterol sample, it is likely that the more polar products observed upon TLC analysis of the acetylated samples were artifacts of the tritiated acetic anhydride reaction (i.e. labelled acetic acid and other undetermined by-products of the reaction). This conclusion is supported by the fact that, whereas considerable mass was visibly coincident with cholesteryl acetate standard on both plates, there was no mass apparent in the region of the more polar labelled products.

About 2 ml of the retina lipid sample were withdrawn and diluted with approximately 10 mg of carrier cholesteryl acetate; the remainder of the sample was stored (-20°C). The acetylated retina lipid sample and the acetylated cholesterol sample were evaporated to dryness under nitrogen and redissolved in about 0.5 ml of hexane-benzene, 84:16.

Both samples were applied to silicic acid-Super Cel columns (100 x 1 cm) and eluted with hexane-benzene, 84:16 (net applied activity: retina acetylated lipids, 1.11 x 10^7 cpm, 2.23 x 10^7 dpm; acetylated cholesterol, 5.78 x 10^7 cpm, 1.86 x 10^8 dpm). Fractions were collected and assayed as usual for radioactivity and carrier mass. The elution profile of the retina sample is shown in Figure IV-19. About 99% of the total recovered radioactivity co-eluted with authentic cholesteryl
FIGURE IV-20

Silica Gel G-Super Cel-silver nitrate column chromatography of the C27 steryl acetates (fractions 49-130) obtained by silicic acid-Super Cel column chromatography of the acetylated nonsaponifiable lipids from whole bovine retinas (see Figure IV-19). Open circles (o — o), radioactivity; closed triangles (▲ — ▲), steryl acetates, determined colorimetrically. The first colorimetric peak is due to cholesteryl acetate; the second colorimetric peak is due to 3β-acetoxy-cholesta-8,14-diene; the third colorimetric peak is due to 3β-acetoxy-5,7-diene. Solvent system: hexane-benzene, 7:3.
acetate. The overall recovery of radioactivity from the column was about 84%. An almost identical elution profile was obtained for the acetylated cholesterol sample.

The fractions which corresponded to the cholesteryl acetate standard (54-150, for the acetylated cholesterol sample) and other possible C₂₇ steryl acetates (49-130, for the retina acetylated lipids) were pooled, concentrated under reduced pressure, dried under nitrogen and redissolved in 10.0 ml of benzene. Aliquots of the acetylated cholesterol sample were assayed for radioactivity and for cholesteryl acetate (colorimetrically); the specific activity was determined to be 8.61 x 10⁶ dpm per mg of cholesteryl acetate. Aliquots of the retina sample were assayed for radioactivity (6.36 x 10⁶ cpm); about half of the sample was stored (−20°C), while the remaining half was diluted with about 5 mg each of carrier cholesteryl acetate, cholesta-8,14-dien-38-yl acetate and cholesta-5,7-dien-38-yl acetate. The sample was then evaporated to dryness under nitrogen and redissolved in 0.5 ml of hexane-benzene, 7:3.

The retina C₂₇ steryl acetate sample was chromatographed on a silica gel G-Super Cel-silver nitrate column (50 x 1 cm), eluting with hexane-benzene, 7:3, and fractions were collected and assayed, as usual, for radioactivity and carrier mass. The radiochromatogram is shown in Figure IV-20. A single peak of radioactivity (fractions 10-28) co-eluted with the carrier cholesteryl acetate, representing about 99% of the recovered activity; the residual activity trailed just after the monoene steryl acetate peak. Although the diene standards were only partially resolved, no radioactivity was detected in the diene region of the chromatogram. The recovery of applied radioactivity was approximately
FIGURE IV-21
Alumina-Super Cel-silver nitrate column chromatography of the C_{27} monoene steryl acetates (fractions 10-28) from the previous Silica Gel G-Super Cel-silver nitrate column (see Figure IV-20). Open circles (--- o), radioactivity; closed triangles (▲ --- ▲), steryl acetates, determined colorimetrically. The first colorimetric peak is due to 3β-acetoxy-cholest-8(14)-ene; the second colorimetric peak is due to 3β-acetoxy-cholest-7-ene; the third colorimetric peak is due to cholesteryl acetate. Solvent system: hexane-benzene, 9:1.

FIGURE VI-22
Re-assay of fractions of 1-70 from the previous alumina-Super Cel-silver nitrate column (see Figure IV-21). Open circles (--- o), radioactivity; closed triangles (▲ --- ▲), steryl acetates, determined colorimetrically. The first colorimetric peak is due to 3β-acetoxy-cholest-8(14)-ene; the second colorimetric peak is due to 3β-acetoxy-cholest-7-ene.
The fractions corresponding to the monoene steryl acetate peak were pooled, diluted with approximately 5 mg each of cholest-8(14)-en-3β-yl acetate and cholest-7-en-3β-yl acetate, evaporated to dryness under nitrogen and redissolved in 0.5 ml of hexane-benzene, 9:1. The sample (6.51 x 10^6 dpm) was applied to an alumina-Super Cel-silver nitrate column (100 x 1 cm) and eluted with hexane-benzene, 9:1. Fractions were collected and assayed as usual for radioactivity and carrier mass. The elution profile (Figure IV-21) showed that essentially all of the radioactivity (ca. 99.8%) co-migrated with the authentic cholesteryl acetate standard (fractions 90-180). Upon assaying five-fold larger aliquots of fractions 1-70 for radioactivity (Figure IV-22), minor radioactive peaks were observed which had the chromatographic behavior of cholestanyl acetate (fractions 26-31; ca. 0.08% of the radioactivity) and cholest-7-en-3β-yl acetate (fractions 50-68; ca. 0.12% of the radioactivity). The overall recovery of applied radioactivity was about 92%.

The fractions which co-migrated with cholesteryl acetate were pooled, diluted with about 55 mg of carrier cholesteryl acetate, evaporated to dryness and recrystallized repeatedly from both methanol and acetone-water, as previously described. The data for the recrystallizations are shown in Table IV-7. As observed previously, considerable variation was apparent in the specific activity after successive recrystallizations.

The cholesterol content of the original retina nonsaponifiable extract was calculated, taking into account the initial total radioactivity of the sample, the recoveries from the individual chromatographic columns, and the specific activity of the acetylated cholesterol standard.
# TABLE IV-7
Co-Crystallization of Authentic Cholesteryl Acetate with Pooled Fractions 90-180 from the Alumina-Super Cel-Silver Nitrate Column

<table>
<thead>
<tr>
<th>RECRYSTALLIZATION</th>
<th>SPECIFIC ACTIVITY (dpm-mg⁻¹ ± S.D.) x 10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>9.63 ± 1.96</td>
</tr>
<tr>
<td>Once from methanol</td>
<td>9.42 ± 1.21</td>
</tr>
<tr>
<td>Twice from methanol</td>
<td>9.44 ± 0.35</td>
</tr>
<tr>
<td>Once from acetone-water</td>
<td>9.99 ± 1.81</td>
</tr>
<tr>
<td>Twice from acetone-water</td>
<td>10.42 ± 1.72</td>
</tr>
<tr>
<td>Three times from acetone-water</td>
<td>8.96 ± 0.44</td>
</tr>
</tbody>
</table>

S.D. = Standard deviation (n = 3)

The results obtained for this retina sample, obtained by both the isotopic and colorimetric methods, are compared in Table IV-8 below.

# TABLE IV-8
Cholesterol Content of Whole Bovine Retinas

<table>
<thead>
<tr>
<th>CHOLESTEROL (mg)</th>
<th>% DRY WEIGHT</th>
<th>% TOTAL STEROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.68, 12.00⁻ᵃ</td>
<td>2.02, 2.07</td>
<td>---</td>
</tr>
<tr>
<td>13.60ᵇ</td>
<td>2.36</td>
<td>&gt; 99</td>
</tr>
</tbody>
</table>

*a* Determined colorimetrically (duplicate assays)

*b* Determined isotopically.

The values obtained by isotopic analyses and colorimetric methods were in reasonable agreement. In this sample of bovine retinas, cholesterol represented about 2% of the total dry weight of the retinas.
and accounted for at least 99% of the total monohydroxysterols of this tissue.

In a separate experiment, three groups of bovine retinas (7 per group) were lyophilized, weighed, and extracted with chloroform-methanol, 2:1, by a modification of the method of Folch et al. (1957), as previously described (see Materials and General Methods). The resulting "total lipid" extracts were evaporated to dryness under nitrogen in tared vials, weighed, and then saponified with 15% potassium hydroxide (50 ml per sample, four hours at reflux). The nonsaponifiable lipids were extracted and washed as usual, and the resulting extracts were evaporated to dryness under nitrogen (after being concentrated under reduced pressure) and redissolved in 10.0 ml of benzene. Aliquots (in duplicate, 0.20 and 0.30 ml each) were assayed colorimetrically for cholesterol. The results of the analyses are displayed in Table IV-9, along with the results obtained previously for the retina lipid composition (samples A and B).

The value obtained colorimetrically for the average cholesterol content of the three groups of retinas (samples C, D and E) was about 23% lower than that previously obtained for the original retina samples (A and B). A likely explanation for this discrepancy is that the extraction of the lyophilized membranes with chloroform-methanol, 2:1, was incomplete and therefore resulted in lower values for apparent cholesterol content. Also, there would be losses inherent in the extraction procedure and work-up (i.e., transferring the extract several times during the procedure, washing the extract, etc.). Therefore, the average values derived for the total lipid content (17.33 ± 1.04%, by weight) and
### TABLE IV-9

Summary: Lipid Composition of Bovine Retinas

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>DRY WT. (mg)</th>
<th>TOTAL LIPIDS</th>
<th>CHOLESTEROL $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT. (mg) % DRY WT.</td>
<td>% DRY WT. % TOTAL LIPID</td>
</tr>
<tr>
<td>A</td>
<td>579.45</td>
<td>---</td>
<td>2.05</td>
</tr>
<tr>
<td>B</td>
<td>636.85</td>
<td>---</td>
<td>2.03</td>
</tr>
<tr>
<td>C</td>
<td>420.10</td>
<td>67.77 16.13</td>
<td>1.36 8.43</td>
</tr>
<tr>
<td>D</td>
<td>354.50</td>
<td>63.44 17.90</td>
<td>1.64 9.15</td>
</tr>
<tr>
<td>E</td>
<td>439.20</td>
<td>78.90 17.96</td>
<td>1.71 9.51</td>
</tr>
<tr>
<td>AVE ± S.D.</td>
<td>---</td>
<td>17.33 ± 1.04 1.76 ± 0.29</td>
<td>9.03 ± 0.55</td>
</tr>
</tbody>
</table>

$^1$ Determined colorimetrically by the method of Abell et al. (1952); isotopically determined value in parentheses.

Cholesterol content (1.76 ± 0.29% by weight) of bovine retinas represent minimum values. Other investigators (Lo Cascio, 1923; Krause, 1938; Brante, 1949) have reported values of 25.7%, 19.7% and 22.9%, respectively, for the total lipid content of bovine retinas, on a dry weight basis. These same investigators, however, reported retina cholesterol values of 1.6%, 1.99% and 1.7% respectively, on a dry weight basis.

**DISCUSSION**

The widespread occurrence of sterols in living organisms suggests their importance as biological constituents. Cholesterol is a component of all eukaryotic cells and is the major sterol in most mammalian tissues (Cook, 1958). Although the exact role of this ubiquitous compound is not
fully understood, a variety of experimental results obtained with both artificial and biological membranes suggest that cholesterol may act to buffer the "fluidity" of membranes at temperatures above and below the transition (i.e. melting) point of the membrane lipids (cf. Demel and DeKruyff, 1976; Green, 1977). The requirement for a fluid membrane appears to be a general principle in many cell types, and the regulation of membrane fluidity appears to be a major locus of control in the dynamic behavior of membrane components and the expression of cellular functions (Gitler, 1972; Singer, 1974; Edidin, 1974; Cherry, 1976). In particular, cholesterol has been found to be an effective agent in regulating a variety of biologically significant properties of membranes, including lipid phase transitions, membrane dimensions, permeability to ions and small molecules, osmotic fragility, and microviscosity (Oldfield and Chapman, 1972; Jain, 1975; Demel and De Kruyff, 1976; Shinitzky and Inbar, 1976; Green, 1977). In addition, cholesterol has been implicated in processes which regulate the activity of membrane-bound enzymes (Papahadjopoulos, 1974, 1976) and the exposure of membrane proteins to the surrounding aqueous environment (Borochov and Shinitzky, 1976; Shinitzky and Rivnay, 1977).

The present study was performed in order to quantitate the sterol composition of bovine rod outer segment membranes and retinas. Previous studies of the lipid composition of retina and ROS membranes employed only thin-layer chromatography and/or a modified Liebermann-Burchard assay for the identification and quantitation of cholesterol (Lo Cascio, 1923; Leinfelder and Salit, 1934; Krause, 1938; Brante, 1949; Sjostrand, 1959; Fleischer and Mc Connell, 1966; Borrgreven et al., 1970; De Grip
et al., 1973; Hendriks et al., 1976), and the dry weight per cent range was found to be about 1.6-2.0 for retinas and about 0.9-4.0 for ROS membranes. As previously discussed, these methods of identification and quantitation of sterols are not valid, however, unless it has been demonstrated that sterols other than cholesterol are not present in appreciable quantities. Prior to this study, however, no information existed as to the sterol composition of bovine retinas or ROS membranes.

A recent study employed gas-liquid chromatography as a means of quantitating cholesterol, comparing the chromatograms of retinal lipid extracts to an authentic standard of cholesterol (Miljanich, 1978). This approach, however, is also not strictly valid, since it was not demonstrated that other C$_{27}$ monohydroxy sterols did not co-migrate with cholesterol under the conditions employed for the analyses.

My results indicate that cholesterol is, by far, the predominant sterol in the bovine retina, accounting for at least 99% of the sterol composition of whole retinas and at least 98% of the ROS membrane sterol pool. In both retinas and ROS membranes, cholesterol was accompanied by a minor component which had the chromatographic properties of cholestanol (ca. < 0.1% of the total sterol content); in addition, whole retinas contained another minor component (ca. 0.12% of the total sterol) which had the chromatographic properties of cholest-7-en-3β-ol. The cholesterol content of bovine retinas and ROS membranes was assayed colorimetrically with a modified Liebermann-Burchard reagent as well as chromatographically by TLC, GLC and column chromatography of the tritium-labeled steryl acetates on silicic acid-Super Cel, silica gel G-Super Cel-silver nitrate and alumina-Super Cel-silver nitrate columns. Whole retinas contained
1.76 ± 0.29 per cent cholesterol, by dry weight, whereas ROS membranes contained 1.68 ± 0.15 per cent cholesterol.

Using the literature values for the cholesterol and phospholipid content of bovine retinas (Lo Cascio, 1923; Krause, 1938; Brante, 1949; Hendriks et al., 1976), and assuming an average molecular weight of 800 for retina phospholipids, the cholesterol:phospholipid molar ratio in whole bovine retinas may be calculated to range in value from 0.242 to 0.305, with the most recent value being 0.254 (Hendriks et al., 1976). Performing this same calculation for bovine ROS membranes, assuming an average phospholipid molecular weight of 845, one obtains a range in values of 0.062 to 0.216 (Sjostrand, 1959; Fleischer and Mc Connell, 1966; Borggreven et al., 1970; De Grip et al., 1973; Hendriks et al., 1976; Miljanich, 1978), with the most recent values being 0.088 (Hendriks et al., 1976) and 0.110 (Miljanich, 1978). Plasma membranes from a variety of mammalian cell types possess cholesterol:phospholipid molar ratios of about 0.6 to 1.0. For instance, the erythrocyte plasma membrane has a typical cholesterol:phospholipid molar ratio of about 0.89 (Asworth and Green, 1966; Nelson, 1967), accounting for up to 25% of the total lipid and membrane area (van Deenen and de Gier, 1964; Cooper et al., 1975). The cholesterol:phospholipid molar ratio of rat liver plasma membranes is about 0.83, accounting for almost 72% of the total liver cholesterol content (de Duve, 1971; Dorling and Le Page, 1973). An extreme case is that of nerve myelin which, in adult animals, has a cholesterol:polar lipid ratio of about 1.39; taking into account the high cerebroside content of this membrane, a cholesterol:phospholipid molar ratio of 0.83 is obtained (Asworth and Green, 1966; Demel et al.,
1973). In general, subcellular membranes which have been derived from
the plasma membrane of the cell reflect the cholesterol:phospholipid
molar ratio of the plasma membrane (Green, 1977), and such membranes
generally have moderately high ratios (i.e. 0.6 to 0.8). It has been
suggested that the ROS disc membranes are derived from the plasma mem-
brane of the rod cell by an invagination and detachment process at the
base of the outer segment (Sjostrand, 1961; Young, 1967, 1974; Laties
et al., 1976). One would expect, then, that the overall composition of
the rod cell plasma membrane and that of the discs would be quite simi-
lar. If this is true, then the rod cell plasma membrane is quite unique
with regard to its cholesterol content, being comparable to the choles-
sterol-poor rat liver mitochondrial membranes and rough endoplasmic reti-
culum (Colbeau, 1971; Zambrano, 1975). It has been suggested that the
paucity of cholesterol, in combination with the high polyunsaturated
fatty acid content of the ROS membranes, is responsible for the high
degree of fluidity of ROS membranes (Trauble and Sackman, 1973; Verma
et al., 1973; Cone, 1974), allowing the considerably rapid rotational
and translational mobility of rhodopsin within the plane of the disc
membrane (Brown, 1972; Cone, 1972; Poo and Cone, 1973, 1974; Liebman and
Entine, 1974).

A cholesterol:rhodopsin molar ratio was calculated, using the ex-
perimentally determined cholesterol content for the ROS disc membranes
and the rhodopsin content, corrected for bleached photopigment by re-
generation with 11-cis retinaldehyde. The calculation of the rhodopsin
content assumed average values for the molecular weight (37,500) and
molar extinction coefficient (41,800) of bovine rhodopsin. The range of
published values for the molecular weight of bovine rhodopsin is considerable, with most values in the range of 35,000 to 40,000 daltons (Hubbard, 1954; Shield et al., 1967; Shichi et al., 1969; Daemen et al., Heitzman, 1972; Papermaster and Dreyer, 1974; Lewis et al., 1974; Frank and Rodbard, 1975; Kossi et al., 1977). Also, there is a range of values reported for the extinction coefficient of bovine rhodopsin at 500 nm (38,300 to 42,800), depending on the type of detergent used to solubilize the protein, with the majority of values in the range of 41,600 to 41,950 (Wald and Brown, 1953; Matthews et al., 1963; Shichi et al., 1969; Shichi 1970; Bridges, 1971; Zorn and Futterman, 1971; Daemen et al., 1972; Rotmans et al., 1972). My analyses of bovine ROS disc membranes revealed a cholesterol:rhodopsin molar ratio of 4.70 ± 0.64. This value is considerably lower than that reported by Miljanich (1978), who found a cholesterol:rhodopsin molar ratio of 8.2 for bovine ROS membranes and 8.7 for frog ROS membranes. A value of 42,000 was used for both the molecular weight and the extinction coefficient in his calculations, but this cannot account for the difference between his values and mine. At present, I have no definite explanation for the discrepancy in our two values, but it is possible that some error resides in the methodology employed for the quantitation of cholesterol by gas-liquid chromatography.

The values reported for the phospholipid:rhodopsin molar ratio of bovine ROS membranes range from 65 to 110, depending on the methodology employed for the quantitation of rhodopsin and the average molecular weight assumed for ROS phospholipid (Collins et al., 1952; Poincellot and Zull, 1969; Anderson and Maude, 1970; Borggreven et al., 1970;
De Grijp et al., 1973; Stubbs et al., 1976; Miljanich, 1978). The more recent reports gave a value of 75 for the phospholipid:rhodopsin molar ratio (Stubbs et al., 1976; Miljanich, 1978). Using this value and the cholesterol:rhodopsin molar ratio determined in the course of this study, the cholesterol:phospholipid molar ratio was calculated to be about 0.063 for my bovine disc membrane preparations. This value is on the low end of the range reported by several other laboratories. Assuming that phospholipids represent 80-90% of the total membrane lipid content (Daemen, 1973), cholesterol only comprises about 5-7 mole per cent of the total membrane lipid.

A variety of physical techniques applied to both artificial and biological membranes have demonstrated that cholesterol concentrations of less than about 10 mole per cent of the total lipid do not cause significant perturbations in the physical properties of membranes or the biological activity of membrane-bound enzymes (cf. Demel and DeKryuyff, 1976; Green, 1977). Theoretically, if cholesterol is distributed asymmetrically in the membrane (i.e. either preferentially in the inner or outer monolayer, or clustered within the plane of the membrane), discrete portions of the membrane could be affected selectively. The evidence for the asymmetric distribution of cholesterol in biological membranes is a matter of great conflict (cf. Gottlieb, 1976; Fisher, 1976; Lenard and Rothman, 1976; Bittman and Rottem, 1976; Lange et al., 1977; Blau and Bittman, 1978; Patzer et al., 1978). Currently, there is no direct evidence for the distribution of cholesterol within the ROS disc membranes or plasma membrane. However, a very recent study employing freeze-fracture electron microscopy in conjunction with filipin treatment of
mouse and frog retinas proposes that cholesterol is preferentially distributed in patches within the plane of the ROS plasma membrane and basal discs, coincident with "particle-free patches" (Andrews and Cohen, 1979). In addition, another study, employing the technique of fluorescence polarization, presents data which indicates that cholesterol preferentially effects the phase transition associated with phosphatidylcholine in bovine ROS discs (Sklar et al., 1979); since phosphatidylcholine is known to be asymmetrically distributed with a preference for the inner monolayer of the disc membrane (Litman, 1974; Raubach et al., 1974b; Smith et al., 1977; Crain et al., 1978), these results are consistent with an asymmetric distribution of cholesterol on the inner monolayer of the disc membrane. In connection with these findings, it has been noted that choline phospholipids interact more strongly with cholesterol than amino phospholipids (Demel et al., 1977). Furthermore, it has been postulated that membranes with regions of high curvature may concentrate cholesterol in these highly-curved regions (De Kruijff et al., 1976). In this regard, it has been found that frogs and cattle selectively incorporate a high molecular weight protein (ca. 238,000 daltons in bovine ROS, 290,000 daltons in frog ROS) into the outer segment incisures and disc margins, the regions of highest curvature in the ROS (Papermaster et al., 1976, 1978). It is possible that cholesterol may also be concentrated in these regions of the disc membranes.

The distribution of phospholipids in the disc membrane (i.e. amino phospholipids predominantly on the outer monolayer, choline phospholipids on the inner monolayer) is exactly opposite of that found in plasma membranes from a variety of cell types (Bretscher, 1972; Gordesky, 1976;
Bergelson and Barsukov, 1977; Rothman and Lenard, 1977), and is consistent with the concept that the discs form by invagination of the rod cell plasma membrane. Such a process would require considerable malleability and deformation of the rod cell membrane, apparently at a particular region of the base of the outer segment. It has been noted that a suitably-buffered membrane cholesterol level is critical to the processes of endocytosis in cultured L-cells (Heiniger et al., 1976) and phagocytosis in macrophages (Dianzani et al., 1976); both processes involve the controlled deformation of the plasma membrane with subsequent detachment of a portion of the membrane, forming an encapsulating vacuole which is interiorised by the cell. Assuming that the processes of endocytosis and phagocytosis are mechanistically similar to the formation of disc membranes, it is possible that the low cholesterol levels in ROS membranes permit the dynamic alteration of the cell membrane to proceed unhindered.

The purity of the ROS disc membrane preparations was assayed by several criteria: electron microscopic analysis, SDS-polyacrylamide gel electrophoresis, spectral ratios (O.D.\textsubscript{250}/O.D.\textsubscript{280} and O.D.\textsubscript{280}/ΔO.D.\textsubscript{498}), and marker enzyme analyses. The results indicated that the membranes contained less than 1% contamination from extraneous organelles or other cell types. The procedures used for membrane isolation are claimed to produce only "free-floating" discs, exclusive of the rod cell plasma membrane envelope (Smith et al., 1975). The appearance of the membranes after floatation on a Ficoll cushion was consistent with this claim. The bovine ROS plasma membrane comprises only 2-3% of the total membrane mass prior to fractionation; thus, "contamination" of the discs with
plasma membrane of the rod cell is minimal from the start. The cholesterol content of the disc membranes determined from this study cannot be accounted for by cross-contamination from extraneous organelles or cell debris, since to do so would require a 12% contamination by a membrane having a cholesterol:phospholipid molar ratio of 1.0; such a level of contamination would be readily apparent by SDS-polyacrylamide gel electrophoresis and the absorption spectra, if not by electron microscopy or marker enzyme assays.

This study represents the first rigorous analysis of retina sterol composition, as well as the first such study on the sterol composition of highly-purified rod outer segment disc membranes.
V. IN VITRO METABOLISM OF MEVALONIC ACID
BY ISOLATED BOVINE RETINAS

INTRODUCTION

The results described in the previous chapter demonstrated that cholesterol accounts for essentially all of the monohydroxy sterol content of the bovine retina. Cholesterol in the retina must be derived from at least one of the following possible sources: (1) endogenous de novo biosynthesis; (2) uptake from the circulating lipoproteins of the blood; (3) intraocular exchange with other ocular tissues; (4) retrograde transport from the brain (i.e. via the optic nerve). In addition, it is possible that the cholesterol found in the adult retina represents an extremely long-lived pool of sterol originally deposited during embryogenesis and/or subsequent differentiation and maturation of the tissue. I have chosen to study only one of these possible sources, namely de novo biosynthesis.

The isolated retina has been used successfully in vitro for over 50 years in the study of retina metabolism, particularly in the study of glucose metabolism, bioenergetics (i.e. cellular respiration and ATP production) and their relationship to retina function (for a review, see Ames, 1965; Cohen and Noell, 1965; cf. Graymore, 1969, 1970). With proper precautions, the retina may be removed from the eye and sustained for several hours in vitro with minimal trauma to the tissue, as reflected by the maintenance of metabolic activity, electrical responsiveness and preservation of native morphology (Ames, 1965). The photo-induced electrical response of the retina in vitro is extremely sensitive to both water and electrolyte balance, glucose concentration and oxygen
tension (Ames and Hasings, 1956; Ames and Gurian, 1963; Ames et al., 1965, 1967). The drastic attenuation of the photoresponse upon partial deprivation of glucose and/or oxygen is both rapid and reversible for up to 60 minutes, but complete removal of either glucose or oxygen results in the irreversible loss of the photoresponse as well as concomitant deterioration of retinal morphology (especially pronounced in the mitochondria).

One of the most impressive hallmarks of the retina is the biosynthesis and continual renewal of photoreceptor membranes (Young, 1967; Young and Droz, 1968; Hall et al., 1969; cf. Young, 1974, 1976). The biosynthesis of rhodopsin and the elaboration of rod outer segment membranes have been achieved in vitro with isolated retinas from frogs (Basinger and Hall, 1973; Bok et al., 1974) and cattle (O'Brien et al., 1972; O'Brien and Muellenberg, 1974, 1975). The rate of ROS membrane synthesis and assembly in vitro in the frog retina is nearly identical with that obtained in vivo (Hall et al., 1969), i.e. about one disk synthesized every 40 minutes (at 20–22°C) per rod. The biosynthesis of glycerolipids has also been demonstrated in vertebrate retinas in vitro (Dudley, 1971; Hall et al., 1973; Bazan and Bazan, 1975, 1976, 1977; Giusto and Bazan, 1975, 1977; Bazan et al., 1976a, b, 1977; Basinger and Hoffman, 1976; Aveldano and Bazan, 1977). The de novo biosynthesis and incorporation of fatty acids into rat photoreceptor membranes in vitro has been observed (Dudley, 1971); however, only saturated and monounsaturated fatty acids were synthesized from acetate (incorporation of labelled acetate was also inhibited by the presence of "cold" citrate in the medium), whereas even the addition of linolenic acid (C\text{18:3w3}, a precursor of
docosahexaenoic acid) failed to stimulate the synthesis of polyunsaturated acids.

This chapter documents my findings concerning the uptake and metabolism of mevalonic acid by isolated bovine retinas.

MATERIALS AND METHODS

Methodology used for the measurement of radioactivity, colorimetric assay, digitonin precipitation and recovery of sterols, extraction and saponification of lipids, thin-layer chromatography, column chromatography on silicic acid-Super Cel, Silica Gel G-Super Cel-silver nitrate and alumina-Super Cel-silver nitrate supports, gas-liquid chromatography and radiochromatography (radio-GLC), preparation of buffers, incubation media and isotopic substrates have been previously described (see Materials and General Methods, Chapter II). Chromatographic standards of sterols, steryl acetates, other neutral lipids, fatty acids and fatty acid methyl esters (FAME) were obtained from the sources previously cited (Chapter II).

In Vitro Incubation System

Bovine retinas were obtained from freshly-enucleated eyes which had been stored in a light-tight container on ice for less than one hour prior to dissection (Blue Ribbon Meat Packing Co.). Retinas were dissected into ice-chilled incubation buffer under dim room illumination and gently teased to remove adhering eyecup debris; the retinas were then transferred with forceps to 50-ml capacity Fernbach flasks containing 20 ml of incubation media (3 retinas per flask). Incubation conditions were similar to those originally described by O'Brien et al. (1972). Retinas
were incubated in dim light for varying periods of time at 37° C under a humidified 95% oxygen-5% carbon dioxide atmosphere (flow rate, one standard cubic foot per hour) in a temperature-controlled Dubnoff metabolic incubator, with continuous gentle agitation (ca. 20-25 oscillations/min.). Incubations were terminated by pouring the contents of the flask into three volumes of ice-chilled PBS (isotonic phosphate-buffered saline, pH 7.0); retinas were individually transferred with forceps through two ice-chilled PBS baths (100 ml each), sequentially, prior to further processing.

**Experiment V-1**

A pilot experiment was performed to test the *in vitro* system and to establish a time course over which the retinas are metabolically active. In this case, the uptake and incorporation of ³H-labelled leucine into partially purified ROS membranes and a "rest of retina" (RR) fraction were monitored as a function of incubation time.

Retinas (3 per flask) were incubated in 20 ml of RPMI-1640 medium containing approximately 250 μCi of L-[4,5⁻³H]-leucine (5.5 x 10⁸ dpm, 0.58 μg, 4.3 nmoles) per flask, under the conditions previously described. The incubation medium contained unlabelled leucine as well (1 mg per flask, 0.38 mM), representing a 1775-fold dilution of the isotopic substrate. Incubations were terminated at 0.5, 1.0, 2.0 and 4.0 hours after initiation, as previously described. Retinas were transferred after initial rinsing to 17 ml capacity ultracentrifuge tubes and homogenized in 4.0 ml of sucrose buffer (1.17 g/ml, containing 1 mM MgCl₂ and 10 mM Tris-acetate, pH 7.4) with a tight-fitting, motor-driven Teflon pestle (10 passes at 1800 rpm for 2 minutes on ice). A cushion of a
dense sucrose solution (1 ml; 76%, w:w) was applied with a syringe to the bottom of each tube and the homogenates were overlaid with decreasingly dense sucrose solutions (1.15 g/ml, 2 ml; 1.13 g/ml, 6 ml; 1.11 g/ml, 3 ml; each containing 1 mM MgCl₂ and 10 mM Tris-acetate, pH 7.4). The resulting discontinuous sucrose gradients were centrifuged for one hour at 25,000 rpm (Beckman SW 27 rotor; Beckman Model L-350 ultracentrifuge; 0-4°C). The material which accumulated at the 1.11/1.13 g/ml interface (crude ROS) and the remaining supernatant layer ("rest of retina", RR) were collected with Pasteur pipets and transferred to 50-ml capacity centrifuge tubes. The crude ROS material was washed five times with 40 ml portions of ice-chilled 67 mM phosphate buffer, pH 7.0 (each centrifugation carried out for 20 minutes at 20,000 rpm, Sorvall SS-34 rotor; Sorvall RC-2B centrifuge, 0-4°C); the "rest of retina" material was diluted 3-fold, centrifuged as above and the resulting pellet was washed four times with buffer as described above.

The washed pellets (ROS and RR material) were each dissolved in 67 mM phosphate buffer, pH 7.0. containing 3% Ammonyx-L0 (by volume). Each sample was vortexed for 5 seconds and allowed to stand at room temperature overnight (ca. 12 hours) in parafilm-sealed Corex centrifuge tubes. The samples were then centrifuged (20 minutes at 20,000 rpm, Sorvall SS-34 rotor, 0-4°C) and aliquots of the resulting supernatants were assayed as follows: (a) 0.5 ml was diluted with 3% Ammonyx-L0 buffer (1:1, by volume, for ROS; 1:10, by volume, for RR) and the absorbance at 280 nm was measured in quartz cuvettes (1-cm light path; Cary 118 spectrophotometer); (b) duplicate 0.10 ml aliquots were assayed for total radioactivity, using the aqueous-soluble scintillation cocktail
previously described (Chapter II).

Results

The incorporation of radioactivity into the ROS and RR material from bovine retinas as a function of incubation time is shown in Table V-1 and Figure V-1.

**TABLE V-1**

Incorporation of L-[4,5-3H]-Leucine into Bovine Retina ROS Membranes and "Rest of Retina" as a Function of In Vitro Incubation Time

<table>
<thead>
<tr>
<th>INCUBATION TIME (Hours)</th>
<th>ROS</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O.D. (280 nm)</td>
<td>dpm/ml (x 10^{-4})</td>
</tr>
<tr>
<td>0.5</td>
<td>2.14</td>
<td>2.92</td>
</tr>
<tr>
<td>1.0</td>
<td>2.55</td>
<td>4.56</td>
</tr>
<tr>
<td>2.0</td>
<td>2.61</td>
<td>5.52</td>
</tr>
<tr>
<td>4.0</td>
<td>1.19</td>
<td>3.51</td>
</tr>
</tbody>
</table>

Increasing amounts of labelled leucine were taken up by the retinas and incorporated into retina ROS and RR material up to about 2 hours of incubation time, after which a plateau was reached (expressed as the "specific activity) of incorporation, dpm per O.D. 280). Therefore, it appears that the retinas were viable for at least two hours under these incubation conditions. A maximum of 0.01% of the initially incubated radioactivity was incorporated into ROS material during the course of the incubations.
FIGURE V-1

Incorporation of L-[4,5-^3H]-leucine into bovine retina ROS membranes (o-o) and the "rest of the retina" (•-•) as a function of incubation time. See text for details.
Experiment V-2

Using the in vitro incubation system described in Experiment V-1, bovine retinas were incubated with 3RS-[5-3H]-mevalonic acid (sodium salt; 1.36 x 10^8 dpm, 9.3 nmoles) for 15, 30, 60 and 120 minutes. After terminating the incubations and rinsing the retinas as previously described, the retinas were homogenized in 10 ml of ice-chilled PBS with a tight-fitting, motor-driven Teflon pestle (10 passes at 1800 rpm for 2 minutes) and centrifuged in 17-ml capacity ultracentrifuge tubes (Beckman SW 27 rotor; one hour at 100,000 x g, 0-4°C). The supernatants were discarded and the pellets were washed twice (as above) with 10 ml portions of ice-chilled PBS; no lipid film was apparent at the air-buffer interface. The washed retinal pellets were resuspended in 5.0 ml of PBS using a tight-fitting, ground-glass tissue grinder (15 passes, by hand). An aliquot (0.5 ml) of each suspension was diluted 1:10, by volume, with 3% Ammonyx-LO buffer, centrifuged in a Corex tube (20 minutes at 20,000 rpm, Sorvall SS-34 rotor) and the absorbance of the supernatant was monitored at 280 nm; duplicate 0.20 ml aliquots were assayed for total radioactivity, as usual.

Approximately 1.1 ml of each retina suspension was extracted by a modification of the method of Folch et al. (1957) with 5.0 ml of chloroform-methanol, 2:1; the lower (organic) layer was withdrawn with a Pasteur pipet and saved, while the upper (aqueous) layer was re-extracted with an equal volume of chloroform. The combined organic extracts were evaporated to dryness under nitrogen and redissolved in 0.10 ml of chloroform. Approximately 0.02 ml of each lipid extract was chromatographed on a Silica Gel G TLC plate (solvent system: hexane-
ether-glacial acetic acid, 80:20:1) along with a standard mixture of neutral lipids (approximately 10 μg each of cholesterol, oleic acid, triolein, methyl oleate and cholesteryl oleate) in an adjacent lane. Plates were exposed to iodine vapors for visualization of mass; sections (0.5 cm each) were scraped into vials and assayed for radioactivity as usual. On a separate plate, the neutral lipid mixture was compared with authentic standards of mevalonic acid, mevalonic acid lactone, farnesic acid, farnesol and squalene.

The remaining 3.0 ml of each retina suspension was saponified by refluxing in 25 ml of 15% potassium hydroxide (in 95% ethanol) for 3 hours, under nitrogen atmosphere. After saponification, 25 ml of water was added and the mixture was chilled on an ice bath and extracted four times with 100 ml portions of petroleum ether. The combined extracts were washed once with 100 ml of 5% tartaric acid, twice with 100 ml portions of water, dried over anhydrous magnesium sulfate, filtered, and the solvent was removed under reduced pressure. The residue was eluted with chloroform and transferred to a vial, evaporated to dryness under nitrogen and desiccated under vacuum over phosphorous pentoxide. The nonsaponifiable lipid (NSL) extract was redissolved in 2.0 ml of benzene and duplicate 0.10 ml aliquots were assayed for radioactivity. An aliquot of each NSL extract was chromatographed on a Silica Gel G TLC plate (in chloroform) with an authentic standard of cholesterol in an adjacent lane. Mass and radioactivity were assayed as previously described. Duplicate aliquots (0.2 ml each) of the remaining NSL extract were precipitated with digitonin and the free sterols were recovered and assayed for radioactivity and cholesterol content, as
described previously (Chapter II). The specific activity (dpm/mg cholesterol) was compared before and after precipitation with digitonin to determine the percentage of NSL material which was digitonin-precipitable.

The remainder of the NSL extract from the two-hour incubation (ca. \(1.35 \times 10^5\) cpm) was diluted with about 11 mg of carrier cholesterol and chromatographed on a silicic acid-Super Cel column (2:1, w:w; 50 x 1 cm, equilibrated and eluted with benzene). Fractions (3.4 ml per 30 min.) were collected automatically, evaporated to dryness under nitrogen and redesolved in 2.0 ml of benzene; aliquots were assayed for radioactivity and cholesterol content as usual. Fractions 79-146 were eluted with benzene-ether, 9:1, so as to elute material more polar than monohydroxy sterols from the column.

**Results**

The uptake of mevalonic acid (MVA) by bovine retinas as a function of incubation time is shown in Table V-2 and Figure V-2.

**TABLE V-2**

Uptake of 3RS-[5-\(^3\)H]-Mevalonic Acid by Bovine Retinas *In Vitro* as a Function of Incubation Time

<table>
<thead>
<tr>
<th>INCUBATION TIME (Min.)</th>
<th>RECOVERED ACTIVITY (dpm x 10^-6)</th>
<th>MVA UPTAKE (nmoles)</th>
<th>PERCENT MVA UTILIZED</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>3.87</td>
<td>0.26</td>
<td>2.8</td>
</tr>
<tr>
<td>30</td>
<td>5.09</td>
<td>0.35</td>
<td>3.7</td>
</tr>
<tr>
<td>60</td>
<td>5.89</td>
<td>0.40</td>
<td>4.3</td>
</tr>
<tr>
<td>120</td>
<td>10.6</td>
<td>0.72</td>
<td>7.8</td>
</tr>
</tbody>
</table>
FIGURE V-2

Uptake of [5-\textsuperscript{3}H]-mevalonic acid by bovine retinas \textit{in vitro} as a function of incubation time. See text for details.

FIGURE V-3

Radio-TLC of the "total lipid" extracts from bovine retinas incubated \textit{in vitro} with [5-\textsuperscript{3}H]-mevalonic acid for (A) 15 minutes, (B) 30 minutes, (C) 60 minutes and (D) 120 minutes. Chromatographic system: Silica Gel G, developed once with hexane-diethyl ether-glacial acetic acid, 80:20:1. Chromatographic standards (in order of increasing $R_f$): cholesterol, oleic acid, triolein, methyl oleate and cholesteryl oleate.
Mevalonic acid was taken up by the retinas continuously over the entire range of incubation times. The uptake appeared to be more rapid during the first 15 minutes of incubation, followed by a slower and linear time course up to two hours.

The radiochromatograms of the "total lipid" extracts of the retinas are shown in Figure V-3. The overall distribution of radioactivity at each time point was very similar; there was little evidence of time-dependent changes in individual labelled components. Four major zones of radioactivity were observed for each time point: (1) at the origin of the plate (ca. 9-13% of the recovered activity, coincident with free mevalonic acid and its lactone; (2) between cholesterol and free fatty acids (ca. 22% of the recovered activity; also the region where farnesol migrates); (3) coincident with and immediately adjacent to free fatty acids (ca. 30-40% of the recovered activity; also the region where farnesonic acid chromatographs); (4) coincident with cholesteryl esters and squalene (ca. 25-35% of the recovered activity). Only minor activity was observed in the region of triglycerides and methyl esters (ca. 1-3% of the recovered activity; standards not fully resolved on these plates). The data indicate that mevalonic acid was incorporated into a multitude of lipid products by the retinas. It is obvious from the behavior of the chromatographic standards employed here that the nature of the products cannot be ascertained from TLC analysis alone. Upon exposure of the plates to iodine vapors, the sample lane exhibited intensely stained bands at the origin (i.e. very polar material) and coincident with the cholesterol and free fatty acid standards. A moderately intense band migrated just behind the cholesterol standard
(possibly due to diglycerides); appreciable mass was not apparent in the triglyceride or methyl ester regions, nor was mass detectable coincident with the cholesteryl ester and squalene standards.

The incorporation of mevalonic acid into nonsaponifiable and digitonin-precipitable material is documented in Table V-3 and Figure V-4.

**TABLE V-3**

Incorporation of 3RS-[5-\(^{3}\)H]-Mevalonic Acid into Nonsaponifiable Lipids (NSL) and Digitonin-Precipitable Material (MPD) by Bovine Retinas

<table>
<thead>
<tr>
<th>INCUBATION TIME (Min.)</th>
<th>TOTAL NSL ACTIVITY (dpm x 10(^{-5}))</th>
<th>SPECIFIC ACTIVITY (dpm/O.D.280)</th>
<th>MPD (% of NSL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.72</td>
<td>1,840</td>
<td>1.8</td>
</tr>
<tr>
<td>30</td>
<td>1.69</td>
<td>4,360</td>
<td>1.2</td>
</tr>
<tr>
<td>60</td>
<td>3.22</td>
<td>9,360</td>
<td>1.0</td>
</tr>
<tr>
<td>120</td>
<td>5.84</td>
<td>15,800</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* NSL activity normalized to total homogenate volume (5.0 ml)

The incorporation of mevalonic acid into retinal nonsaponifiable lipids was essentially linear over the two-hour time course of incubation. The percentage of nonsaponifiable material which was precipitable by digitonin was extremely small (<2%) and the relative amount of MPD decreased with increasing incubation time.

The radiochromatograms obtained by TLC analysis of the nonsaponifiable material are shown in Figure V-5. Regardless of incubation time, the amount of radioactivity which was coincident with the cholesterol
FIGURE V-4

Incorporation of [5-^3H]-mevalonic acid into bovine retina nonsaponifiable lipids (NSL) and digitonin-precipitable material as a function of in vitro incubation time. Closed circles (•—•), specific activity (dpm per O.D. \( \text{\text{.A}} \)) of the nonsaponifiable material; open triangles (Δ—Δ), percentage of the total NSL radioactivity which was precipitable with digitonin.

FIGURE V-5

Radio-TLC of the nonsaponifiable lipids obtained from bovine retinas incubated in vitro with [5-^3H]-mevalonic acid for (A) 15 minutes, (B) 30 minutes, (C) 60 minutes and (D) 120 minutes. Chromatographic system: Silica Gel G, developed once with chloroform. The chromatographic standard is cholesterol.
standard was very minor. Most of the activity migrated in the regions expected of hydrocarbons (near the solvent front) and "methyl sterols" (i.e. C_{28}, C_{29} and C_{30} monohydroxy sterols; the region between the cholesterol standard and the solvent front). This behavior is consistent with the low percentage of digitonin-precipitable material; however, since the efficiency of precipitation of even lanosterol is typically 35-45%, the data suggest that the labelled components which were observed to migrate in the region expected of "methyl sterols" on the TLC system employed were predominantly non-sterol components. In addition, the data show that the labelled material previously observed to co-migrate with the authentic cholesterol ester standard upon TLC analysis of the "total lipid" extracts was not due to cholesteryl esters but represents hydrocarbon material (most probably squalene or a related isoprenoid hydrocarbon).

The elution profile obtained by silicic acid-Super Cel column chromatography of the NSL extract from the two-hour incubation is shown in Figure V-6. Several labelled components were observed; the major component eluted with the solvent front (fractions 10-15, ca. 45% of the recovered activity); a minor component co-eluted with the cholesterol standard (fractions 48-60, ca. 4% of the recovered activity); at least five other components (two major and three minor) eluted in the region between the solvent front peak and the cholesterol standard. Increasing the solvent polarity only affected the elution of an additional 4% of the total recovered activity; overall recovery of applied activity was 89%.
FIGURE V-6

Silicic acid-Super Cel column chromatography of the non-saponifiable lipids obtained from a 2-hour incubation of bovine retinas with [5-3H]-mevalonic acid. Fractions 1-78 were eluted with benzene; the arrow denotes the solvent change to benzene-ether, 9:1. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), cholesterol, determined colorimetrically.
Discussion

Under the incubation conditions described, bovine retinas were found to be capable of taking up mevalonic acid from the incubation medium and converting it to a variety of lipid products. A major portion (ca. 30-40%) of the "total lipid" activity resided in components which exhibited the chromatographic behavior of free fatty acids or isoprenoid acids on TLC. Even after two hours of incubation, nonsaponifiable components represented only about 5.5% of the total MVA uptake; the major nonsaponifiable constituent had the chromatographic properties of a hydrocarbon (most likely squalene or a related isoprenoid hydrocarbon). An extremely minor amount of radioactivity was incorporated into digitonin-precipitable material (<2% of the activity incorporated into nonsaponifiable material), indicating that only marginal sterol biosynthesis was performed under these conditions.

Reports from another laboratory have previously demonstrated that bovine retinas maintained for up to four hours in a similar in vitro system as employed here can support the uptake and incorporation of simple precursors into visual pigment and the assembly of new ROS membranes (O'Brien et al., 1972; O'Brien and Muellenberg, 1974, 1975; O'Brien, 1976). These authors incubated two retinas per 10 ml of modified Krebs-Ringer bicarbonate buffer, supplemented with glucose (20 mM), Casamino acids (0.1 mg), penicillin-G (300 units) and streptomycin sulfate (350 units). The RPMI-1640 medium used in the current experiment is a relatively "rich" defined tissue culture medium and was employed with the aim of more effectively preserving the normal morphology of the retina during the incubations (Dr. Paul J. O'Brien, personal communica-
tion).

Due to the number of unknown variables in this system, it is very difficult to assess the relative contribution of de novo biosynthesis of cholesterol to the overall cholesterol content of the retina, based on the results described above. One must take several gross assumptions (some of which are tenuous) in even attempting to evaluate whether or not the amount of sterol synthesized by the retinas from mevalonic acid in this experiment was sufficient to account for the cholesterol content of newly-formed disks. First, one must assume that all the "digitonin-precipitable material" formed represented cholesterol. Second, one must assume no dilution of the labelled precursor, i.e. that all the newly-synthesized cholesterol molecules were derived from the labelled substrate (although we have no idea what the actual dilution might be in this tissue). Third, one must assume that all the newly-formed sterol was synthesized by the rod cells (although we have no idea which cells of the retina take up and metabolize mevalonic acid, nor do we know the relative contribution of each cell type to the overall biosynthesis of cholesterol by the retina) and utilized for ROS membrane biosynthesis. Fourth, one must assume that disk membrane synthesis, in fact, occurred in all rod cells in this preparation and at a rate comparable to that expected in vivo (although we have no evidence for this); based on values determined for the turnover time of ROS membranes in other mammals (Young, 1967, 1971), a value of 10 days may be reasonably assumed. Using the dimensions for the bovine ROS reported by Bonting (1969) and the number of disks per bovine ROS (i.e. about 2 µm x 22 µm, 990 disks/ROS), the approximate turnover rate may be calculated to be $10^2$ disks/day.
or roughly 4 disks/hour, at 37° C. Fifth, one must assume that the cholesterol content and the cholesterol:rhodopsin molar ratio of the newly formed disks are the same as found for the overall ROS membrane population (see results of the previous chapter). Sixth, if one assumes that the packing of rhodopsin in the bovine disk membranes is the same as it is in the frog ROS membranes (Daemen, 1973), there are about 6-7 x 10^4 rhodopsin molecules per disk; since the cholesterol:rhodopsin molar ratio determined for bovine ROS membranes is about 5 (see previous chapter), there must be about 3-3.5 x 10^5 cholesterol molecules per disk. Employing all the assumptions specified above, and further assuming that there are about 10^8 rod cells per bovine retina, it is evident that the retina must produce on the order of 10^{14} molecules of ROS cholesterol per retina per hour at 37° C, or about 0.2 nmole of cholesterol per retina per hour, to account for the synthesis of new ROS membranes at the specified rate. Experimentally, it was found that after two hours of incubation at 37° C (3 retinas per flask), the retinas accumulated 5.84 x 10^5 dpm in the nonsaponifiable material, of which about 0.5% was precipitable with digitonin (2.92 x 10^3 dpm). Using the known specific activity of the mevalonic acid incubated (1.48 x 10^7 dpm/nmole), the fact that one mole of cholesterol synthesized requires the metabolism of six moles of mevalonic acid, and assuming that all the digitonin-precipitable activity was due to cholesterol, the rate of cholesterol synthesis may be calculated to be 3.3 x 10^{-5} nmole per retina per hour under the conditions employed. Comparing this value with the rate required for disk synthesis, it is apparent that the experimental rate of synthesis of cholesterol was about four orders of magnitude too low to account for the synthesis of
new disks. However, it must be kept in mind that this value is only a gross approximation as certain of the assumptions may not be valid. Furthermore, the in vitro conditions employed here may not be conducive to sterol biosynthesis and may not accurately reflect the biosynthetic capability of the retina. Also, it is possible that the synthesis of disk membranes may not require the synthesis or incorporation of cholesterol, even though cholesterol is found to be a routine component of the disks in apparently stoichiometric amounts relative to rhodopsin.

Experiment V-3

The metabolism of mevalonic acid by bovine retinas in vitro was examined further, using three different incubation media (see Materials and General Methods, Chapter II): (a) RPMI-1640 tissue culture medium (bicarbonate buffered); (b) Krebs-Ringer bicarbonate (KR-B); (c) Krebs-Ringer phosphate (KR-P). As previously described, all three media contained glucose (10.0 mM in the Krebs-Ringer media, 11.1 mM in the RPMI-1640 medium) and were supplemented with penicillin-G (potassium salt, 8 µg/ml) and streptomycin-sulfate (12 µg/ml). As usual, all three media were allowed to warm to room temperature (from 4°C) with continuous aeration with oxygen-carbon dioxide (95:5). The pH and osmolarity of the media were then measured. Whereas the bicarbonate-buffered media had very similar pH and osmolarity, the KR-P medium was more acidic (by one pH unit) and had a higher osmolarity. In comparison, the Krebs-Ringer bicarbonate system used for frog retina incubations (Basinger and Hall, 1973; Bok et al., 1974; Basinger and Hoffman, 1976) was pH 7.3 and 302 mOsm after saturation with oxygen-carbon dioxide, 95:5.
<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>pH</th>
<th>OSMOLARITY (mOsm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td>7.35</td>
<td>283</td>
</tr>
<tr>
<td>KR-B</td>
<td>7.45</td>
<td>282</td>
</tr>
<tr>
<td>KR-P</td>
<td>6.46</td>
<td>300</td>
</tr>
</tbody>
</table>

* Osmolarity measured with a Model 3W Advanced Osmometer (Advanced Instruments, Inc., Needham Heights, MA);

variation in measured values is ± 2 mOsm (n = 3).

Freshly dissected retinas were incubated for 3 hours at 37°C as previously described (3 retinas per flask, in 20 ml of medium) with 3RS-[5-³H]-mevalonic acid (5.5 x 10⁸ dpm, 37 nmoles, 1.9 µM). The incubations were terminated and the retinas were rinsed and then homogenized in ice-cold PBS, exactly as described in Experiment V-2. Aliquots of each homogenate (from ca. 5 ml total volume) were withdrawn for absorbance measurements (O.D.₂₈₀; 0.2 ml), assay of total radioactivity (0.2 ml), saponification (3.0 ml) and total lipid extraction (1.0 ml), as described in Experiment V-2.

Aliquots of each total lipid extract were analyzed by TLC (silica gel G; hexane-ether-glacial acetic acid, 80:20:1) along with a mixed neutral lipid standard, as previously described. In addition, an aliquot of the total lipid extract from the incubation using the KR-P medium was analyzed by TLC on a system which resolves cholesteryl esters from squalene (benzene-petroleum ether, 3:7). After assaying the total radioactivity recovered in the nonsaponifiable extracts, each NSL extract was diluted with about 10 mg of carrier cholesterol and approximately half of each sample was chromatographed on a silicic acid-Super Cel
column (2:1, w/w; 50 x 1 cm, eluted with benzene). Fractions were collected and analyzed as usual for radioactivity and for carrier mass. The remaining half of each NSL sample was precipitated with digitonin and the free sterols were recovered, as previously described; aliquots were assayed in triplicate before and after digitonin precipitation for total radioactivity and sterol mass. A portion of the material recovered after digitonin precipitation of the NSL material from the incubation with the KR-P medium was analyzed by TLC (in chloroform), along with authentic standards of cholesterol and lanosterol/dihydrolanosterol. Selected fractions obtained by silicic acid-Super Cel chromatography of the NSL material from the incubation with the KR-P medium were analyzed further by TLC, radio-GLC and/or further chromatography on columns of Silica Gel G-Super Cel-silver nitrate and alumina-Super Cel-silver nitrate (described in detail in the Results section of this experiment), with appropriate standards.

A cursory examination of the labelled saponifiable material from these incubations was also performed. The aqueous-ethanolic phase which remained after extraction of the nonsaponifiable material (stored for several days at -20°C) was removed from the freezer and immediately acidified by dropwise addition of concentrated HCl, with continuous rapid stirring, to pH 1-2. The acidified mixtures (ca. 400 ml total volume) were each extracted three times with 100 ml portions of diethyl ether and once with 100 ml of benzene (to recover the last traces of ether-soluble material from the aqueous phase). The combined organic extracts were washed once with 100 ml of water, dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure and
transferred to vials. The extracts were then evaporated to dryness under nitrogen and desiccated under vacuum over phosphorous pentoxide for several hours; the saponifiable residues were then redissolved in 5.0 ml portions of chloroform and aliquots were assayed for total radioactivity and by radio-TLC (silica gel G; hexane-ether-glacial acetic acid, 80:20:1), as previously described.

The saponifiable material obtained from the incubation with the Krebs-Ringer phosphate medium was derivatized with freshly-generated diazomethane (one hour at room temperature, in methanol-ether, 1:9), as previously described; the solvent was removed under a stream of nitrogen and the residue was redissolved in hexane and analyzed by radio-TLC, as above. Authentic standards of oleic acid and farnesoic acid (ca. 50 µg each) were derivatized simultaneously under the same conditions and analyzed by TLC along with the labelled material (in adjacent lanes of the same TLC plate). The labelled methyl esters were purified by preparative TLC (Silica Gel G; hexane-ether-glacial acetic acid, 80:20:1); a standard of methyl oleate was chromatographed in a lane near one edge of the plate. After developing in the given solvent system, the plate was dried under a stream of nitrogen and then partially covered with a clean glass plate so as to expose only the lane containing the methyl oleate standard; the plate was then briefly exposed to iodine vapors to visualize the standard and the band corresponding to labelled methyl esters was scraped from the plate and eluted with 50 ml of hexane. After filtration, the silica gel residue was eluted twice more with 10 ml portions of dichloromethane and the combined eluants were evaporated under nitrogen and redissolved in approximately 20 µl of hexane. Radio-GLC analysis
of a portion of the sample (6 μl, 2.25 x 10⁴ cpm) was performed on a 3% OV-1 column (column temperature, 170°C isothermal; carrier flow rate, 66 ml/min.), collecting and assaying one-minute samples of effluent gas as previously described. Under identical conditions, a mass tracing of the sample (ca. 1 μl) was recorded just prior to the radio-GLC analysis and compared with an authentic mixture of fatty acid methyl esters (16:0, 18:0, 18:1, 18:2, 18:3 and 20:0 species, where the number preceding the colon represents the number of carbons in the fatty acid chain and the number after the colon designates the number of double bonds in the molecule).

Results

The uptake and incorporation of mevalonic acid by bovine retinas in vitro and subsequent conversion to lipid products under the three different incubation conditions employed is documented in Table V-4. All three incubation media gave comparable results; about 2.8 ± 0.7 per cent of the incubated substrate was taken up by the retinas over the three-hour incubation period and approximately 52.6 ± 6.6 per cent of the incorporated substrate was converted to lipid products. The apparent variation in the quantitative values cannot be attributed with reliability to the nature of the buffer or other media constituents since this amount of variability would probably be encountered in three consecutive incubations using a single incubation medium. In this particular experiment, the uptake and conversion of mevalonic acid was highest in the Krebs-Ringer phosphate system on the basis of both total incorporated activity and specific activity (dpm/O.D. 280), for reasons which may or may not be a function of the incubation medium itself.
<table>
<thead>
<tr>
<th>INCUBATION MEDIUM</th>
<th>MEVALONIC ACID (MVA) INCORPORATION</th>
<th>TOTAL LIPIDS&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TOTAL ACTIVITY (dpm x 10&lt;sup&gt;-7&lt;/sup&gt;)</td>
<td>SPECIFIC ACTIVITY (dpm/O.D. 280 x 10&lt;sup&gt;-6&lt;/sup&gt;)</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>1.37</td>
<td>2.05</td>
</tr>
<tr>
<td>KR-B</td>
<td>1.32</td>
<td>2.23</td>
</tr>
<tr>
<td>KR-P</td>
<td>1.98</td>
<td>2.91</td>
</tr>
</tbody>
</table>

<sup>1</sup> Incubations carried out for 3 hours at 37° C (3 retinas per 20 ml of medium per flask) under a humidified oxygen-carbon dioxide (95:5) atmosphere; each flask contained 5.5 x 10<sup>8</sup> dpm (50 nmoles) of labelled mevalonic acid.

<sup>2</sup> RPMI-1640 tissue culture medium (pH 7.35, 283 mOsm); KR-b, Krebs-Ringer bicarbonate (pH 7.45, 282 mOsm); KR-P, Krebs-Ringer phosphate (pH 6.46, 300 mOsm).

<sup>3</sup> Chloroform-soluble material, extracted by a modification of the method of Folch et al. (1957).
Thin-layer chromatography of the "total lipid" extracts from each incubation gave remarkably similar radiochromatograms, as shown in Figure V-7. As observed in Experiment V-2, four major zones of radioactivity were apparent on each plate: (1) at the origin (ca. 9-12% of the recovered activity); (2) the region between cholesterol and the free fatty acid standard (ca. 22-30% of the recovered activity); (3) the region just ahead of and coincident with the free fatty acid standard (ca. 25-35% of the recovered activity); (4) the region coincident with cholesteryl esters and hydrocarbons (ca. 18-25% of the recovered activity).

Only minor activity was apparent in the region coincident with triglyceride and fatty acid methyl ester standards (ca. 2-4% of the recovered activity), as well as coincident with the cholesterol standard (ca. 3-7% of the recovered activity). It is evident that mevalonic acid was incorporated into a variety of lipid products, as previously observed, but the exact nature of the products cannot be determined from this analysis alone. Again, the apparent variation in the distribution of labelled components cannot be attributed with certainty to differences in the physical and chemical properties and constituents of the incubation media. Analysis of an aliquot of the total lipid extract obtained from the incubation using the KR-P medium on another TLC system (benzene-petroleum ether, 3:7) clearly demonstrated that the activity which previously appeared to co-migrate with the cholesteryl ester standard (Figure V-7C) is not due to steryl esters but displays the chromatographic behavior of a hydrocarbon (Figure V-8), most likely squalene or a related isoprenoid; in both instances, the component in question represented 18-20% of the total lipid activity.
FIGURE V-7
Radio-TLC of the "total lipid" extracts derived from bovine retinas incubated \textit{in vitro} with [5-\textsuperscript{3}H]-mevalonic acid in (A) RPMI-1640 medium, (B) Krebs-Ringer bicarbonate medium, and (C) Krebs-Ringer phosphate medium for three hours. Chromatographic system: Silica Gel G, developed once with hexane-diethyl ether petroleum ether, 80:20:1. Chromatographic standards (in order of increasing $R_f$): cholesterol, oleic acid, triolein, methyl oleate and cholesteryl oleate.

FIGURE V-8
Radio-TLC of the "total lipid" extract derived from bovine retinas incubated \textit{in vitro} for three hours with [5-\textsuperscript{3}H]-mevalonic acid in Krebs-Ringer phosphate medium. Chromatographic system: Silica Gel G, developed once with benzene-petroleum ether, 3:7. Chromatographic standards (in order of increasing $R_f$): oleic acid (and cholesterol), triolein (and methyl oleate), cholesteryl oleate and squalene.
The incorporation of mevalonic acid into nonsaponifiable lipids (NSL) and digitonin-precipitable material (MPD) is shown in Table V-5. It is evident from these data that nonsaponifiable material accounted for the majority of the lipids synthesized from mevalonic acid in these incubations (76.9 ± 7.6 per cent of the total lipid activity); a striking feature of these incubations, as opposed to previous results, is the appreciable synthesis of digitonin-precipitable material (19.2 ± 4.4 per cent of the total nonsaponifiable lipid activity), approximately 10-fold greater than the highest relative incorporation observed in the previous experiment.

**TABLE V-5**

*In Vitro* Incorporation of 3RS-[5-3H]-Mevalonic Acid into Nonsaponifiable Lipids (NSL) and Digitonin-Precipitable Material (MPD) by Bovine Retinas

<table>
<thead>
<tr>
<th>INCUBATION MEDIUM</th>
<th>TOTAL ACTIVITY (dpm x 10^-6)</th>
<th>PER CENT of MVA UPTAKE</th>
<th>PER CENT of TOTAL LIPID</th>
<th>MPD (PER CENT of NSL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td>5.73</td>
<td>41.9</td>
<td>78.3</td>
<td>14.6</td>
</tr>
<tr>
<td>KR-B</td>
<td>5.02</td>
<td>37.9</td>
<td>83.7</td>
<td>22.4</td>
</tr>
<tr>
<td>KR-P</td>
<td>7.98</td>
<td>40.4</td>
<td>68.7</td>
<td>20.6</td>
</tr>
</tbody>
</table>

Thin-layer radiochromatography of the digitonin-precipitable material obtained from the incubation using the KR-P medium (Figure V-9) showed that the majority of this material exhibited the chromatographic behavior of
methylated monohydroxy sterols, whereas relatively little of the recovered activity co-migrated with the cholesterol standard.

The radiochromatograms obtained by chromatography of the NSL extracts on silicic acid-Super Cel columns are shown in Figures V-10 through V-12. All three samples exhibited very similar elution profiles. Unfortunately, the lack of baseline resolution of the components prevented accurate quantitative analysis of the distribution of radioactivity. The sample from the incubation with the RPMI-1640 medium (Figure V-10; applied activity, $1.71 \times 10^6$ dpm; recovery, 87%) contained several prominent components which eluted in the following fractions: 4-8 (ca. 22%), 12-18 (ca. 17%), 19-26 (ca. 27%), 30-35 (ca. 16%) and 36-44 (ca. 12%). The cholesterol standard eluted in fractions 32-45; therefore, there was some overlap between radioactive components and the $C_{27}$ sterol standard. Two minor components (ca. 4% each) eluted just after the cholesterol standard; the remainder of the recovered activity eluted as unresolved components which attended the major components listed above. Similarly, the sample from the incubation with the KR-b medium (Figure V-11; applied activity, $1.50 \times 10^6$ dpm; recovery 95%) showed major components at fractions 5-10 (ca. 26%), 13-20 (ca. 11%), 21-28 (ca. 28%), 29-38 (ca. 13%), 39-46 (ca. 5%) and 47-60 (ca. 5%); the cholesterol standard eluted with fractions 35-46. The sample from the incubation with the KR-P medium (Figure V-12; applied activity, $2.39 \times 10^6$ dpm; recovery, 97%) revealed labelled constituents at fractions 5-9 (ca. 27%), 12-19 (ca. 15%), 20-26 (ca. 25%), 27-35 (ca. 17%), 36-43 (ca. 7%), 44-56 (ca. 6%) and 60-72 (ca. 2%); the cholesterol standard eluted with fractions 32-44. Thus, in contrast to the elution profile of the NSL material in Experi-
FIGURE V-10
Silicic acid-Super Cel column chromatography of the non-saponifiable lipids obtained by incubating bovine retinas with $[5^{-3}H]$-mevalonic acid in vitro in RPMI-1640 medium. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), cholesterol, determined colorimetrically.

FIGURE V-11
Silicic acid-Super Cel column chromatography of the non-saponifiable lipids obtained by incubating bovine retinas with $[5^{-3}H]$-mevalonic acid in vitro in Krebs-Reinger bicarbonate medium. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), cholesterol, determined colorimetrically.

FIGURE V-12
Silicic acid-Super Cel column chromatography of the non-saponifiable lipids obtained by incubating bovine retinas with $[5^{-3}H]$-mevalonic acid in vitro in Krebs-Ringer phosphate medium. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), cholesterol, determined colorimetrically.
ment V-2, these radiochromatograms showed a more extensive distribution of labelled components and more incorporation into material which co-eluted with the cholesterol standard; also, the solvent front peak in these radiochromatograms did not contain the majority of the NSL activity, although it was a prominent component in each sample. The fractions corresponding to the components listed above were pooled and stored in vials under nitrogen at -20° C for further study. Due to the relatively larger amount of labelled material recovered from the sample obtained from the incubation with the KR-P medium, further analysis was conducted on the pooled fractions from that sample only.

Analysis of Nonsaponifiable Components Obtained from the Incubation of Retinas with the Krebs-Ringer Phosphate Medium

The material from fractions 5-10 was analyzed by radio-TLC on plates of Silica Gel G in two different solvent systems (petroleum ether; chloroform), along with standards of squalene and cholesterol. The radiochromatograms are shown in Figure V-13. Approximately 80-85% of the labelled material co-migrated with the squalene standard on both TLC systems. Another aliquot of this material (2.88 x 10^4 dpm) was analyzed by radio-GLC on a 1% OV-17 column (column temperature, 230° C; carrier flow rate, 66 ml/min.). The chromatogram of an authentic sample of squalene was recorded under identical conditions before and after analysis of the labelled material. It was observed that the mass tracing was non-Gaussian and therefore the apparent retention time of the squalene sample was a function of the amount of squalene injected. Care was taken, therefore, to co-inject the same amount of carrier squalene with the labelled sample as was used for the mass tracings. Effluent gas was
FIGURE V-13
Radio-TLC of fractions 5-10 from the silicic acid-Super Cel column chromatography of nonsaponifiable lipids obtained by incubating bovine retinas in vitro with [5-³H]-mevalonic acid in Krebs-Ringer phosphate medium (see Figure V-12). Chromatographic system: Silica Gel G, developed once with either (A) petroleum ether or (B) chloroform. The chromatographic standards (in order of increasing $R_f$) are cholesterol and squalene.

FIGURE V-14
Radio-GLC of fractions 5-10 from the silicic acid-Super Cel column chromatography of nonsaponifiable lipids obtained by incubating bovine retinas in vitro with [5-³H]-mevalonic acid in Krebs-Ringer phosphate medium (see Figure V-12). Chromatographic system: 1% OV-17, 230°C isothermal; carrier flow rate, 66 ml/min. The mass peak is due to squalene.
collected as previously described at one-minute intervals and the radio-
activity was determined as usual. The radiochromatogram is shown in
Figure V-14. At least 99% of the recovered radioactivity co-migrated
with the squalene standard under the conditions employed; the overall
recovery was about 76%. This analysis was repeated on the remainder of
the sample, yielding essentially identical results.

A portion of the material from fractions 20-26 was analyzed by
radio-TLC (Silica Gel C; benzene-diethyl ether, 4:1), along with authen-
tic standards of cholesterol, lanosterol/dihydrolanosterol and squalene.
The radiochromatogram (Figure V-15) showed that the majority of the
activity co-migrated with the C₃₀ sterols. Another aliquot of this
sample was analyzed by radio-GLC on a 1% OV-17 column (column tempera-
ture, 255°C; carrier flow rate, 66 ml/min.). Mass tracings of a com-
mercial lanosterol sample (a 3:2 molar mixture of lanosterol and dihy-
drolanosterol) were recorded before and after analysis of the labelled
material. The labelled sample (5.72 x 10⁴ dpm) was co-injected with
about 5 µg of the commercial lanosterol standard and the effluent gas
was collected at one-minute intervals and analyzed for radioactivity as
previously described. The radiochromatogram is shown in Figure V-16.
Approximately 92% of the recovered radioactivity was coincident with
the second (i.e. lanosterol) mass peak, whereas no activity was observed
to co-migrate with the first (i.e. dihydrolanosterol) mass peak; the
overall recovery of applied radioactivity was about 88%. Under the
conditions employed, the lanosterol peak (retention time, 26.46 min.)
and the dihydrolanosterol peak (retention time, 21.42 min.) were fully
resolved. Analysis of a second aliquot of this sample under identical
FIGURE V-15
Radio-TLC of fractions 20-26 obtained by silicic acid-Super Cel-column chromatography of the non-saponifiable lipids derived from an incubation of bovine retinas with $[5-^3H]$-mevalonic acid in Krebs-Ringer phosphate medium (see Figure V-12). Chromatographic system: Silica Gel G, developed once with benzene-diethyl ether, 4:1. Chromatographic standards (in order of increasing $R_f$): cholesterol, lanosterol and squalene.

FIGURE V-16
Radio-GLC of fractions 20-26 obtained by silicic acid-Super Cel-column chromatography of the non-saponifiable lipids derived from an incubation of bovine retinas with $[5-^3H]$-mevalonic acid in Krebs-Ringer phosphate medium (see Figure V-12). Chromatographic system: 1% OV-17, 255°C isothermal; carrier flow rate, 66 ml/min. The first mass peak is due to 24,25-dihydrolanosterol; the second mass peak is due to lanosterol.
conditions yielded the same results.

The remainder of the material from fractions 20-26 (3.06 x 10^5 dpm) was hydrogenated over freshly-prepared Raney nickel (ca. 1 gram of catalyst) along with 10 mg of carrier commercial lanosterol in benzene (24 hours at 40 p.s.i. of hydrogen). Catalyst was removed by vacuum filtration through a bed of Super-Cel; after rinsing the Super-Cel with two 25 ml portions of benzene, the combined eluants were concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and desiccated under vacuum over phosphorous pentoxide for several hours. The recovered material was redissolved in 4.0 ml of benzene and assayed for radioactivity (2.08 x 10^5 dpm; recovery, 68%). Approximately half of the sample (1.02 x 10^5 dpm) was applied to a Silica Gel G-Super Cel-silver nitrate chromatographic column (50 x 1 cm; equilibrated and eluted with hexane-benzene 3:12) along with about 5 mg of carrier commercial lanosterol; fractions were collected automatically, evaporated to dryness under nitrogen, redissolved in 2.0 ml of benzene and aliquots were removed for measurement of radioactivity and colorimetric determination of sterol mass (fractions: 3.7 ml per 20 minutes). The elution profile (Figure V-17) exhibited baseline resolution of the dihydrolanosterol (fractions 62-98) and lanosterol (fractions 99-160) standards. Approximately 78% of the recovered radioactivity co-chromatographed with the dihydrolanosterol peak; no activity co-migrated with the lanosterol peak. A very broad and irregular peak of radioactivity eluted just after the lanosterol peak (fractions 160-250; ca. 22% of the recovered radioactivity). The overall recovery of applied activity from this column was only 52%. The results indicated that the
FIGURE V-17
Silica Gel G-Super Cel-silver nitrate column chromatography of the hydrogenated material derived from fractions 20-26 of the previous silicic acid-Super Cel column (see Figure V-12). Solvent system: hexane-benzene, 3:1. Open circles (○ — ○), radioactivity; closed triangles (▲ — ▲), sterols, determined colorimetrically. The first colorimetric peak is due to 24,25-dihydrolanosterol; the second colorimetric peak is due to lanosterol.

FIGURE V-18
Alumina-Super Cel-silver nitrate column chromatography of fractions 62-110 from the previous Silica Gel G-Super Cel-silver nitrate column (see Figure V-17). Solvent system: hexane-benzene, 4:1; arrow denotes solvent change to hexane-benzene, 7:3. Open circles (○ — ○), radioactivity; closed triangles (▲ — ▲), 24,25-dihydrolanosterol, determined colorimetrically.
material which previously exhibited the chromatographic properties of lanosterol was quantitatively converted to material with the chromatographic properties of dihydrolanosterol; the more polar material was probably generated during the hydrogenation and/or handling procedures and was not analyzed further.

The fractions corresponding to the dihydrolanosterol peak (fractions 62-110; $3.98 \times 10^4$ dpm) were pooled and applied to an alumina–Super Cel–silver nitrate column (100 x 1 cm; equilibrated and eluted with hexane-benzene, 4:1). After fraction 43, the column was eluted with hexane-benzene, 7:3; fractions (5.0 ml each) were collected, evaporated to dryness under nitrogen, redissolved in 2.0 ml of benzene and assayed for radioactivity and sterol mass as previously described. The elution profile (Figure V-18) showed a single, slightly asymmetric peak of radioactivity which was coincident with the dihydrolanosterol mass peak (fractions 103-165). It can be seen that, although the radioactivity and colorimetry peaks were scaled so as to be exactly coincident, there was some broadening of the radioactivity peak which suggested the presence of more than one labelled component. Recovery of activity was nearly 98%.

Fractions 99-160 were pooled and acetylated in 10 ml of dry pyridine-acetic anhydride (1:1) for 48 hours at room temperature under nitrogen, and the reaction mixture was worked up in the manner previously described (see Materials and General Methods). The acetylated material ($3.12 \times 10^4$ dpm) was applied to an alumina-Super Cel-silver nitrate column (100 x 1 cm; equilibrated and eluted with hexane-benzene, 95:5). The solvent was changed to hexane-benzene, 9:1, at fraction 40, then to
FIGURE V-19

Alumina-Super Cel-silver nitrate column chromatography of the acetylated material derived from pooled fractions 99-160 from the previous alumina-Super Cel-silver nitrate column (see Figure V-18). Solvent system: hexane-benzene, 95:5; first arrow, solvent changed to hexane-benzene, 9:1; second arrow, solvent changed to hexane-benzene, 4:1, third arrow, solvent changed to benzene. Open circles (o — o), radioactivity; closed triangles (△ — △), 3β-acetoxy-lanost-8-ene, determined colorimetrically.
hexane-benzene, 4:1, at fraction 120, and finally to straight benzene at fraction 173. Fractions (4.8 ml per 35 minutes) were collected, evaporated to dryness under nitrogen, redissolved in 2.0 ml of benzene; even-numbered fractions were assayed in their entirety for radioactivity while odd-numbered fractions were assayed colorimetrically (0.2 ml aliquots each) for dihydrolanosteryl acetate. The elution profile (Figure V-19) exhibited an exact correspondence between the dihydrolanosterol mass peak and the major peak of radioactivity (fractions 55-115; 87% of the recovered activity). Only after changing the solvent system to straight benzene was another radioactive component observed (fractions 182-210; 13% of the recovered activity). The overall recovery of applied activity was about 96%. Considering the gross difference in chromatographic behavior of the major and minor labelled components on this column, it is unlikely that the minor component was responsible for the broadening of the radioactivity peak on the previous column; more likely, this material represented either unreacted material or polar products which resulted from the work-up of the acetylation mixture and subsequent handling.

The remaining half of the hydrogenated material derived from fractions 20-26 of the silicic acid-Super Cel column (1.06 x 10^5 dpm) was chromatographed on a Silica Gel G-Super Cel-silver nitrate column prepared from the same batch of column support as previously employed (50 x 1 cm; equilibrated and eluted with hexane-benzene, 7:3); about 15 mg of carrier commercial lanosterol was applied with the labelled sample. The solvent was changed to hexane-benzene, 1:1, at fraction 97. Fractions (3.2 ml per 20 minutes) were collected, evaporated to
FIGURE V-20

Silica Gel G-Super Cel-silver nitrate column chromatography of the remaining portion of the material pooled from fractions 20-26 obtained by silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from incubation of bovine retinas with [5-^3H]-mevalonic acid in Krebs-Ringer phosphate medium (see Figure V-12). Solvent system: hexane-benzene, 7:3; arrow denotes solvent change to hexane-benzene, 1:1. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), sterol, determined colorimetrically. The first colorimetric peak is due to lanosterol.
dryness under nitrogen, redissolved in 2.0 ml of benzene and aliquots were withdrawn for measurement of radioactivity and colorimetric assay of sterol mass as usual. The elution profile is shown in Figure V-20. Carrier dihydrolanosterol (fractions 43-62) and lanosterol (fractions 63-100) resolved well on this column. About 67% of the recovered radioactivity co-eluted with the dihydrolanosterol peak, with a distinct shoulder on the trailing edge of the peak; the dihydrolanosterol mass peak was symmetrical, in contrast, without a detectable shoulder. No activity was coincident with the lanosterol mass peak, although an irregular peak of radioactivity (ca. 33% of the recovered radioactivity) eluted just after the lanosterol peak. The recovery of applied radioactivity from this column was only 56%. The results are in reasonable agreement with the results obtained from the previous chromatographic analysis of this sample and indicate that more than one labelled product was coincident with the dihydrolanosterol standard, as previously suggested. The identity of the unlabelled carrier material (fractions 73-88) was confirmed as lanosterol by GLC analysis (3% OV-17; column temperature, 270°C; flow rate, 60 ml/min.) in comparison with a mixed standard of lanosterol and dihydrolanosterol.

Fractions 36-44 from the silicic acid-Super Cel column (i.e. the material which co-eluted with the cholesterol standard) were pooled, diluted with 50 mg of carrier cholesterol and processed via the dibromide, as described previously. The specific activity (dpm per mg of cholesterol) of the sample was as follows: before, 1700 dpm/mg; after, 250 dpm/mg. These results suggest that only 15% of the material which exhibited similar chromatographic behavior as cholesterol on a silicic
acid–Super Cel column also behaved chemically like cholesterol by this criterion. Hence, since fractions 36-44 only represented about 7% of the total recovered activity of the NSL sample, the cholesterol-like material only accounted for about 1% of the total nonsaponifiable material from this incubation.

Analysis of Saponifiable Material

The following amounts of saponifiable material were recovered from the incubations: RPMI-1640, $2.48 \times 10^6$ dpm; KR-B, $1.09 \times 10^6$ dpm; KR-P, $2.70 \times 10^6$ dpm. For reasons unknown, the recovery of saponifiable material was markedly variable. Radio-TLC analysis of the saponifiable material from the KR-P incubation was performed before and after esterification with diazomethane. The radiochromatograms are presented in Figures V-21A and V-21B, respectively. Before esterification, the majority of the radioactivity co-migrated with the oleic acid standard (ca. 55-60%); after esterification (but prior to purification by preparative TLC), a corresponding amount of radioactivity co-migrated with the methyl oleate standard. Under identical esterification conditions, authentic standards of oleic acid and farnesolic acid were converted quantitatively to the corresponding methyl esters. The identity of the polar material observed in both radiochromatograms is not known; under these chromatographic conditions, both free mevalonic acid and its lactone remain at the origin of the plate. Most likely these polar constituents represent oxidative degradation products.

After purification of the methyl esters by TLC, the sample was examined by radio-GLC as described previously. The radiochromatogram (Figure V-22) revealed that about 49% of the recovered radioactivity
FIGURE V-21

Radio-TLC of the saponifiable lipids obtained from the incubation of bovine retinas with [5-³H]-mevalonic acid in Krebs-Ringer phosphate medium. Chromatographic system: Silica Gel G, developed once with hexane-diethyl ether-glacial acetic acid, 80:20:1. (A) before and (B) after esterification with diazomethane. The chromatographic standards (in order of increasing Rf) are oleic acid and methyl oleate.

FIGURE V-22

Radio-GLC of the methyl esters derived from the saponifiable lipids obtained from the incubation of bovine retinas with [5-³H]-mevalonic acid in Krebs-Ringer phosphate medium. Chromatographic system: 3% OV-17, 170°C isothermal; carrier flow rate, 66 ml/min. The arrow denotes the position expected for the elution of a C₂₀:₀ fatty acid methyl ester.
eluted just after a mass component which had the chromatographic behavior of methyl myristate but before a major mass component which had the chromatographic behavior of methyl palmitate. At least three other labelled components eluted after the C₁₈ methyl ester species (the major mass components of the chromatogram, under these conditions) but before the position expected for a saturated C₂₀ methyl ester. Notably, there was no radioactivity coincident with the major methyl ester components derived from the endogenous fatty acids of the retina. The overall recovery of radioactivity was about 76%. Under the chromatographic conditions employed, the long-chain polyunsaturated methyl esters (which may account for up to 30% of the total acyl species) did not elute from the column. It was also observed that the isomers of the methyl farnesoate chromatographic standard eluted in the region of C₁₄ and C₁₅ fatty acid methyl esters, coincident with the major portion of recovered radioactivity.

Discussion

The results of this experiment demonstrate that bovine retinas have the ability to incorporate exogenous mevalonic acid into a variety of lipid products in vitro. Although the constituents and properties (i.e. pH and osmolarity) of the three incubation media employed here were quite different, the results obtained from incubation of the retinas in these media were remarkably comparable and within the normal variability expected for such in vitro systems. In this set of incubations, about 75% of the labelled lipids was due to nonsaponifiable components. The major labelled nonsaponifiable components exhibited the chromatographic properties of squalene and C₃₀ sterols. Analysis of the C₃₀ sterol-like
material from the Krebs-Ringer-phosphate incubation indicated that the majorities of this material was represented by lanosterol; at least one other component with very similar properties was also apparent in this material. All three incubations produced small amounts of material which behaved like C\textsubscript{27} monohydroxy sterols upon analysis by silicic acid-Super Cel column chromatography. However, dibromide purification of this material derived from the incubation in the Krebs-Ringer phosphate medium revealed that only about 1\% of the total activity incorporated into nonsaponifiable material behaved like cholesterol by chemical as well as chromatographic criteria. Digitonin-precipitable material accounted for 15-22\% of the total nonsaponifiable activity, depending on the incubation system (in contrast to previous results). Considering the above results, it is most likely that the nonsaponifiable components which eluted from the silicic acid-Super Cel columns between squalene and the cholesterol standard were primarily due to a mixture of methylated monohydroxy sterols, although the analysis of most of these components was not pursued. Even though considerably more digitonin-precipitable material was synthesized in these incubations than previously observed, if one assumes that 1\% of the total NSL activity from the KR-P incubation was due to cholesterol and also uses the assumptions considered in the previous experiment (i.e. with regard to ROS membrane turnover, etc.), the \textit{in vitro} rate of cholesterol synthesis in this incubation may be calculated to be approximately $1 \times 10^{-4}$ nmole/retina/hour (which is still considerably less than the rate of 0.2 nmoles/retina/hour apparently required for ROS membrane synthesis in bovine retina, under these conditions). However, due to the caveats previously discussed,
this estimate of retina cholesterol synthesis may not be accurate and the contribution of de novo cholesterol biosynthesis to ROS membrane biogenesis may not be assessed with confidence.

A substantial portion of the activity incorporated into retina total lipids exhibited the chromatographic properties of free fatty acids (by radio-TLC). Analysis of this material obtained from the KR-P incubation revealed that it was able to be derivatized with diazomethane. However, radio-GLC analysis of the derivatized "fatty acid-like" material revealed that the labelled components did not correspond to the fatty acid composition of the bovine retina (cf. Bartley et al., 1962; Hands and Bartley, 1963). Considering the radiochromatographic results and the nature of the labelled substrate, it is evident that the fatty acid-like material represented a mixture of isoprenoid acids. The formation of isoprenoid acids by rat liver preparations has been reported previously (Christophe and Popjak, 1961; Popjak, 1959; Popjak et al., 1959). Such acids are structurally related to and derived from certain allyl pyrophosphates known to be intermediates in the biosynthesis of cholesterol. However, it has also been reported that tissues of neural ectoderm origin (i.e. central and peripheral nervous tissue and skin) have the ability to convert mevalonic acid to fatty acids via the so-called "trans-methyl-gluconate shunt" (Popjak, 1959; Edmond and Popjak, 1974), whereas liver lacks such ability. This shunt involves the diversion of mevalonate metabolites normally utilized for isoprenoid biosynthesis to reform HMG-CoA, which may then be acted upon by a mitochondrial lyase (Clinkenbeard et al., 1975) to form acetoacetate and acetyl-CoA. Dudley (1971) has demonstrated that the rat retina can synthesize labelled fatty acids
from $^{14}$C-labelled sodium acetate \textit{in vivo} and \textit{in vitro} (either \textit{de novo} or by chain elongation); the labelled fatty acids synthesized \textit{in vivo} were representative of the normal fatty acid composition of that tissue. Therefore, if the trans-methylglutaconate shunt were operative in bovine retina, one would expect to find labelled material which corresponded to the known fatty acids of the tissue upon incubation with labelled mevalonic acid. However, such was not the case in this experiment. Isoprenoid acids have not been reported as normal endogenous components of vertebrate retinas and the relevance of the \textit{in vitro} formation of such acids to \textit{in vivo} metabolism of mevalonic acid and isoprenoid biosynthesis in the retina remains an interesting but unresolved question.
VI. IN VITRO METABOLISM OF CHOLESTEROL
PRECURSORS BY BOVINE RETINA HOMOGENATES:
SMALL-SCALE INCUBATIONS

INTRODUCTION

For over 20 years, cell-free preparations of rat liver have been used for the study of cholesterol biosynthesis (Bucher, 1953; Frantz and Bucher, 1954; Bucher and McGarrah, 1956; cf. Popjak, 1969). The metabolism of de novo substrates as well as higher intermediates of the cholesterol biosynthetic pathway by such preparations has contributed heavily to the present understanding of sterol biosynthesis (Popjak and Cornforth, 1960; Bloch, 1965; Clayton, 1965; Frantz and Schroepfer, 1967; Schroepfer et al., 1972). In particular, the incorporation of mevalonic acid (3,5-dihydroxy-3-methylpentanoic acid) into sterols and other nonsaponifiable lipids proceeds with great efficiency in such preparations. Only the 3R-enantiomer is metabolised (Tavormina and Gibbs, 1956; Tavormina et al., 1956). The early work of Bucher and co-workers (as cited above) established the composition of the buffer and the conditions for the preparation and incubation of cell-free homogenates of rat liver which were conducive to sterol biosynthesis from $^{14}$C-labelled sodium acetate. Such parameters have been maintained and respected by numerous researchers who have utilized the in vitro system with little or no modification for the successful demonstration of the convertibility of a wide range of precursors to cholesterol. The original procedure called for homogenizing freshly-excised rat livers in 2.5 volumes of ice-cold 0.08 M potassium phosphate buffer
(pH 7.4) containing 4.8 mM MgCl₂ and 30 mM nicotinamide; homogenization was performed with a large-clearance (0.5 mm gap), smooth-walled glass Potter-Elvehjem homogenizer with a motor-driven Teflon pestle for 1-1.5 minutes at 300 rpm, on ice (Bucher, 1953; Frantz and Bucher, 1954). It was reported that increasing the duration of the homogenization or utilizing a tight-fitting pestle would result in almost total loss of incorporation of acetate into cholesterol in "cell-free" preparations. It has been confirmed in this laboratory that the homogenization step is extremely critical for the efficient incorporation of acetate and mevalonic acid for the synthesis of digitonin-precipitable material by rat liver homogenates (Raulston, 1979). We routinely use a loose-fitting, Teflon-on-glass homogenizer for the homogenization of freshly-excised, minced rat livers in 2.5 volumes (i.e. 2.5 ml per gram wet weight) of 0.1 M potassium phosphate buffer (pH 7.4) containing 30 mM nicotinamide and 5 mM MgCl₂·6H₂O at 660 rpm for 90 seconds (0-4°C). Both the "cell-free" (500 x g) supernatant preparation (Bucher, 1953; Frantz and Bucher, 1954) and the 9,000 x g supernatant preparation (Bucher and McGarrah, 1956; cf. Popjak, 1969) were reported to require the addition of exogenous pyridine nucleotides and ATP for the conversion of acetate to sterols. Examination of the 9,000 x g supernatant by phase contrast microscopy revealed the presence of mostly "microsomes", with few (if any) mitochondria observed (Bucher and McGarrah, 1956). In this laboratory, we routinely use the following cofactors when incubating aqueous-soluble substrates with the 10,000 x g supernatant: NAD (1 mM), NADP (1 mM), glucose-6-phosphate (G-6-P; 3 mM) and ATP (5 mM). In most instances, the incubation of such rat
liver homogenates under aerobic conditions at 37°C with either acetate or mevalonic acid leads to efficient conversion of the substrate to cholesterol within 1-3 hours of incubation.

In contrast, the "optimal" conditions established for the de novo synthesis of nonsaponifiable lipids in vitro by rat brain homogenates are considerably different (Kelley et al., 1969; Ramsey et al., 1971a,b). These authors report that the addition of nicotinamide or NADP to the incubation system results in the inhibition of the conversion of acetate and mevalonic acid to nonsaponifiable material and digitonin-precipitable material by rat brain homogenates. Furthermore, micromolar levels of cofactors NADH, ATP, G-6-P, magnesium ions and reduced glutathione are apparently required for efficient utilization of the de novo substrates and the exact concentration of each cofactor varies as a function of the type of preparation (i.e. whole brain homogenate, "cell-free" homogenate, or "soluble + microsomes" preparation) and the particular substrate employed. In addition, effective utilization of such substrates by the rat brain preparations requires 12-20 hours of incubation (under aerobic conditions, at 37°C); even so, the majority of the utilizable substrate is converted to isoprenoid hydrocarbons, with lesser formation of "methyl sterols" (i.e. C28, C29 and C30 monohydroxy sterols) and very minor production of C27 sterols (in contrast to the metabolism of these substrates by the rat liver preparations). It is evident that the biosynthetic properties of the rat liver and rat brain homogenate preparations are quite distinct and this fact presents complications when comparing and interpreting results derived from incubations involving these in vitro systems. It has been demonstrated that the
rat brain preparations, like the liver homogenates, require both the soluble and microsomal fractions of the cells for conversion of acetate and mevalonic acid to digitonin-precipitable material (Ramsey et al., 1971a, b). Unlike the liver preparations, however, the brain homogenates from both immature and adult rats appear to be defective in the conversion of squalene to cholesterol (Kelley et al., 1969; Ramsey et al., 1971a, b). These authors have suggested that the "defect" is microsomal in origin. However, it has been reported that rat brain homogenates lack an essential factor which is necessary for sterol biosynthesis and which is present in the 105,000 x g supernatant fraction of rat liver (Shah, 1972; Shah and Johnson, 1974). These authors speculated that the "essential factor" may be synonymous with one or more soluble proteins which have been reported to stimulate the conversion of squalene and certain sterol intermediates to cholesterol in rat liver preparations (Scallen et al., 1971, 1975; Ritter and Demsey, 1970, 1971, 1973; cf. Dempsey, 1974).

This chapter presents a series of preliminary "pilot" experiments designed to investigate various aspects of the in vitro biosynthesis of nonsaponifiable lipids by bovine retina S10 homogenates, with particular emphasis on the synthesis of digitonin-precipitable material (i.e. sterols) from mevalonic acid. In many instances, the incubation results are compared with those obtained from parallel incubations of rat liver S10 homogenates with the given substrate, primarily as an "enzymatic control".
MATERIALS AND METHODS

The preparation of incubation buffer, cofactor stocks, isotopic substrates and $S_{10}$ homogenates of rat livers and bovine retinas has been described previously (see Materials and General Methods). Routinely, incubations were performed in 70 ml capacity screwtop test tubes; each tube typically contained 1.5 ml of $S_{10}$ homogenate, 0.1 ml of concentrated cofactor stock and 0.1 ml of an isotopic substrate solution; the final volume was adjusted to 2.0 ml with buffer. Tubes were flushed with oxygen for 10 seconds, vortexed for 3-5 seconds and then incubated (sealed) at 37° C for various amounts of time, with continuous agitation (Dubnoff Metabolic Incubator, temperature-controlled; 60-80 oscillations/min.).

Incubations were terminated, in general, by the addition of 1 ml of an ethanolic cholesterol solution (4 mg/ml) plus 3 ml of 15% ethanolic potassium hydroxide. Saponification, work-up and extraction of the nonsaponifiable material was performed as previously described (see Materials and General Methods). After determination of the amount of incorporation of the given substrate into nonsaponifiable material, the extracted lipids were analyzed further by thin-layer chromatography and/or digitonin precipitation, as described previously.

The protein concentration of the $S_{10}$ homogenates was determined by the Biuret method, as previously detailed.

Experiment VI-1

Portions of bovine retina $S_{10}$ homogenate (1.5 ml; 10.9 mg protein) and rat liver $S_{10}$ homogenate (1.5 ml; 57.2 mg protein) were incubated, in triplicate, with 0.1 ml of concentrated cofactor stock and 0.1 ml of
a sodium 3RS-[5-3H]-mevalonate solution (9.79 x 10^7 dpm; 8.9 nmoles), at 37° C for 3 hours (final incubation volume adjusted to 2.0 ml with buffer). In separate tubes, portions of liver and retina homogenates (1.5 ml each) were each placed into rapidly-boiling water for 5 minutes, then allowed to cool to room temperature and incubated with the appropriate cofactors and substrate as described above. After the addition of carrier cholesterol (4 mg/tube), the incubation mixtures were saponified and the nonsaponifiable lipids were extracted, as previously described. The extracted lipids were dissolved in 10.0 ml of benzene and aliquots were assayed, in triplicate, for radioactivity and cholesterol (as previously described). Approximately 20 μl from one retina extract and one liver extract were applied to silica gel G TLC plates, along with authentic standards of cholesterol, lanosterol/dihydrolanosterol and squalene (ca. 10 μg each), and developed once with chloroform. The sample lanes were divided into one-centimeter sections, scraped into vials and the radioactivity was measured as usual; chromatographic standards were visualized by spraying the plates with molybdic acid reagent and heating for 15 minutes at 110-120° C. The remainder of the nonsaponifiable lipid extracts were evaporated to dryness under nitrogen, redissolved in 10 ml of acetone–absolute ethanol (1:1, v/v) and precipitated with digitonin, as previously described. After splitting the digitonides (see Materials and General Methods), the free sterols recovered were dissolved in 10.0 ml of benzene and aliquots were assayed, in triplicate, for radioactivity and cholesterol.

**Results**

The incorporation of 3RS-[5-3H]-mevalonate into nonsaponifiable
TABLE VI-1
The Incorporation of Mevalonic Acid into Nonsaponifiable Lipids (NSL) and Digitonin-Precipitable Material by S_{10} Homogenates Prepared from Bovine Retinas and Rat Livers

<table>
<thead>
<tr>
<th>HOMOGENATE</th>
<th>ACTIVITY (dpm x 10^{-7})</th>
<th>SPECIFIC ACTIVITY (dpm/mg Protein x 10^{-5})</th>
<th>PER CENT INCORPORATION</th>
<th>DIGITONIN-PRECIPITABLE MATERIAL (Per Cent of NSL Activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retina</td>
<td>1.95</td>
<td>18.0</td>
<td>19.9</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>1.61</td>
<td>14.9</td>
<td>16.5</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>1.58</td>
<td>14.6</td>
<td>16.2</td>
<td>0.68</td>
</tr>
<tr>
<td>Ave. ± S.D.</td>
<td>1.72 ± 0.21</td>
<td>15.8 ± 1.9</td>
<td>17.5 ± 0.21</td>
<td>0.62 ± 0.07</td>
</tr>
<tr>
<td>Control</td>
<td>0.002</td>
<td>---</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>2.57</td>
<td>4.5</td>
<td>26.3</td>
<td>96.1</td>
</tr>
<tr>
<td></td>
<td>2.75</td>
<td>4.8</td>
<td>28.1</td>
<td>90.0</td>
</tr>
<tr>
<td></td>
<td>2.45</td>
<td>4.3</td>
<td>25.0</td>
<td>93.9</td>
</tr>
<tr>
<td>Ave. ± S.D.</td>
<td>2.59 ± 0.15</td>
<td>4.5 ± 0.3</td>
<td>26.5 ± 1.6</td>
<td>93.3 ± 3.1</td>
</tr>
<tr>
<td>Control</td>
<td>0.004</td>
<td>---</td>
<td>0.04</td>
<td>0.00</td>
</tr>
</tbody>
</table>

\(^a\) Initial substrate, sodium 3RS-[5-^{3}H]-mevalonate (9.79 x 10^{7} dpm, 8.9 nmoles); see text for details of incubation conditions.
FIGURE VI-1

Radio-TLC of the nonsaponifiable lipids obtained from incubations of rat liver (A) and bovine retina (B) S₁₀ homogenates with [5-³H]-mevalonic acid. Chromatographic system: Silica Gel G, developed once with chloroform. Chromatographic standards (in order of increasing $R_f$): cholesterol, lanosterol and squalene.
lipids and digitonin-precipitable material in bovine retina and rat liver homogenates is shown in Table VI-1. While the absolute incorporation of mevalonate into retina nonsaponifiable material was only about 66% of the liver control, the specific activity was about 3.5-fold greater in the retina homogenates than in the liver system. However, only about 0.6% of the retina nonsaponifiable lipid activity was precipitable by digitonin, whereas the liver homogenate extracts contained labelled components which were about 93% precipitable by digitonin.

The radiochromatograms obtained from the nonsaponifiable extracts of retina and liver incubations are shown in Figure VI-1. In the liver radiochromatogram, about 94% of the total activity co-migrated with the cholesterol standard. In contrast, less than 1% of the total activity in the retina extract co-chromatographed with the cholesterol standard. Approximately 47% of the activity migrated in the region between the cholesterol and lanosterol/dihydrolanosterol standards, while about 39% migrated in the region between the C_{30} sterol standard and the squalene standard. The majority of the remaining activity in the retina extract remained at or near the origin.

Discussion

The results of this experiment indicate that, under the given incubation conditions, the bovine retina S_{10} homogenate was able to convert appreciable amounts of mevalonate to nonsaponifiable lipids, but these products were essentially nonprecipitable by digitonin and therefore do not represent typical 3\beta-hydroxy sterols. In contrast, the rat liver homogenate converted about 1.5 times more mevalonate to nonsaponifiable
lips of which about 93% exhibited the chromatographic behavior and chemical properties of C27 monohydroxy sterols. It is important to note that, although the retina and liver homogenates were prepared and incubated under essentially the same conditions, the liver homogenate had a protein concentration about 5.3-fold greater than that of the retina homogenate. Although a large proportion of the total retina nonsaponifiable activity exhibited the chromatographic behavior expected of desmethyl sterols (i.e. C28 and C29 monohydroxy sterols), such sterols should have been precipitable by digitonin (although to a lesser extent than cholesterol) under the conditions employed. It is quite likely that even the minor amount of activity which appeared to be precipitable by digitonin in the retina extracts may have been due to non-sterol products which were occluded by or adsorbed to the digitonide formed with the carrier cholesterol. Considering the nature of the substrate, the solubility of the products in hexane after saponification and their chromatographic properties, the components synthesized by the incubated retina homogenates appear to be isoprenoids, but their structures are unknown. Figure VI-2 shows the chromatographic behavior of several nonsaponifiable isoprenoid compounds. It may be seen that farnesol (a C15 primary, open-chain isoprenol) migrates in the region between cholesterol and lanosterol on the TLC systems used, whereas dolichol (a mixture of long-chain primary isoprenols, 85-105 carbons in length) migrates midway between lanosterol and squalene. Also, geraniol (a C10 primary, open-chain isoprenol) nearly co-migrates with cholesterol, while phytol (a C20 primary, open-chain isoprenol) almost co-migrates with the C30 sterol standard. It should be appreciated
FIGURE VI-2

Thin-layer chromatograms of some nonsaponifiable metabolites of mevalonic acid. Chromatographic system: Silica Gel G, developed once with either benzene-diethyl ether-petroleum ether, 7:3:2 (A) or with chloroform (B). The following compounds (in order of increasing $R_f$ in a given lane) were chromatographed: (1) cholesterol, lanosterol and squalene; (2) dolichol and coenzyme Q10; (3) farnesol and nerolidol; (4) geraniol and phytol; (5) nerol. Due to the presence of at least two isomers, the farnesol standard exhibited two unresolved bands.
from these data that the criterion of co-migration on TLC is insufficient for identification and characterization of organic compounds, especially when derived from a complex and heterogenous source such as an incubation mixture.

Experiment VI-2

Retina S₁₀ homogenates (1.5 ml; 5.7 mg protein) were incubated as before with 0.1 ml of concentrated cofactor stock and 0.1 ml of a solution of sodium 3RS-[5-³H]-mevalonate (3.80 x 10⁷ dpm; 3.46 nmoles; 1.72 µM), plus 0.01 ml of antibiotic stock solution (penicillin-G, 4 mg/ml; streptomycin sulfate, 6 mg/ml; in distilled water). Incubations were carried out in duplicate for each time point (0.5, 1.0, 3.0, 6.0, 10.0, 16.0 and 24.0 hours), at 37° C. Termination, saponification and extraction of the incubation mixtures were performed as previously described. After measurement of the radioactivity incorporated into the nonsaponifiable lipids and colorimetric determination of cholesterol (each performed on triplicate aliquots from each sample), one extract from each time point was precipitated with digitonin while the other was used for radiochromatographic analysis (insufficient material was present in the 0.5- and 1.0-hour samples for chromatographic analysis). The digitonides were split and the recovered free sterols were re-dissolved in 10.0 ml of benzene and assayed for total radioactivity and colorimetrically for cholesterol content, as before.

Results

The incorporation of mevalonate into nonsaponifiable lipids and digitonin-precipitable material as a function of incubation time is
**TABLE VI-2**

Incorporation of Mevalonate into Nonsaponifiable Lipids and Digitonin-Precipitable Material by Retina Homogenates as a Function of Incubation Time

<table>
<thead>
<tr>
<th>INCUBATION TIME (Hours)</th>
<th>NSL ACTIVITY* (dpm x 10^-6)</th>
<th>PER CENT CONVERSION</th>
<th>DIGITONIN-PRECIPITABLE** ACTIVITY (% of NSL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.003</td>
<td>0.01</td>
<td>---</td>
</tr>
<tr>
<td>1.0</td>
<td>0.08</td>
<td>0.22</td>
<td>0.7</td>
</tr>
<tr>
<td>3.0</td>
<td>2.89</td>
<td>7.60</td>
<td>0.5</td>
</tr>
<tr>
<td>6.0</td>
<td>5.91</td>
<td>15.57</td>
<td>0.2</td>
</tr>
<tr>
<td>10.0</td>
<td>5.55</td>
<td>14.62</td>
<td>0.6</td>
</tr>
<tr>
<td>16.0</td>
<td>7.19</td>
<td>18.92</td>
<td>0.7</td>
</tr>
<tr>
<td>24.0</td>
<td>8.30</td>
<td>21.86</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Average of duplicate incubations for each time point.

** Results obtained from one of two samples for each time point.
FIGURE VI-3

Incorporation of [5-\(^3\)H]-mevalonic acid into nonsaponifiable lipids (NSL) and digitonin-precipitable material by bovine retina S10 homogenates as a function of incubation time. Closed circles (● --- ●), nonsaponifiable lipid specific activity (cpm per mg protein); open triangles (Δ --- Δ), percent of the NSL radioactivity which is precipitable with digitonin.
shown in Table VI-2 and Figure VI-3. The amount of labelled substrate incorporated into nonsaponifiable lipids increased almost linearly up to about 6 hours of incubation, after which time the rate of utilization of substrate decreased and finally appeared to approach a plateau by 16 to 24 hours. In contrast, the incorporation of substrate into material which was precipitable by digitonin remained extremely low and nearly constant throughout the entire time course of the incubations (i.e. ca. 0.5 ± 0.2 per cent of the total nonsaponifiable lipid activity was precipitable by digitonin.

The radiochromatograms obtained from the nonsaponifiable material of each incubation are shown in order of increasing incubation time (from 3.0 to 24.0 hours) in Figure VI-4 (Silica Gel G; chloroform). At 3 hours of incubation, the activity was distributed into two major zones, as observed in the previous experiment; about 36% of the activity migrated in the region between cholesterol and lanosterol/dihydrolanosterol, while about 48% migrated in the region between lanosterol/dihydrolanosterol and squalene (Figure VI-4A). By 6 hours of incubation, the percentage of activity distributed in these two major zones had nearly equalized (32% and 38%, respectively); new components were observed near the origin (ca. 8% of the activity) and co-migrating with the squalene standard (ca. 6%) (Figure VI-4B). A further increase in the percentage of activity near the origin (ca. 14%) was noted by 10 hours of incubation (Figure VI-4C), with a concomitant decrease in the relative percentage of activity in the region between cholesterol and the C_{30} sterol standard (to about 28%). The distribution of activity in the other regions remained essentially unchanged. The relative percentage of activity in
FIGURE VI-4

Radio-TLC of the nonsaponifiable lipids obtained from incubations of bovine retina S10 homogenates with [5-\(^3\)H]-mevalonic acid for 3 hours (A), 6 hours (B), 10 hours (C), 16 hours (D) and 24 hours (E). Chromatographic system: Silica Gel G, developed once with chloroform. Chromatographic standards (in order of increasing R\(_f\)): cholesterol, lanosterol and squalene.
the component near the origin (ca. 28%) increased further by 16 hours of incubation (Figure VI-4D), with a slight increase in the percentage of material which migrated between the cholesterol and lanosterol standards (ca. to 32%) and a slight decrease in the percentage of activity which migrated between C_{30} sterols and squalene (to about 21%). Finally, by 24 hours of incubation (Figure VI-4E), the distribution of labelled components had apparently stabilized, with only a slight decrease in the percentage of activity near the origin (to about 24%) and a slight increase in the activity adjacent to the squalene region (to about 26%); the remainder of the radiochromatogram appeared essentially unaltered.

The appearance of a very polar, hexane-extractable nonsaponifiable lipid component in these incubations which increased with increasing incubation time was intriguing. One possibility for its formation was the nonenzymatic conversion of squalene-2,3-epoxide to the corresponding 2,3-diol during basic hydrolysis of the mixtures. To test this hypothesis, an authentic standard of ^3H-labelled squalene-2,3-epoxide (a gift of Dr. Terence J. Scallen, University of New Mexico Medical School; formed by chemical epoxidation of squalene, biosynthesized from tritiated mevalonate; 101.3 cpm/nmole, 92.8 µg) was subjected to the identical conditions of saponification, work-up and extraction as the incubation mixtures. The recovered activity (ca. 92% of the initial material) was applied to a TLC plate, along with authentic standards of cholesterol, lanosterol/dihydrolanosterol, coenzyme Q_{10} and squalene (ca. 10 µg each), and the plate was developed once with benzene-ether-petroleum ether, 7:3:2. Prior to sectioning and assaying the plate for distribution of radioactivity, the plate was exposed to iodine vapors
FIGURE VI-5

Radio-TLC of an authentic sample of $^3$H-labelled squalene-2,3-epoxide before (A) and after (B) saponification with 15% ethanolic KOH. Chromatographic system: Silica Gel G, developed once with benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards (in order of increasing $R_f$): cholesterol, lanosterol, coenzyme Q₁₀ and squalene. On this chromatographic system, squalene-2,3-epoxide cochromatographs with coenzyme Q₁₀.
to visualize the standards as well as the squalene-2,3-epoxide mass (or its derivatives). The results are shown in Figure VI-5. Before hydrolysis (Figure VI-5A), at least 98% of the total activity and mass co-migrated with the CoQ_{10} standard on the TLC system employed. After saponification (Figure VI-5B), the majority of the recovered activity and mass (ca. 91%) appeared unchanged, while at least two other components of more polar nature (ca. 3-4% each) were also observed. Thus, the polar constituent observed in the retina homogenate extracts did not appear to be formed as an artifact of saponification from squalene-2,3-epoxide, since the epoxide was not significantly convertible to such polar material under the conditions employed routinely for saponification.

Discussion

Bovine retina homogenates synthesized nonsaponifiable products from mevalonate continuously over the duration of the incubation period (24 hours), with the majority of the synthesis taking place within the first 10 hours of the incubation. However, even over extended incubation times, the amount of digitonin-precipitable material synthesized from mevalonate was very minor, representing less than 1% of the total incorporation of substrate into nonsaponifiable material. With increasing incubation time, increasing amounts of a very polar component were synthesized which became a major constituent of the labelled nonsaponifiable material. This component was shown not to be derived from squalene-2,3-epoxide as an artifact of the saponification procedure. It is possible that the polar component was a product of autoxidation of other accumulated labelled components (and, as such, could represent
more than one kind of molecule), although the precursor-product relationship was not well defined. A minor component have the chromatographic properties of squalene was observed after 6 hours of incubation; the relative percentage of activity in this component apparently reached a level by 6 hours (ca. 6%) which did not change appreciably over longer incubation times. The results of this experiment indicate that increasing the duration of the incubation does not result in an enhancement of incorporation of mevalonate into sterol products in bovine retina homogenates. The biosynthetic activity of this preparation is apparently relatively stable under the incubation conditions employed, with respect to nonsaponifiable lipid biosynthesis. Based on this finding, a routine incubation time of 24 hours was adopted for further such incubations using the retina S_{10} homogenate system.

Experiment VI-3

The following experiment was performed to examine the effect of varying the level of cofactors and antibiotics on the synthesis of nonsaponifiable lipids and digitonin-precipitable material by retina homogenates, using sodium 3RS-[5-^{3}H]-mevalonate as the substrate. The S_{10} homogenates were prepared with 100 mM potassium phosphate, pH 7.4 (no nicotinamide or magnesium ions). Each incubation mixture contained 1.4 ml of retina homogenate (6.2 mg protein), 0.10 ml of tritiated mevalonate stock (2.95 \times 10^{7} \text{ cpm}; 5.3 \text{ nmols}; 2.65 \mu M), adjusted to a final incubation volume of 2.00 ml with appropriate cofactors and buffer. Each incubation condition was performed in duplicate, and all assays were performed in triplicate. The following incubation media were tested:
(A) "complete liver medium" (100 mM potassium phosphate, pH 7.4, plus 30 mM nicotinamide, 5 mM MgCl₂·6H₂O, 1 mM NAD, 1 mM NADP, 3 mM glucose-6-P and 5 mM ATP); (B) medium A minus nicotinamide; (c) media A minus nicotinamide and NADP; (D) 100 mM potassium phosphate, pH 7.4, plus 5 mM MgCl₂·6H₂O; (e) medium D plus 30 mM nicotinamide; (F) 100 mM potassium phosphate, pH 7.4; (G) medium A plus penicillin-G (20 μg/ml) and streptomycin sulfate (30 μg/ml); (H) 100 mM potassium phosphate, pH 7.4, plus 25 μM MgCl₂·6H₂O, 14 μM NADH, 2 μM ATP, 7 μM glucose-6-P and 16 μM glutathione; (I) boiled control (homogenate in medium A, heated in rapidly-boiling water for 10 minutes). All incubations were carried out at 37°C for 24 hours in sealed tubes (flushed with oxygen prior to incubation). Termination of the incubation, saponification, work-up and extraction of nonsaponifiable material and precipitation of sterols with digitonin were performed as described previously.

Results

The effect of variations in the incubation medium on incorporation of mevalonate into nonsaponifiable lipids and digitonin-precipitable material is exhibited in Table VI-3. Omitting magnesium ions, NAD, NADP, ATP or glucose-6-P from the medium, or using micromolar levels of these cofactors (as suggested by the results of Kelley et al., 1969) gave results which were essentially indistinguishable from those obtained with the boiled control. The presence of exogenous nicotinamide (at the level of 30 mM) actually decreased the incorporation of mevalonate into nonsaponifiable material by 50% (relative to medium B). A dramatic stimulation of nonsaponifiable lipid biosynthesis was observed (about 1.8-fold, relative to medium A) when penicillin-G and streptomycin
TABLE VI-3

The Effects of Altering the Concentrations of Incubation Media Constituents on the Incorporation into Nonsaponifiable Lipids (NSL) and Digitonin-Precipitable Material by Bovine Retina Homogenates

<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>ACTIVITY (dpm x 10^-6)</th>
<th>% of MAXIMUM</th>
<th>ACTIVITY (dpm x 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.80</td>
<td>50.3</td>
<td>0.09</td>
</tr>
<tr>
<td>B</td>
<td>7.55</td>
<td>100.0</td>
<td>0.28</td>
</tr>
<tr>
<td>C</td>
<td>5.83</td>
<td>77.2</td>
<td>0.28</td>
</tr>
<tr>
<td>D</td>
<td>0.002</td>
<td>0.03</td>
<td>ND</td>
</tr>
<tr>
<td>E</td>
<td>0.002</td>
<td>0.03</td>
<td>ND</td>
</tr>
<tr>
<td>F</td>
<td>0.002</td>
<td>0.02</td>
<td>ND</td>
</tr>
<tr>
<td>G</td>
<td>6.74</td>
<td>89.2</td>
<td>0.22</td>
</tr>
<tr>
<td>H</td>
<td>0.002</td>
<td>0.03</td>
<td>ND</td>
</tr>
<tr>
<td>I</td>
<td>0.002</td>
<td>0.02</td>
<td>ND</td>
</tr>
</tbody>
</table>

a A, "complete liver medium"; B, medium A minus nicotinamide; C, medium B minus NADP; D, 100 mM potassium phosphate buffer, pH 7.4, plus 5 mM MgCl_2·6H_2O; E, medium D plus 30 mM nicotinamide; F, 100 mM potassium phosphate buffer, pH 7.4; G, medium A plus penicillin-G (20 μg/ml) and streptomycin sulfate (30 μg/ml); H, 100 mM potassium phosphate, pH 7.4, plus 25 μM MgCl_2·6H_2O, 14 M NADH, 2 μM ATP, 7 μM G-6-P and 16 μM glutathione; I, boiled control (medium A); see text for details.

b Average of duplicate incubations.

c Values normalized to the activity from medium B (100%).

d ND, not determined.
sulfate were included in the incubation medium (medium G). However, none of the modifications of the incubation medium routinely used for the rat liver homogenate system (medium A) significantly enhanced the incorporation of mevalonate into digitonin-precipitable material.

Aliquots of the nonsaponifiable lipids obtained from incubations employing media A, B and G were chromatographed on TLC plates, along with authentic standards of cholesterol, 4a-methyl cholesterol, lanosterol/dihydrolanosterol and squalene; the plates were developed once in benzene-ether-petroleum ether, 7:3:2, and the plates were assayed for radioactivity distribution and for mass as usual. The results are shown in Figure VI-6. Comparing the three major areas of radioactivity (shaded regions), the deletion of nicotinamide from the incubation medium (Figure VI-6B) appeared to increase the percentage of radioactivity in the very polar material (from about 42% to about 50%) as well as in the components which migrated in the "methyl sterols" region (from about 12% to about 24%), relative to the distribution observed in the radiochromatogram from the incubation with medium A (Figure VI-6A). Adding antibiotics to medium A (Figure VI-6C) caused a slight decrease in the percentage of the very polar activity (from about 42% to about 36%) and significant increase in the percentage of activity which migrated in the "methyl sterols" region (from about 12% to about 24%). The percentage of radioactivity in the region of the chromatograms immediately adjacent to and including the region of squalene remained essentially constant in each case (ca. 32% of the total nonsaponifiable activity). The diffuse distribution of activity over the entire plate in each case precluded more quantitative
FIGURE VI-6
Radio-TLC of the nonsaponifiable lipids obtained from incubations of bovine retina S10 homogenates with [5-3H]-mevalonic acid in (A) the usual medium used for incubations of rat liver S10 homogenates, (B) the usual medium minus nicotinamide, and (C) the usual medium plus penicillin-G and streptomycin sulfate. Chromatographic system: Silica Gel G, developed once with benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards (in order of increasing $R_f$): cholesterol, 4α-methyl cholesterol, lanosterol and squalene. See text for details.
comparisons. Since the identities of the labelled components remain unknown, further interpretation of the above data is not warranted. The lack of appreciable activity in the other samples precluded further analysis.

Discussion

The results of this experiment demonstrate that the cofactor requirements of the bovine retina S_{10} homogenate preparation, with regard to nonsaponifiable lipids biosynthesis from mevalonate, are quite different from those of the liver homogenate system. Nicotinamide, at the level of 30 nM, definitely inhibits the incorporation of mevalonate into retina nonsaponifiable lipids; this cofactor is included routinely in incubations of the rat liver homogenate preparation, presumably to attenuate degradation of purine nucleotides during the incubation (cf. Bucher and McGarrahan, 1956). A similar effect of nicotinamide on the incorporation of mevalonate into nonsaponifiable lipids and digitonin-precipitable material in rat brain homogenates had been observed previously (Kelley et al., 1969). Omitting NADP from the retina homogenate, however, was detrimental to the utilization of mevalonate, in contrast to the results reported for the rat brain "cell-free" system (Kelley et al., 1969). The "optimal" incubation medium for the rat brain homogenate preparation consisted of the following components: 0.15 M potassium phosphate, pH 7.4, 24.6 μM MgCl_2·6H_2O, 14.1 μM NADH, 1.7 μM ATP, 6.6 μM glucose-6-P and 16.3 μM glutathione (Kelley et al., 1969). Using a similar medium for incubation of the retina homogenate (medium C), the incorporation of mevalonate into nonsaponifiable material was essentially indistinguishable from the boiled control;
therefore, the cofactor requirements determined for the rat brain preparation also appear to be quite different from those of the retina system. In striking contrast to the results presented here, the rat brain homogenate system (under "optimal" incubation conditions) synthesized substantial amounts of digitonin-precipitable material (ca. 38% of the nonsaponifiable lipid activity), although cholesterol (identified by TLC, GLC, and dibromide purification of the digitonin-precipitable material) only accounted for about 2-3% of the digitonin-precipitable material. Even under such "optimal" conditions and extended incubation times (i.e. 20 hours), the major amount of nonsaponifiable activity was distributed in isoprenoid hydrocarbons (mostly squalene), with lesser amounts of "lanosterol-like" activity and very little "cholesterol-like" components (Kelley et al., 1969). The only common feature shared by both rat brain and bovine retina homogenate systems was the apparent requirement for extended incubation times (i.e. 16-24 hours for retina preparations, 12-20 hours for brain preparations), with regard to "optimal" incorporation of mevalonate into nonsaponifiable lipids. Unlike the brain homogenates, however, extended incubation times did not enhance the incorporation of mevalonate into digitonin-precipitable material by retina homogenates.

A striking finding in these incubations was the apparent stimulation of retina nonsaponifiable lipid synthesis in the presence of penicillin-G and streptomycin sulfate. The reason for the antibiotic effect on the system is not understood at this time. Kelley et al. (1969) reported that such antibiotics had no effect on the ability of rat brain homogenates to incorporate mevalonic acid into nonsaponifiable
or digitonin-precipitable material. It is known that the rate of permeation of organic acids through the cell membrane is a function of the lipid solubility of the acid and relies on diffusion, in the absence of a specific transport mechanism (Collander et al., 1931). Highly dissociated carboxylic acids, such as the citric acid cycle intermediates, have very poor lipid solubilities and penetrate intact yeasts very poorly, if at all (Lynen, 1939; Lynen and Kalb, 1955; Oura et al., 1959). The presence of a hydroxyl group on such carboxylic acids (as is present on mevalonic acid) decreases the lipid solubility and membrane permeability of such solutes substantially (Oura et al., 1959; Soumalainen et al., 1969; cf. Soumalainen and Oura, 1971). For these reasons, it is highly unlikely that microorganisms are involved in the observed effect of antibiotics on the retina incubation system since such microorganisms (i.e. bacteria, yeasts, etc.) would be expected to be relatively impermeable to mevalonic acid. In particular, yeasts are unlikely to be involved since they generally prefer much different incubation conditions for growth than employed here (i.e. pH 4.0-6.0, vigorous aeration and agitation of the medium) and the viability of many strains of yeasts at 37° C is quite variable (Morris, 1958); for instance, S. cerevisiae have an optimal growth temperature of about 28° C and the growth rate rapidly declines above 30° C (Richards, 1934).

It is apparent from these results that the bovine retina S10 homogenate preparation has requirements for magnesium ions, ATP, NAD (or NADH), NADP (or NADPH) and glucose-6-phosphate, with regard to the synthesis of nonsaponifiable lipids using mevalonic acid as a precursor. No attempt was made here to systematically investigate the "optimum"
levels of each cofactor in the incubation system and conditions have not been found which support the synthesis of appreciable amounts of digitonin-precipitable material by the retina homogenate preparation in vitro.

Experiment VI-4

The following experiment was performed to examine the relative effectiveness of incorporation of three de novo substrates (mevalonate, acetate and β-hydroxybutyrate) into nonsaponifiable lipids by bovine retina homogenates and to assess the possible contribution of substrate metabolism by microorganisms in these incubations.

Sodium 3RS-[2-3H]-mevalonate was prepared from the corresponding lactone (176 mCi/mmol) as previously described (ca. 73.6 μCi in 1.1 ml of a 5% sodium bicarbonate solution). Sodium [2-14C]-acetate (1 mCi; 55 mCi/mmol) was dissolved in 10.0 ml of incubation buffer (100 mM potassium phosphate, pH 7.4, containing 5 mM MgCl2·6H2O); 0.05 ml of this stock was diluted to 5.0 ml with incubation buffer. Potassium D (-)-β-[3-14C]-hydroxybutyrate (0.2 ml of a 1:1, v/v, ethanol-water solution, 0.1 mCi/ml; 18.4 mCi/mmol) was evaporated at room temperature under a stream of nitrogen and dissolved in 5.0 ml of incubation buffer.

Each incubation tube contained 1.5 ml of retina homogenate (7.1 mg protein; prepared in incubation buffer, as previously described, without nicotinamide), 0.1 ml of concentrated cofactor stock (final concentrations: NAD, 1 mM; NADP, 1 mM; G-6-P, 3 mM; ATP, 5 mM; in incubation buffer) and 0.4 ml of the desired isotopic substrate (initial activity: [2-3H]-mevalonate, 3.10 x 10^7 dpm; [2-14C]-acetate, 9.68 x 10^6 dpm; [3-14C]-hydroxybutyrate, 3.24 x 10^6 dpm; initial concentration, ca.
20 μM each). The final incubation volume was adjusted to 2.01 ml with 0.01 ml of either penicillin/streptomycin stock (20 μg/ml) penicillin-G, 30 μg/ml streptomycin sulfate, final incubation concentrations; in incubation buffer), cycloheximide (final incubation concentration, 50 μg/ml; in incubation buffer) or incubation buffer. Each incubation condition was performed in quadruplicate (24 hours at 37°C, as described previously). The following controls were also performed (in duplicate): (1) buffer control (without homogenates or antibiotics); (2) incubation buffer inoculated with wild-type yeast {S. cerevisiae, D273-10B; supplied by Dr. Neil Morgan, Rice University); (3) yeast-inoculated incubation buffer, containing 50 μg/ml of cycloheximide. The controls were incubated with [2-3H]-mevalonate (20 μM; 3.10 x 10^7 dpm).

At the end of the incubation period, 0.025 ml aliquots were withdrawn from tubes representative of each incubation condition and plated onto agar culture slabs containing yeast extracts, mineral salts medium and 2% ethanol as a carbon source (plates prepared and samples applied by Dr. Neil Morgan); the plates were allowed to incubate in the dark at room temperature for 48 hours, and then examined for the presence of yeast and/or bacterial colonies (examination supervised by Dr. Neil Morgan). The incubation mixtures were then terminated, saponified and the nonsaponifiable material extracted as previously described. The nonsaponifiable material from selected extracts was analyzed by TLC (silica gel G; benzene-ether-petroleum ether, 7:3:2), as previously described; the sterols (including the carrier cholesterol added prior to saponification) were then precipitated with digitonin, as described previously.
<table>
<thead>
<tr>
<th>SUBSTRATE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ADDITIONS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NSL ACTIVITY (Ave. dpm + S.D. x 10&lt;sup&gt;-6&lt;/sup&gt;)</th>
<th>SUBSTRATE CONVERSION (Ave. %) (% of Maximum)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>DIGITONIN-PRECIPITABLE&lt;sup&gt;d&lt;/sup&gt; MATERIAL (% of NSL ± S.D.)</th>
</tr>
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<tbody>
<tr>
<td>MVA</td>
<td>Buffer</td>
<td>2.77 ± 0.48</td>
<td>8.96 ± 65.09</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Pen/Strep</td>
<td>4.26 ± 0.29</td>
<td>13.75 ± 100.00</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Cyclohex</td>
<td>2.96 ± 0.25</td>
<td>9.54 ± 69.45</td>
<td>0.33 ± 0.03</td>
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<tr>
<td>BHB</td>
<td>Buffer</td>
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<td>0.12 ± 0.83</td>
<td>N.D.</td>
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<tr>
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<td>Pen/Strep</td>
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<td>0.20 ± 1.34</td>
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<tr>
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<td>0.11 ± 0.76</td>
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<td>Cyclohex</td>
<td>0.0004 ± 0.0002</td>
<td>0.004 ± 0.03</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

<sup>a</sup> MVA, sodium [2-<sup>3</sup>H]-mevalonate (3.10 x 10<sup>7</sup> dpm); BHB, D(-)-β-[3-<sup>14</sup>C]-hydroxybutyrate (3.24 x 10<sup>6</sup> dpm); Acetate, sodium [2-<sup>14</sup>C]-acetate (9.68 x 10<sup>6</sup> dpm); all substrate concentrations ca. 20 μM.

<sup>b</sup> Pen/Strep, penicillin-G (20 μg/ml) plus streptomycin sulfate (30 μg/ml); Cyclohex, cycloheximide (50 g/ml); all tubes contain 7.1 mg of retina protein.

<sup>c</sup> Values normalized to substrate conversion in the incubation containing MVA and Pen/Strep.

<sup>d</sup> N.D., not determined.
Results

The incorporation of the labelled substrates into nonsaponifiable material and digitonin-precipitable material is exhibited in Table VI-4. Of the three substrates examined, only mevalonate was efficiently incorporated into nonsaponifiable material by the retina homogenates. Acetate showed essentially negligible incorporation, while \( \gamma \)-hydroxybutyrate exhibited marginal but definite incorporation into nonsaponifiable material. In each case, the incorporation of activity into nonsaponifiable material in the presence of cycloheximide was the same as in the absence of antibiotics; however, in the presence of penicillin/streptomycin, there was a significant increase in the total amount of nonsaponifiable activity, using mevalonate as the substrate (about 1.5-fold; 0.001 \( \leq p \leq 0.01 \), \( n = 4 \)) (the apparent effect using the other substrates was not considered significant). It was apparent that neither cycloheximide nor penicillin/streptomycin exerted a significant effect on the synthesis of digitonin-precipitable material (the apparent decrease in digitonin-precipitable activity in the presence of penicillin/streptomycin was not statistically significant; 0.05 \( \leq p \leq 0.1 \), \( n = 4 \)). As previously observed, incubation buffer alone did not convert significant amounts of mevalonate to nonsaponifiable material.

Examination of the plated homogenate mixtures after 48 hours of incubation on agar supports revealed that the penicillin/streptomycin mixture effectively prevented the growth of bacteria (also, no yeast were observed on the "bacteria-free" plates); in the absence of penicillin/streptomycin, massive bacterial colonies were observed on the plate, but no yeast colonies were observed. Even in the yeast-inoculated control
(without cycloheximide), very minor yeast proliferation was apparent and the size of the colonies was much smaller than normally observed for this strain of yeast grown under the specified conditions. Regardless of the presence or absence of bacteria or yeast in the incubation media, these controls failed to convert appreciable amounts of mevalonate to nonsaponifiable products (average NSL activity <2000 dpm; substrate conversion <0.006%).

Radiochromatograms obtained from the nonsaponifiable extracts of selected retina homogenates, where mevalonate was the isotopic substrate, are shown in Figure VI-7. The presence of the antibiotics had little effect on the overall relative distribution of labelled components in the nonsaponifiable material; however, an increase in the percentage of activity incorporated into the "very polar component" (relative to the incubation in the absence of the antibiotics) was noted. In all cases, significant amounts of activity were incorporated into material which co-migrated with the squalene standard as well as in the region immediately adjacent to the squalene standard. The percentage of activity in the "methyl sterols" region was relatively unaffected by the presence of antibiotics (ca. 18-21% of the total nonsaponifiable material). Less than 2% of the total nonsaponifiable activity was observed to co-migrate with the cholesterol standard, regardless of the presence or absence of antibiotics; this minor activity was most probably due to "trailing" of the material which migrated just ahead of the cholesterol standard (i.e. in the "methyl sterols" region).

Although incubation buffer alone supported bacterial growth, little or no conversion of mevalonate to nonsaponifiable products was observed.
FIGURE VI-7

Radio-TLC of the nonsaponifiable lipids obtained from incubations of bovine retina $S_{10}$ homogenates with $[5-^3\text{H}]$-mevalonic acid (A) in the absence of antibiotics, (B) in the presence of penicillin-G and streptomycin sulfate and (C) in the presence of cycloheximide. Chromatographic system: Silica Gel G, developed once with benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards (in order of increasing $R_f$): cholesterol, lanosterol and squalene.

FIGURE VI-8

Radio-TLC of the saponifiable lipids obtained from the buffer control incubation with $[5-^3\text{H}]$-mevalonic acid. Chromatographic system: Silica Gel G, developed once with hexane-diethyl ether-glacial acetic acid, 80:20:1. Chromatographic standards (in order of increasing $R_f$): oleic acid and squalene. Under these conditions, mevalonic acid and its lactone remain at the origin of the plate.
The saponified aqueous phase from the buffer control (no homogenate, no antibiotics) was acidified by dropwise addition of concentrated HCl (on ice, with continuous stirring) and extracted three times with two volumes of ether; the combined extracts were washed once with 0.2 volumes of water and then dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure and evaporated to dryness under nitrogen. The residue was redissolved in 5.0 ml of chloroform and an aliquot (ca. 10-15 μl) was applied to a TLC plate, along with authentic standards of oleic acid, mevalonic acid lactone and squalene, and developed once in hexane-ether-glacial acetic acid, 80:20:1. The sample lane was then sectioned into one-centimeter sections, scraped into scintillation vials and the radioactivity of each section was measured as usual; the standard lane was sprayed with molybdic acid reagent and charred for 30 minutes in an oven to visualize mass. The radiochromatogram (Figure VI-8) showed that all the recovered radioactivity remained at the origin, with no evidence of other labelled components in the region of free fatty acids or other regions of the plate.

Discussion

The results of this experiment demonstrate that mevalonate is a preferred substrate for nonsaponifiable lipid biosynthesis by bovine retina homogenates, in comparison with acetate and β-hydroxybutyrate. The extremely low incorporation of acetate into nonsaponifiable material in these incubations is not surprising, since acetate (or its CoA ester) is a common precursor for a large variety of biological molecules, many of which are water-soluble. In addition, it is possible that the
formation of labelled acetyl-CoA in this system was limiting, due to insufficient free CoA and/or acetyl-CoA synthetase activity. Even if labelled acetyl-CoA was formed, there may have been metabolic "blocks" in the conversion of acetyl-CoA to mevalonate (for a review of these enzymatic steps, see Popjak and Cornforth, 1960; Bloch, 1965; Clayton, 1965). The enzymatic conversion of β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) to mevalonate, as mediated by the enzyme HMG-CoA reductase, is known to be a very salient locus of regulation of sterol biosynthesis (as well as terpenoid biosynthesis, in general) in animal tissues, plants and yeast (for a review, see Rodwell et al., 1973). The presence of HMG-CoA reductase activity in retina has not been reported in the literature, and the conversion of acetate or acetyl-CoA to mevalonate has not been reported for this tissue. This problem alone would require extensive investigation and is not within the scope of this thesis. Ramsey et al. (1971a,b) have reported that both sodium [2-14C]-acetate and [2-14C]-mevalonic acid are incorporated into nonsaponifiable material in vitro in newborn, 15-day-old and adult rat brains, with a distinct preference for incorporation of mevalonic acid. Although both substrates exhibited significant metabolism to nonsaponifiable material, most of the activity was not precipitable by digitonin (in the case of in vitro incubations) and the major labelled nonsaponifiable component exhibited the chromatographic behavior of squalene by TLC; material which exhibited the chromatographic behavior of "methyl sterols" was also observed, with lesser amounts of material which behaved like cholesterol.

Ketone bodies (β-hydroxybutyrate and acetoacetate) have been
implicated as important precursors in brain lipid metabolism, especially in the developing animal (Edmond, 1974; Patel and Owen, 1976; Yeh et al., 1977). In particular, Edmond (1974) has shown that, in the developing rat, β-hydroxybutyrate is the preferred substrate for brain sterol and fatty acid biosynthesis \textit{in vivo} (as well as in spinal cord and skin), in comparison to acetoacetate, acetate and mevalonate, when introduced by subcutaneous injection. Yeh et al. (1977) have reported that the majority of the metabolized ketone bodies in developing rat brain are incorporated into polar lipids (predominantly phospholipids) and the utilization of ketone bodies for lipid biosynthesis in brain decreases as the animal develops to adulthood. In their proposed scheme for ketone body utilization by brain tissue, Yeh et al. (1977) suggest that β-hydroxybutyrate must first be converted to acetoacetate by β-hydroxybutyrate dehydrogenase, a mitochondrial enzyme (Klee and Sokoloff, 1967; Page et al., 1971) before it can be metabolized further to lipids (ultimately via acetyl-CoA). In this regard, it is curious that incubations of the retina S10 homogenates with labelled β-hydroxybutyrate exhibited a definite (although very minimal) incorporation of the substrate into nonsaponifiable material, even though this preparation is putatively devoid of mitochondria and also did not incorporate the corresponding amount of labelled acetate into nonsaponifiable material in parallel incubations. The reasons for the observed levels of incorporation of acetate and β-hydroxybutyrate into nonsaponifiable lipids by the retina homogenates are not understood at this time. The incorporation of these substrates into digitonin-precipitable material was not examined, due to the paucity of activity in the total nonsaponifiable
material derived from these substrates.

Although it was evident that bacterial growth could be substantial, especially over 24 hours of incubation, incubations in which bacteria proliferated did not appear to metabolize mevalonate significantly. The apparent stimulation of nonsaponifiable lipid biosynthesis from mevalonate by the penicillin/streptomycin mixture was observed again in these incubations. Yeast did not appear to proliferate in these incubations, even when the medium was inoculated with wild-type S. cerevisiae (in fact, bacteria proliferated in these inoculated incubations). As suggested before, the pH and temperature of the incubation medium was not suited to rapid growth of this strain of yeast. Even in the yeast-inoculated incubations, no metabolism of mevalonate was apparent. These results suggest that the metabolism of mevalonate by the retina homogenates is due to the endogenous biosynthetic capacity of the tissue and is not due to metabolism of the substrate by microorganisms, even over extended incubation times.

The presence of cycloheximide in the incubation medium had no observable effect on the overall incorporation of mevalonate into nonsaponifiable lipids or digitonin-precipitable material, and the relative distribution of labelled components in the nonsaponifiable material appeared to be minimally affected. Since cycloheximide is a known inhibitor of eukaryotic protein synthesis, the results of this experiment and those of Experiment VI–2 seem to indicate that the metabolism of mevalonate by the retina S_{10} preparation is performed by a relatively stable enzyme pool (i.e. mevalonate was metabolized continuously over a period of 24 hours of incubation, in the absence of new protein synthesis).
The lack of an effect by cycloheximide further supports the suggestion that the presence of yeasts is not an important consideration in these incubations.

**Experiment VI-5**

In previous incubations, radiochromatograms of the nonsaponifiable lipids derived from retinal metabolism of mevalonate exhibited three major regions of radioactivity: (1) activity near the origin (i.e. the "very polar component"); (2) activity in the "methyl sterols" region; (3) activity in the region approximately midway between the regions of C$_{30}$ sterols and squalene. The following experiment examined the conversions of these radioactive products to cholesterol by rat liver homogenates.

A preparative incubation was performed by incubating an S$_{10}$ retina homogenate (5.0 ml, 4.7 mg protein/ml) with sodium [2-$^3$H]-mevalonate (ca. 7 x 10$^6$ cpm; 6.3 μCi) and appropriate cofactors (no nicotinamide, no antibiotics) for 24 hours at 37°C in a 125-ml capacity erlenmeyer flask (final incubation volume, 5.45 ml). The incubation was terminated by the addition of an equal volume of 15% ethanolic potassium hydroxide and the mixture was saponified under nitrogen for 3 hours at 75-80°C. The nonsaponifiable lipids were extracted as previously described and concentrated under nitrogen to about 0.5-1.0 ml (in hexane); the entire sample was applied to a silica gel G TLC plate (20 cm x 20 cm x 0.25 mm) and developed once in benzene-ether-petroleum ether, 7:3:2 (standards of cholesterol, lanosterol/dihydrolanosterol and squalene were run in an adjacent lane of the plate). A two-centimeter wide lane of the plate was sectioned into one-centimeter portions along
the length of the plate and the sections were assayed as usual for radioactivity; the standards were visualized by exposure to iodine vapors, briefly. The resulting radiochromatogram (Figure VI-9) exhibited the expected three major zones of radioactivity (referred to hereafter as components A, B and C, in the order of decreasing polarity, respectively). The regions of the TLC plate corresponding to these three components (shaded areas, Figure VI-9) were scraped into erlenmeyer flasks and each sample was eluted with 75 ml of chloroform; after filtration (Whatman #2 paper), the silica gel residues were eluted three times more with 25-ml portions of chloroform. Solvent was removed under reduced pressure and the samples were redissolved in 10.0 ml of benzene. Aliquots were removed for measurement of recovered radioactivity and for TLC analysis (recovered activity: component A, $3.42 \times 10^5$ dpm; component B, $1.69 \times 10^5$ dpm; component C, $2.56 \times 10^5$ dpm).

Each sample was evaporated to dryness under nitrogen in a 20 ml capacity glass vial and redissolved in 0.5 ml of propylene glycol. Portions of a rat liver homogenate (4.0 ml, 37.3 mg protein/ml; prepared as described previously) were added to each vial, along with 0.5 ml of a concentrated cofactor stock (prepared as previously described to yield the optimal levels of NAD, NADP, G-6-P and ATP for this system) and the mixture was vortexed (10 seconds at low speed, 0-4°C) to insure complete mixing of the substrates. The incubation mixtures were transferred by pipet to 125-ml capacity screwtop erlenmeyer flasks and were then flushed with oxygen for 10 seconds, sealed and incubated for 3 hours at 37°C with continuous shaking (80 oscillations/min.). The incubations were terminated by the addition of 0.1 ml of an ethanolic cholesterol
FIGURE VI-9
Preparative radio-TLC of the nonsaponifiable lipids obtained from an incubation of a bovine retina S_{10} homogenate with [2-\textsuperscript{3}H]-mevalonic acid. Chromatographic system: Silica Gel G, developed once with benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards (in order of increasing R_{f}): cholesterol, lanosterol and squalene. The shaded regions correspond to the areas from the TLC plate: component "NSL-A" (cross-hatched), component "NSL-B" (stippled) and component "NSL-C" (solid).

FIGURE VI-10
Radio-TLC of the individual nonsaponifiable components obtained by preparative TLC of the nonsaponifiable lipids from an incubation of a bovine retina S_{10} homogenate with [2-\textsuperscript{3}H]-mevalonic acid (see Figure VI-9). (A) component "NSL-A"; (B) component "NSL-B"; (C) component "NSL-C". Chromatographic system: Silica Gel G, developed once with benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards (in order of increasing R_{f}): cholesterol, lanosterol and squalene.

FIGURE VI-11
Radio-TLC of the nonsaponifiable products obtained from incubations of rat liver S_{10} homogenates with (A) component "NSL-A", (B) component "NSL-B" and (C) component "NSL-C" (see Figure VI-10). Chromatographic system: Silica Gel G, developed once with benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards (in order of increasing R_{f}): cholesterol, lanosterol and squalene.
solution (4 mg/ml, in absolute ethanol) plus 5.0 ml of 15% ethanolic potassium hydroxide; saponification and extraction of the nonsaponifiable material was carried out as previously described. The nonsaponifiable material was redissolved in 5.0 ml of benzene and aliquots were assayed for total radioactivity and for distribution of radioactivity by thin-layer chromatography, as before.

Results

The isolation of nonsaponifiable components A, B and C by preparative TLC is shown in Figure VI-10. By comparison with Figure VI-9, it is apparent that each of the major zones of radioactivity was recovered relatively intact, with little evidence of decomposition. The results of the incubation of the three components with the rat liver homogenate are shown in Figure VI-11. While component A appeared to be relatively unchanged by the incubation (Figure VI-11A), the radiochromatograms of the nonsaponifiable lipids recovered from incubations of components B and C (Figure VI-11B and VI-11C, respectively) indicated that extensive metabolism of these components had taken place. About 60% of the recovered nonsaponifiable activity from component B appeared to be unchanged, while the majority of the remaining activity trailed to the origin, suggesting degradation of the material. In addition to the apparent degradation of the recovered nonsaponifiable material derived from component C, about 32% of the activity was observed to migrate in the "methyl sterols" region (i.e. like component B); roughly 34% of the activity appeared to be unchanged. No distinct trend toward the conversion of the components to material with the chromatographic properties of cholesterol was apparent.
It was found that the overall recoveries of nonsaponifiable activities from the incubations was extremely poor: component A, $3.4 \times 10^4$ dpm (10.0% recovery); component B, $1.3 \times 10^4$ dpm (8.1% recovery); component C, $1.2 \times 10^4$ dpm (4.8% recovery). This suggested that the gross majority of the material in each incubation was converted to hexane-insoluble (i.e. polar) products by the liver homogenate (after saponification).

Discussion

The results of this experiment suggest that the nonsaponifiable products derived by incubation of bovine retina homogenates with mevalonate are not appreciably convertible to material which has the chromatographic properties of $C_{27}$ monohydroxy sterols, upon incubation with rat liver homogenates. This suggests (but does not prove) that these components are not intermediates in cholesterol biosynthesis, although they are metabolites of mevalonate. There was some indication that component C was converted to material having the chromatographic properties of component B; however, the identity of the constituents of each "component" is not known and the probable multiplicity of constituents in each "component" precludes further interpretation of these results. It is possible that the nonsaponifiable components formed by the retina homogenate from mevalonate are structurally related to known intermediates in the cholesterol biosynthetic pathway; for instance, farnesol is not a known precursor of cholesterol, but it is derived from farnesyl pyrophosphate which is a known intermediate in terpene and sterol biosynthesis (cf. Christophe and Popjak, 1961; Popjak et al., 1961, 1962).

It is obvious that a further understanding of the metabolism of
mevalonate by the retina homogenates would require rigorous structural
analysis of the nonsaponifiable components, establishing the multiplicity
of products, their chemical structures and possible relationship to the
sterol biosynthetic pathway.

Experiment VI-6

The following experiment was performed as a preliminary investiga-
tion to examine the possibility that an inhibitor of sterol biosynthesis
is present in the retina S_{10} preparation. The S_{10} homogenates of rat
liver and bovine retinas were prepared as previously described in 0.1 M
potassium phosphate buffer (pH 7.4) containing 5 mM MgCl_2·6H_2O (no nico-
tinamide). Each incubation mixture contained 1.5 ml of the appropriate
homogenate, the usual complement of cofactors, 0.1 ml of a sodium
3RS-[5-^3H]-mevalonate solution (2.29 x 10^7 cpm; 4.12 nmols, 2.06 μM)
and 0.01 ml of an antibiotic stock solution (final incubation concentra-
tions: penicillin-G, 20 μg/ml; streptomycin sulfate, 30 μg/ml), in a
final incubation volume of 2.01 ml. Incubations were carried out for
24 hours at 37^o C under the usual aerobic conditions and were terminated
and worked up in the usual fashion. Retina homogenates (9.0 mg protein/
tube) and liver homogenates (42.8 mg protein/tube) were each incubated
in duplicate; a "mixed homogenate" containing both liver homogenate (3
parts, by volume; 25.7 mg protein) and retina homogenate (2 parts, by
volume; 3.6 mg protein) was incubated in triplicate. A buffer control
(no homogenate) was also run. An aliquot of the nonsaponifiable
material obtained from one of each of the incubation systems was analyzed
by ratio-TLC (Silica Gel G; developed once in benzene-ether-petroleum
ether, 7:3:2) along with authentic standards of cholesterol, 4α-methyl
cholesterol, commercial lanosterol and squalene, as previously described. After measurement of the total radioactivity in the nonsaponifiable extracts, sterols were precipitated with digitonin and the digitonin-precipitable radioactivity was measured, as previously described.

Results

The incorporation of mevalonic acid into nonsaponifiable material and digitonin-precipitable material by the incubation systems described above is documented in Table VI-5. The absolute amount of radioactivity incorporated into retina nonsaponifiable material was about 28% of that found in the liver nonsaponifiable material, although the specific activity of incorporation (cpm per mg protein) was about 1.3-fold greater in the retina incubations than in the liver incubations (the retina homogenates contained only about 21% as much total protein as the liver homogenates). As previously observed, the incorporation of radioactivity into digitonin-precipitable material by the retina homogenates was nearly negligible (ca. 0.3% of the total nonsaponifiable activity); in contrast, about 76% of the nonsaponifiable activity was precipitable with digitonin in the liver incubation system. The absolute amount of activity recovered in the nonsaponifiable lipids extracted from the mixed homogenate incubations was about 49% of that obtained from the liver homogenate incubations; however, the mixed homogenate contained only about 49% as much liver protein as the liver homogenate and the specific activity of incorporation was comparable to that of the liver homogenate system. There was a marked decrease in the relative incorporation of mevalonic acid into digitonin-precipitable material by the mixed homogenate (ca. 77% of the value obtained for the liver homogenate
### TABLE VI-5

The Incorporation of Mevalonic Acid into Nonsaponifiable Lipids (NSL) and Digitonin-Precipitable Material by Bovine Retina and Rat Liver S10 Homogenates and Mixed Homogenates

<table>
<thead>
<tr>
<th>HOMOGENATE SYSTEM</th>
<th>TOTAL NSL ACTIVITY (cpm)</th>
<th>SPECIFIC ACTIVITY (cpm/mg protein)</th>
<th>DIGITONIN-PRECIPITABLE MATERIAL (% of NSL Activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retina</td>
<td>306,000</td>
<td>34,000</td>
<td>0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>1,105,000</td>
<td>25,800</td>
<td>76.3</td>
</tr>
<tr>
<td>Mixed Homogenate</td>
<td>540,000</td>
<td>21,000*</td>
<td>58.5</td>
</tr>
<tr>
<td>Buffer Control</td>
<td>200</td>
<td>---</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Each incubation was carried out under aerobic conditions at 37°C for 24 hours in a final incubation volume of 2.01 ml; initial level of sodium 3RS-[5-³H]-mevalonate, 20.6 μCi (4.12 nmoles); Retina, 9.0 mg total protein; Liver, 42.8 mg total protein; Mixed Homogenate, 25.7 mg liver protein plus 3.6 mg retina protein.

* Specific activity value based on liver protein only.
incubations), suggesting that the retina homogenate depressed the synthesis of sterols by the liver components of the mixed homogenate. There was essentially no conversion of mevalonic acid by the buffer control.

The radiochromatograms of the nonsaponifiable material obtained from each type of incubation system are shown in Figure VI-12. The sample from the liver homogenate incubation (Figure VI-12A) contained a large proportion of the total activity in material which co-migrated with the cholesterol standard (ca. 78%); only minor amounts of activity (ca. 4%) migrated in the regions coincident with and immediately adjacent to the squalene standard. About 6% of the nonsaponifiable activity was observed in the "methyl sterols" region of the chromatogram. In contrast, the major component of nonsaponifiable activity in the retina sample resided near the origin of the plate (the "very polar component"), accounting for about 64% of the total activity (Figure IV-12B). Roughly 14% of the nonsaponifiable activity migrated in the "methyl sterols" region, while less than 4% of the activity chromatographed in the region of the cholesterol standard. About 2% of the activity co-migrated with the squalene standard, while about 6% of the activity chromatographed immediately adjacent to the squalene standard. The radiochromatogram of the sample taken from the mixed homogenate incubation (Figure VI-12C) showed about 74% of the total nonsaponifiable activity migrating in the region of the cholesterol standard, with about 5% of the activity migrating immediately ahead in the "methyl sterols" region. Nearly 7% of the total nonsaponifiable activity co-migrated with the squalene standard, while about 12% immediately preceded the squalene-like compo-
FIGURE VI-12

Radio-TLC of the nonsaponifiable lipids derived from incubations of [5-\textsuperscript{3}H]-mevalonic acid with (A) rat liver $S_{10}$ homogenate, (B) bovine retina $S_{10}$ homogenate, and (C) a 3:2, v/v, mixture of rat liver and bovine retina $S_{10}$ homogenates. Chromatographic system: Silica Gel G, developed once with benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards (in order of increasing $R_f$): cholesterol, 4α-methyl cholesterol, lanosterol and squalene. Note the lack of the "very polar component" (arrow) in the incubations containing rat liver homogenate.
nent on the plate. Since only about 58-59% of the nonsaponifiable ac-
tivity from the mixed homogenate incubation was found to be precipitable
with digitonin, it was evident that some portion of the activity which
was observed to co-migrate with cholesterol on the TLC plate was not due
to 3β-hydroxy sterols. Strikingly, the "very polar component" was com-
pletely absent from the radiochromatogram obtained from the extract of
the mixed homogenate.

Discussion

The above results suggest that some factor or factors present in
the bovine retina S10 homogenate depressed the synthesis of digitonin-
precipitable material (i.e. sterols) by the liver homogenate. Although
the absolute amount of nonsaponifiable activity synthesized from mevalonic
acid appeared to be a function of the amount of liver protein present in
the incubation (comparing the results obtained from the liver incubations
and those of the mixed homogenate incubations), the relative percentage
of nonsaponifiable activity which was also precipitable with digitonin
was markedly lower in the mixed homogenate incubations. The mechanism
by which this apparent depression of sterol biosynthesis was effected is
not clear from this preliminary experiment. It is possible that the
retina homogenate contained an "inhibitor" (i.e. one or more small mole-
cules or even proteins) which either affected the enzymes of sterol
biosynthesis directly or else competed with the liver enzymes for
mevalonic acid (or one or more of its metabolites). In the mixed homo-
ogenate, retina protein only accounted for about 12% of the total pro-
tein in the incubation, yet the percentage decrease in the proportion
of nonsaponifiable activity which was also precipitable with digitonin
was about 23% (relative to that of the liver homogenate incubations). This amount of retina protein was not sufficient to effect the negligible levels of sterol biosynthesis observed in the incubation of the retina S_{10} homogenates alone. In addition, the conspicuous absence of the "very polar component" in the nonsaponifiable material obtained from the mixed homogenate incubations suggested that either the component was not synthesized or else it was metabolized further to other products.

It is not clear from this experiment that the cause of the apparently negligible biosynthesis of digitonin-precipitable material by the retina S_{10} homogenate preparation is due primarily to the presence of an "inhibitor" (which apparently is released upon homogenization of the tissue). It cannot be ruled out that the lack of sterol biosynthesis in the retina preparations is simply due to the "demise" of the biosynthetic capabilities of the tissue as degradative processes ensue after death of the animal or as a result of the enucleation of the eye and/or subsequent processing of the retina prior to the actual incubation. Alternatively, the retina S_{10} homogenates may possess some endogenous "defect" in their sterol biosynthetic apparatus. In regard to this last point, it has been proposed that rat brain homogenates possess a "defect" in sterol biosynthetic capabilities which is primarily *microsomal* in origin (Kelley *et al.*, 1969; Ramsey *et al.*, 1971a, b). It has also been suggested that rat brain tissue lacks one or more *soluble* factors which are required for sterol biosynthesis and which are normally present in the 105,000 x g supernatant fraction of rat liver (Shah, 1972; Johnson and Shah, 1974). Similar "defects" may be present in the bovine retina. It is entirely possible that both microsomal "defects" and the absence
of certain soluble factors underlie the severely attenuated level of sterol biosynthesis by the retina preparations; however, the nature of the "inhibition" of retina sterol biosynthesis is still unknown.

Experiment VI-7

In order to determine whether the "inhibitor" apparently present in the 10,000 x g supernatant fraction of bovine retina has a specific subcellular location (i.e. soluble vs. microsomal), the following experiment was performed. Fresh bovine retinas and rat livers were processed as previously described to obtain the $S_{10}$ homogenates. In this experiment, the incubation buffer contained 30 mM nicotinamide and 5 mM MgCl$_2\cdot$6H$_2$O in 100 mM potassium phosphate (pH 7.4), but no antibiotics were included. Also, the rats used in this experiment had been maintained for several weeks on a "normal" (as opposed to a "cholesterol-free") lab chow diet and were sacrificed between 11:00 A.M. and 11:30 A.M. (as opposed to 8:30 - 9:00 A.M., as routinely done). The retina $S_{10}$ homogenate ($S_{10R}$; ca. 20 ml) was centrifuged for 90 minutes at 105,000 x g at 0-4°C (Beckman Ti 65 rotor; Spinco Model L ultracentrifuge, 40,000 rpm). The resulting translucent supernatant ($S_{105R}$; 20.0 ml, 6.6 mg protein per ml) was transferred to another tube, on ice, with a Pasteur pipet; the pellet was resuspended in incubation buffer by homogenization with a Ten-Broeke homogenizer (10 passes, by hand, on ice), forming the microsomal fraction ($P_{105R}$; 7.5 ml, 6.8 mg protein per ml).

In 70-ml capacity screwtop test tubes, increasing amounts of either $S_{105R}$, $P_{105R}$ or incubation buffer were added to decreasing amounts of the liver $S_{10}$ homogenate ($S_{10L}$ volumes: 2.0, 1.9, 1.8, 1.6 and 1.2 ml, respectively), maintaining a constant incubation volume of
2.4 ml. Each tube contained 0.2 ml of concentrated cofactor stock (providing the usual levels of ATP, NAD, NADP and G-6-P) and 0.2 ml of potassium 3RS-[2-3H]- mevalonate solution (1.86 x 10^7 dpm; 48.2 nmoles, 20.1 μM). The tubes were flushed with oxygen for 10 seconds, sealed and then incubated at 37°C for 3.5 hours with continuous agitation. In addition, aliquots of both the S_{105R} and P_{105R} preparations (0.8 ml each) were boiled for 20 minutes in a hot-water bath prior to addition to liver homogenate samples ("boiled controls"). All incubations were performed in duplicate. Termination, saponification, extraction of nonsaponifiable lipids and precipitation of sterols with digitonin were performed as previously described. Assay of radioactivity and carrier sterol mass was performed in duplicate for each sample.

Results

The effect of adding either the soluble (S_{105R}) or microsomal (P_{105R}) fraction of bovine retina to the rat liver homogenate, with regard to total nonsaponifiable lipid biosynthesis, is shown in Figure VI-13. A substantial decrease in total nonsaponifiable activity was observed upon addition of either retina fraction, over and above the effect exerted by decreasing the absolute amount of liver homogenate (compare with the buffer control). The bulk of the overall effect was incurred by the addition of up to 0.4 ml of retina material or incubation buffer, after which increasing volumes had little effect on the synthesis of nonsaponifiable material (up to 0.8 ml). Even though the P_{105R} additions contained slightly more protein than the corresponding volumes of the S_{105R} preparation, the largest effect was noted upon addition of the soluble retina fraction. In the most extreme case
FIGURE VI-13
The effect of increasing amounts of bovine retina material on the incorporation of [2-3H]-mevalonic acid into nonsaponifiable lipids (NSL) by rat liver S10 homogenates. Closed circles (● — ●), the 105,000 xg resuspended pellet (P105) obtained from a bovine retina S10 homogenate; closed triangles (▲ — ▲), the 105,000 xg supernatant (S10S) obtained from a bovine retina S homogenate; open circles (○ — ○), buffer control. See text for details.

FIGURE VI-14
The effect of increasing amounts of bovine retina material on the incorporation of [2-3H]-mevalonic acid into digitonin-precipitable nonsaponifiable lipids (NSL) by rat liver S10 homogenates. Closed circles (● — ●), the 105,000 xg resuspended pellet (P105) obtained from a bovine retina S10 homogenate; closed triangles (▲ — ▲), the 105,000 xg supernatant (S10S) obtained from a bovine retina S10 homogenate; open circles (○ — ○), buffer control. See text for details.
(i.e. adding 0.8 ml of retina material to the liver homogenate), the
correction of either the soluble or the microsomal retina protein to
the total incubated protein represented only about 8%, whereas these
fractions decreased the total nonsaponifiable activity by 67% and 53%,
respectively. The corresponding volume of incubation buffer added to
the liver homogenate produced a 37% decrease in the incorporation of
mevalonic acid into nonsaponifiable lipids, in reasonable agreement with
the fact that the amount of liver protein in these tubes was only 60% of
that present in the absence of additions to the incubation system.
Thus, the observed effect with buffer alone was not due to simply a
dilution of the total protein concentration, but was a result of the
decrease in the absolute amount of liver homogenate in the incubations.
Both boiled controls gave results similar to those obtained with the
corresponding volume of incubation buffer alone, suggesting that the
effect produced by addition of the retina material was due to a heat-
labile factor (or factors).

The effect of adding the retina material to the liver homogenate,
with regard to the production of digitonin-precipitable material, is
shown in Figure VI-14. Comparing the per cent of nonsaponifiable
material which was also precipitable with digitonin in each case, the
addition of either the soluble or microsomal retina fraction caused a
significant decrease in the synthesis of sterols by the liver homogenate.
Again, the effect was more pronounced with the soluble fraction than
with the corresponding volume of resuspended retina microsomes. The
addition of increasing amounts of incubation buffer (concomitantly
decreasing the total amount of liver homogenate in the incubations) did
not significantly affect the percentage of digitonin-precipitable material; addition of the "boiled controls" gave results which were comparable to those obtained by adding the corresponding amount of incubation buffer alone. These data support the suggestion that the effect exerted by the retina material on the liver homogenate system was due to a heat-labile factor (or factors). The effect was even more pronounced on the synthesis of digitonin-precipitable material than on the overall synthesis of nonsaponifiable lipids. In the most extreme case, addition of the soluble or microsomal retina fractions decreased the percentage of digitonin-precipitable activity by 63% and 43% respectively, relative to the incubation containing only liver homogenate (i.e. no additions). In these incubations, the maximal percentage of nonsaponifiable activity which was precipitable with digitonin was only about 50% in contrast to the levels previously observed (i.e. 90-95%) using the rat liver $S_{10}$ homogenate system with mevalonic acid as a precursor. Thus, the liver homogenate biosynthetic activity with regard to sterol biosynthesis was already depressed in the absence of retina material. The reasons behind this observation are not understood, but may reflect the inherent variability in these in vitro preparations.

Discussion

Although decreasing the absolute amount of liver homogenate in the incubations resulted in an overall decline in the total amount of nonsaponifiable lipid synthesis from mevalonic acid, the percentage of the nonsaponifiable activity which was also precipitable with digitonin was not appreciably affected in the absence of retina material (i.e. the absolute amount of digitonin-precipitable material declined in proportion
to the overall decrease in the nonsaponifiable material). In contrast, marked decreases in the digitonin-precipitable material were observed upon addition of either the soluble or microsomal fraction of bovine retina which were even greater than the overall decreases in nonsaponifiable lipid biosynthesis produced by these fractions. This suggested that factors in both the soluble and microsomal fractions of the retina selectively affected the biosynthesis of sterols. The nature of these "factors" remains unknown, although they appear to be heat-labile components (i.e. perhaps proteins). It is still unclear whether these factors act directly on the sterol biosynthetic enzymes (i.e. as ligands or perhaps by enzymatic modification) or whether they simply compete with those enzymes for common substrates and thus divert precursors from the sterol biosynthetic pathway. These "factors" seem to be released upon homogenization of the retina (and subsequent fractionation), since intact bovine retinas incubated in vitro with mevalonic were capable of synthesizing a variety of cholesterol precursors (including sterols) as well as small amounts of cholesterol-like material (see Chapter V). The relevance of these "factors" to the normal physiology of the retina in vivo remains unclear.
VII. THE METABOLISM OF MEVALONIC ACID BY
BOVINE RETINA S_{10} HOMOGENATES

INTRODUCTION

Previous incubations of bovine retina 10,000 x g supernatant preparations (S_{10} homogenates) have been performed on a small scale, i.e. 2.0 ml total incubation volume, containing 5-10 mg of protein. Even over extended incubation periods (24 hours), less than 22% of the incubated substrate was converted to nonsaponifiable lipids and only minor conversion to monohydroxy sterols was observed. In one case, it was found that about 70% of the radioactivity present in saponifiable compounds exhibited the chromatographic properties of "fatty acid-like" material; however, a consideration of the nature of the substrate (mevalonic acid), the nature of the S_{10} homogenate and the lack of chromatographic coincidence between the labelled material and the major fatty acid constituents of the retina (by radio-GLC analysis) indicated that the "fatty acid-like" material most probably represented isoprenoid acids.

In an attempt to more fully characterize the saponifiable and nonsaponifiable components derived from mevalonic acid upon incubation with bovine retina S_{10} homogenates, the following experiments were performed.

EXPERIMENT VII-1

The S_{10} homogenate was prepared as previously described from 20 freshly-dissected bovine retinas in 40 ml of incubation buffer (100 mM potassium phosphate, pH 7.4, containing, 30 mM nicotinamide, 5 mM MgCl_{2}, 6H_{2}O, 40 µg/ml penicillin-G and 40 µg/ml streptomycin sulfate). The
homogenate was distributed into three 125-ml capacity screw-top erlenmeyer flasks (12.0 ml each; 134.3 mg protein per flask), and stored on ice.

A concentrated cofactor stock solution was prepared, as described previously, immediately prior to use in the incubations and contained the following constituents (in incubation buffer, adjusted to pH 7.4 with 5N KOH): NAD, 12.95 mg/ml; NADP, 13.93 mg/ml; G-6-P, 14.39 mg/ml; ATP, 51.44 mg/ml. One ml of this stock provided the following final concentrations of each cofactor: NAD and NADP, 1 mM; G-6-P, 3 mM; ATP, 5 mM.

The potassium salt of 3RS-[2-3H]-mevalonic acid (176 Ci/mol) was prepared from the lactone, as previously described, in incubation buffer (3.0 ml total; ca. 0.1 mCi/ml). Each flask contained 1.0 ml of the isotopic substrate stock solution, providing the following levels of mevalonic acid:

<table>
<thead>
<tr>
<th>FLASK</th>
<th>TOTAL RADIOACTIVITY (dpm)*</th>
<th>3RS-[2-3H]-MEVALONIC ACID (µM)</th>
<th>(µMOLES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.06 x 10^8</td>
<td>46.4</td>
<td>0.79</td>
</tr>
<tr>
<td>B</td>
<td>2.94 x 10^8</td>
<td>44.6</td>
<td>0.76</td>
</tr>
<tr>
<td>C</td>
<td>3.03 x 10^8</td>
<td>46.0</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* Determined by assaying triplicate aliquots (0.10 ml each) of the final incubation system.

The final incubation volume of each flask was adjusted to 17.0 ml with incubation buffer. After addition of all the incubation components (adding the labelled substrate last), each flask was flushed with oxy-
gen for 10 seconds, sealed and then incubated for 24 hours at 37°C in a Dubnoff metabolic incubator with continuous gentle agitation (60 oscillations per minute).

The incubation of flasks B and C was terminated by the addition of 17 ml of 15% ethanolic potassium hydroxide, followed by saponification, work-up and extraction of the nonsaponifiable lipids as described previously (see Materials and General Methods, Chapter II). After transferring the nonsaponifiable material to vials, each extract was evaporated to dryness under nitrogen and redissolved in 10.0 ml of benzene. The remaining ethanolic-aqueous phases (containing the saponifiable material) were chilled to -20°C and then acidified immediately by dropwise addition of concentrated HCl (4.12 ml per flask), with continuous stirring, to pH ~1-2. The acidified mixtures were each extracted four times with 50 ml portions of dichloromethane and the combined extracts were washed once with 100 ml of water, then dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in 10.0 ml of chloroform.

Incubation of flask A was terminated by the addition of 70 ml of methanol, followed by extraction of the "total lipids" by a modification of the method of Bligh and Dyer (1959), as described previously. The aqueous-methanolic mixture was transferred to a separatory funnel and then adjusted with methanol and chloroform so as to obtain a ternary monophase having the final composition of chloroform:methanol:water, 60:45:1 (v/v). The resulting monophase was adjusted with water until a separation into two distinct phases was effected; the lower
phase was collected, while the upper phase was re-extracted with 70 ml of chloroform. Again, the resulting lower phase was collected and the combined lower phases were dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in 10.0 ml of chloroform.

The general methods employed for the analysis of lipids by radio-TLC, radio-GLC and chromatography on columns of silicic acid-Super Cel and Silica Gel G-Super Cel-silver nitrate, and the precipitation of sterols with digitonin have been described previously. The results of the analyses of the nonsaponifiable, saponifiable and "total lipid" extracts are discussed in the following section.

RESULTS

The incorporation of radioactivity into "total lipids", nonsaponifiable lipids and saponifiable material by the bovine retina homogenates is documented in Table VII-1. These results indicated that approximately 40% of the incubated substrate was converted to lipid products in these incubations. Since only the 3R-isomer of mevalonic acid is biologically "active", the maximum theoretical conversion is 50%; therefore, these incubations yielded about 80% of the theoretical conversion expected for the given substrate. It is clear from these results that the great majority of metabolized substrate was converted to saponifiable components (i.e. about three-fourths of the usable substrate).

Analysis of "Total Lipid" and Saponifiable Lipid Extracts

Aliquots of the "total lipid" extract (A-TL) and the saponifiable
TABLE VII-1
Incorporation of 3RS-[2-3H]-Mevalonic Acid into Lipid Products by Bovine Retina S10 Homogenates

<table>
<thead>
<tr>
<th>FLASK</th>
<th>COMPONENT(^1)</th>
<th>RECOVERED(^2) ACTIVITY (dpm)</th>
<th>PER CENT INCUBATED ACTIVITY</th>
<th>PER CENT RECOVERED ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>TL</td>
<td>1.15 x 10^8</td>
<td>37.5</td>
<td>100.0</td>
</tr>
<tr>
<td>B</td>
<td>NSL</td>
<td>8.78 x 10^6</td>
<td>3.0</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>SL</td>
<td>1.11 x 10^8</td>
<td>37.7</td>
<td>92.6</td>
</tr>
<tr>
<td>C</td>
<td>NSL</td>
<td>1.12 x 10^7</td>
<td>3.7</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>SL</td>
<td>1.10 x 10^8</td>
<td>36.3</td>
<td>90.7</td>
</tr>
</tbody>
</table>

\(^1\) Abbreviations: TL, total lipids; NSL, nonsaponifiable lipids; SL, saponifiable lipids

\(^2\) Average values based on triplicate aliquots (0.05 ml each from 10.0 ml total sample volume)

Lipid extracts (B-SL and C-SL) were applied to Silica Gel G TLC plates along with authentic standards of dipalmitin, cholesterol, oleic acid, triolein, methyl oleate and squalene. The plates were developed once with hexane-diethyl ether-glacial acetic acid (80:30:1, v/v), then divided horizontally into one-centimeter sections and scraped into scintillation vials for measurement of radioactivity; the standards were visualized by spraying the standard lane with 50% methanolic sulfuric acid and charring for one hour. The radiochromatograms are shown in Figure VII-1. About 73% of the activity from the "total lipid" extract (Figure VII-1A) co-migrated with the free fatty acid standard, while about 6% of the activity co-migrated with the triglyceride standard;
FIGURE VII-1

Radio-TLC of the total lipids (A) and the saponifiable lipids (B and C) extracted after 24 hours of incubation of retina S10 homogenates with [2-3H]-mevalonic acid. A, sample A-TL; B, sample B-SL; C, sample C-SL. Chromatographic system: Silica Gel G, developed once with hexane-diethyl ether-glacial acetic acid, 80:20:1, v/v. Chromatographic standards (in order of increasing Rf): dipalmitin, cholesterol, oleic acid, triolein, methyl oleate and squalene.
the remainder of the activity was very polar, with about 11% of the total activity remaining at the origin of the plate. Saponification apparently removed the minor component which co-migrated with the triglyceride standard, as evident from the chromatograms shown in Figures VII-1B and VII-1C. In the case of the saponifiable samples, the gross majority of the total activity co-migrated with the free fatty acid standard (ca. 80% of B-SL, ca. 85% of C-SL; Figures VII-1B and VII-1C, respectively); most of the residual activity remained at the origin of the plates (ca. 13% of B-SL, ca. 10% of C-SL).

Portions of both saponifiable extracts were esterified with diazomethane, as previously described, and the reaction products were analyzed by radio-TLC (Silica Gel G; developed twice with hexane-diethyl ether-glacial acetic acid, 94:4:2, v/v). The radiochromatograms are shown in Figure VII-2. The material which previously co-migrated with the oleic acid standard was apparently quantitatively converted to the corresponding methyl ester and co-migrated with the methyl oleate standard.

The methyl esters obtained from samples B-SL and C-SL were purified by chromatography on a Unisil column (4 grams; 21 x 1 cm column, equilibrated and eluted with hexane-diethyl ether, 95:5). Fractions (3.15 ml per 4-minute fraction) were collected and assayed for total radioactivity as usual (0.3 ml aliquots per fraction). Fractions 4-10 (corresponding to the methyl esters) were pooled, concentrated under reduced pressure, transferred to vials, evaporated to dryness under nitrogen and redissolved in 2.0 ml of benzene. Duplicate aliquots from each purified methyl ester sample (i.e. B-SLME and C-SLME, re-
FIGURE VII-2


FIGURE VII-3

spectively) were assayed for total radioactivity (B-SLME, $3.66 \times 10^5$ dpm; C-SLME, $5.62 \times 10^6$ dpm). Analysis of both samples by radio-TLC, as before, revealed that greater than 99% of the total radioactivity behaved chromatographically like methyl esters. An aliquot of each methyl ester sample was further analyzed by radio-TLC on a Silica Gel G - 5% silver nitrate plate, along with authentic standards of fatty acid methyl esters ($C_{20:0}$, $C_{20:1}$, $C_{20:2}$, $C_{20:3}$ and $C_{22:6}$) and methyl farnesoate (plates developed twice with hexane-diethyl ether-glacial acetic acid, 94:4:2). The radiochromatograms are shown in Figure VII-3. The radioactivity was distributed between two components; the major component (ca. 70% of B-SLME, ca. 76% of C-SLME) in both samples co-migrated with the $C_{20:3}$ FAME standard, while the minor component (ca. 28% of B-SLME, ca 21% of C-SLME) migrated just ahead of the $C_{20:2}$ FAME standard (between the $C_{20:2}$ and $C_{20:1}$ standards, in the region of the methyl farnesoate isomers). Under these conditions, the FAME standards were well resolved from one another; in addition, the methyl farnesoate standard was resolved into two components.

A portion of each methyl ester sample was hydrogenated as described previously (over 2 mg platinum oxide catalyst; hydrogen bubbled through the sample, dissolved in 5 ml of 2:1 hexane-ethanol, for 15 minutes at room temperature). The hydrogenated samples were then analyzed as described above by radio-TLC; the radiochromatogram of hydrogenated sample B-SLME is shown in Figure VII-3C. At least 98% of the total radioactivity in the hydrogenated samples co-migrated with the completely saturated FAME standard. The hydrogenated samples were further analyzed by radio-GLC on a 3% OV-17 column (isothermal column
temperature, 170°C; carrier flow rate, 66 ml/min.), collecting effluent
gas at one-minute intervals and measuring the radioactivity as described
previously. The samples were co-injected with authentic standards of
methyl palmitate (C_{16:0}) and methyl stearate (C_{18:0}) and the elution
profile of the labelled components was compared with that of the FAME
standards. The radiochromatogram obtained from hydrogenated sample
B-SLME (injected activity, 2.25 \times 10^5 cpm) is shown in Figure VII-4.
The radioactivity was distributed into only two components (overall
recovery, 91%), neither of which corresponded to the FAME standards.
About 30% of the recovered activity eluted just after the solvent
peak, even before the position calculated for a 14-carbon saturated
n-fatty acid methyl ester; the remaining 70% of the total activity
eluted between the C_{16:0} and C_{18:0} FAME standards. Since the major
fatty acid components of the retina, after hydrogenation, would include
both palmitic and stearic acid (Bartley et al., 1962), it was apparent
from these results the labelled saponifiable constituents did not
correspond to the typical retina n-fatty acids. Furthermore, it was
apparent from the above results that only two kinds of acids were syn-
thesized, differing in chain length. From the relative retention times
of the two radioactive components, it was apparent that the hydrogena-
ted saponifiable constituents represented C_{15} and C_{20} isoprenoid acids
(Popják and Cornforth, 1960), with the major species accounting for
about 70% of the activity in sample B-SLME. The results obtained from
hydrogentated sample C-SLME were quite similar, with about 22% of the
total activity distributed in the shorter-chain species and 78% of the
activity exhibiting the chromatographic behavior of a C_{20} isoprenoid
acid.
FIGURE VII-4

Radio-GLC of hydrogenated sample 3-SLME. Chromatographic system: 3% OV-17, 170°C isothermal. The first mass peak is due to methyl palmitate, the second mass peak is due to methyl stearate. The arrows denote the expected positions of C_{14:0} (first) and C_{20:0} (second) fatty acid methyl esters.
The above results suggested that the bovine retina homogenates converted the majority of the usable mevalonic acid to isoprenoid acids which differed in chain length and in unsaturation. However, it was not apparent from the above data whether or not each of the two kinds of acids (i.e. of each chain length) was also composed of species differing in the number and position of double bonds. To answer this question (at least in part), sample B-SLME was further fractionated on columns of Silica Gel G-Super Cel-silver nitrate (50 x 1 cm), essentially per the procedures used for the separation of diene steryl acetates. A trial run was performed on the remainder of sample B-SLME (7.60 x 10^5 dpm), using a column equilibrated and eluted with toluene (fraction volume, 5.3 ml; flow rate, 0.13 ml/min.); the solvent was changed to chloroform-toluene, 1:9, at fraction 108 and then to 1:4 chloroform-toluene at fraction 165. Aliquots of even-numbered fractions were assayed for radioactivity as usual. The elution profile is shown in Figure VII-5. Prior to the first solvent change, a sharp peak of radioactivity eluted, corresponding to about 31% of the recovered activity (fractions 19-30). Two other minor peaks (ca. 1% of the total recovered activity each) eluted just before and just after this relatively non-polar major component. After the first solvent change, about 50% of the recovered activity eluted as two partially resolved components (fractions 106-141 and 142-158, with a relative activity ratio of 9:1). The second solvent change eluted another component (fractions 162-190) representing about 25% of the recovered activity. The overall recovery of applied radioactivity from the column was about 83%.
FIGURE VII-5

Silica Gel G-Super Gel-silver nitrate column chromatography of sample 8-SLME. Arrows denote the solvent changes to toluene-chloroform, 9:1 (first) and to toluene-chloroform, 4:1 (second).
FIGURE VII-6

Radio-GLC of fractions 19-30 obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME (see Figure VII-5). Chromatographic system: 3% OV-17, 135°C isothermal. The mass peak is due to all-trans methyl farnesoate.
The material from fractions 19-30 was pooled and analyzed further by radio-GLC on a 3% OV-17 column (isothermal column temperature, 135°C; carrier flow rate, 66 ml/min.), along with an authentic standard of all-trans methyl farnesoate. The radiochromatogram is shown in Figure VII-6. Greater than 98% of the recovered radioactivity co-migrated with the all-trans methyl farnesoate standard. These results suggested that essentially all of the activity which chromatographed like a C\textsubscript{15} isoprenoid acid was distributed in the all-trans isomer of farnesoic acid; therefore, the remainder of the activity was apparently due to isomeric C\textsubscript{20} isoprenoid acids. The overall recovery of injected activity from the GLC column was about 96%.

The remainder of sample B-SL was esterified with diazomethane and the methyl esters were purified by Unisil column chromatography as described previously. The methyl esters (2.19 x 10\textsuperscript{7} dpm) were applied to a second Silica Gel G-Super Cel-silver nitrate column, equilibrated and eluted with benzene (fraction volume, 6.8 ml; flow rate, 0.14 ml/min.); the solvent was changed to chloroform-benzene, 5:95, at fraction 56, just after the elution of the C\textsubscript{15} isoprenoid peak. The elution profile is shown in Figure VII-7. The first major peak of radioactivity eluted with fractions 13-30, representing about 26% of the total recovered activity; this peak exhibited marked "tailing" for over 40 fractions. After the solvent change, two other major peaks eluted from the column, again exhibiting only partial resolution. The first peak (fractions 72-95, ca. 38% of the activity) was relatively symmetrical and narrow, while the second peak (fractions 96-170, ca. 31% of
FIGURE VII-7

Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME. Arrow denotes solvent change to benzene-chloroform, 95:5.
the activity) was much more broad and tailed substantially. The overall recovery of applied activity was about 86%.

Fractions 13-30 were pooled (4.30 x 10^6 dpm) and analyzed further by radio-GLC before and after hydrogenation. About 5% of the sample was hydrogenated over PtO_2 as previously described and the recovered material was checked by radio-TLC on argentated plates; greater than 98% of the total activity co-migrated with the saturated FAME standard. The radiochromatogram of the sample prior to hydrogenation (3% OV-17; isothermal column temperature, 135°C) was essentially identical to that shown in Figure VII-6. Another portion of the unhydrogenated sample (7.97 x 10^4 cpm) was chromatographed on a 12% DEGS column (isothermal column temperature, 135°C; carrier flow rate, 66 ml/min.), along with a mixture of methyl farnesoate isomers. The radiochromatogram is shown in Figure VII-8. Under the conditions employed, the four isomers of methyl farnesoate were well resolved, yielding the following relative retention times (relative to the all-trans isomer; absolute retention time, 31.32 min.); 0.638, 0.753, 0.912 and 1.000; the first mass peak was due to the all-cis isomer, the last peak was due to the all-trans isomer and the intermediate two peaks were due to the cis, trans- and trans, cis- isomers (order of elution not known). Greater than 98% of the recovered activity co-migrated with the all-trans isomer of methyl farnesoate. About 1% of the activity co-migrated with the second mass peak (relative retention time, 0.753). The overall recovery of radioactivity was quantitative. The radiochromatogram of the hydrogenated material (1.00 x 10^4 cpm; 3% OV-17; isothermal column temperature, 115°C) is shown in Figure VII-9. About 99% of the recovered radioacti-
FIGURE VII-8
Radio-GLC of peak I (fractions 13-30) isolated by Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME (see Figure VII-7). Chromatographic system: 12% DEGS, 135°C isothermal.
Chromatographic standards (in order of increasing retention time): cis,cis-methyl farnesoate, "cis-trans" isomers of methyl farnesoate (second and third peaks) and all-trans methyl farnesoate.

FIGURE VII-9
Radio-GLC of the hydrogenated material from peak I (fractions 13-30) isolated by Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME (see Figure VII-7). Chromatographic system: 3% OV-17, 115°C isothermal. The mass peak is due to methyl-3,7,11-trimethyl-dodecanoate.
DETECTOR RESPONSE

RETENTION TIME (min)

RADIOACTIVITY (DPM x 10^{-3})
vity co-migrated with the methyl perhydrofarnesoate standard; the recovery of applied activity was about 97%. These data confirmed that the material in fractions 19-30 was derived from a C\textsubscript{15} isoprenoid acid having the chromatographic properties of all-trans farnesoic acid.

A similar set of analyses was performed on portions of the material obtained from pooled fractions 74-95 (total recovered activity, 7.29 \times 10^6 dpm). The radiochromatogram of the sample obtained by chromatography on a 3% OV-17 column (isothermal column temperature, 180°C; injected radioactivity, 2.43 \times 10^5 cpm) is shown in Figure VII-10. The following chromatographic standards of C\textsubscript{20} isoprenoid acid methyl esters (in order of their elution from the GLC column) were employed: methyl phytanate (saturated), \textit{trans}-\Delta^2-methyl phytenate (monounsaturated) and all-trans methyl geranylgeranoate (tetraunsaturated). Greater than 98% of the recovered radioactivity co-migrated with the all-trans methyl geranylgeranoate standard; the overall recovery of applied activity was about 99%. Essentially identical results were obtained when another portion of the sample (1.78 \times 10^5 cpm) was analyzed on a 12% DEGS column (Figure VII-11). Under the conditions employed (isothermal column temperature, 170°C; carrier flow rate, 66 ml/min.), the methyl and ethyl esters of all-trans geranylgeranoic acid were only partially resolved, but greater than 98% of the recovered radioactivity co-migrated with the methyl ester of all-trans geranylgeranoic acid. The recovery of applied activity was 96%. Chromatographic analysis of the hydrogenated material from fractions 74-95 on a 3% OV-17 column (column temperature, 160°C) yielded the radiochromatogram shown in Figure VII-12. Greater than 99% of the recovered acti-
FIGURE VII-10
Radio-GLC of peak II (fractions 74-95) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME (see Figure VII-7). Chromatographic system: 3% OV-17, 180°C isothermal. Chromatographic standards (in order of increasing retention time): methyl phytanate, trans-$\Delta^2$-methyl phytenate, all-trans methyl geranylgeranoate and all-trans ethyl geranylgeranoate.

FIGURE VII-11
Radio-GLC of peak II (fractions 74-95) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME (see Figure VII-7). Chromatographic system: 12% DEGS, 170°C isothermal. Chromatographic standards (in order of increasing retention time): trans-$\Delta^2$-methyl phytenate, all-trans methyl geranylgeranoate and all-trans ethyl geranylgeranoate.

FIGURE VII-12
Radio-GLC of the hydrogenated material from peak II (fractions 74-95) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME (see Figure VII-7). Chromatographic system: 3% OV-17, 160°C isothermal. The mass peak is due to methyl phytanate.
vity co-chromatographed with the fully-saturated C_{20} isoprenoid acid methyl ester standard (methyl phytanate). The recovery of applied activity (4.04 x 10^{6} cpm) was quantitative. These results confirmed that the material obtained from fractions 74-95 was derived from a C_{20} isoprenoid acid having the chromatographic properties of all-trans geranylgeranoic acid.

Using the GLC columns and conditions employed for the analysis of fractions 74-95 discussed above, the material from pooled fractions 96-210 (total recovered activity, 6.35 x 10^{6} dpm) was analyzed by radio-GLC. The radiochromatogram obtained by chromatography on the 3\% OV-17 column is shown in Figure VII-13. About 82\% of the recovered activity eluted just after the trans-\Delta^{2}-methyl phytanate standard, while about 16\% of the activity (probably due to cross-contamination with the preceding peak of radioactivity from the argonated chromatographic column) co-migrated with the all-trans methyl geranylgeranoate standard. The recovery of activity from this GLC column was essentially quantitative. Analysis on the 12\% DEGS column yielded the radiochromatogram shown in Figure VII-14 (applied activity, 2.05 x 10^{5} cpm). About 76\% of the recovered activity eluted just after the monounsaturated C_{20} isoprenoid standard, while about 6\% of the activity co-migrated with a minor impurity of the all-trans methyl geranylgeranoate standard having the chromatographic properties of the 2-cis-6,10-trans- isomer of methyl geranylgeranoate. As before, about 16\% of the recovered activity co-chromatographed with the all-trans isomer of methyl geranylgeranoate. Recovery of applied activity was about 94\%. Analysis of the sample by radio-TLC on a Silica Gel G-5\% silver nitrate
FIGURE VII-13
Radio-GLC of peak III (fractions 96-210) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME (see Figure VII-7). Chromatographic system: 3% OV-17, 180°C isothermal. Chromatographic standards (in order of increasing retention time): methyl phytanate, \textit{trans}-\Delta^2\text{-methyl phytanate}, all-\textit{trans} methyl geranylgeranoate and all-\textit{trans} ethyl geranylgeranoate.

FIGURE VII-14
Radio-GLC of peak III (fractions 96-210) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME (see Figure VII-7). Chromatographic system: 12% DEGS, 170°C isothermal. Chromatographic standards (in order of increasing retention time): \textit{trans}-\Delta^2\text{-methyl phytanate}, all-\textit{trans} methyl geranylgeranoate and all-\textit{trans} ethyl geranylgeranoate.

FIGURE VII-15
Radio-GLC of the hydrogenated material from peak III (fractions 96-210) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample B-SLME (see Figure VII-7). Chromatographic system: 3% OV-17, 160°C isothermal. The mass peak is due to methyl phytanate.
plate revealed that about 5% of the total activity remained at the origin (apparently due to polar degradation products) while 95% co-chromatographed with an authentic FAME standard. The radiochromatogram of the hydrogenated sample obtained from fractions 96-210 is shown in Figure VII-15 (3% OV-17; column temperature, 160°C). At least 98% of the recovered radioactivity co-migrated with the authentic standard of methyl phytanate; the recovery of applied activity (8.82 x 10^4 cpm) was about 98%. These data suggested that the material from fractions 96-210 was derived from a C_{20} isoprenoid acid which differed from all-trans geranylgeranoic acid in the number and/or arrangement of its double bonds. Since the material in fractions 96-210 eluted after the material which had the chromatographic properties of all-trans methyl geranylgeranoate on the Silica Gel G-Super Cel-silver nitrate column but eluted before the all-trans methyl geranylgeranoate standard on the GLC columns, it was evident that the C_{20} isoprenoid acid contained at least one cis double bond (although the arrangement and number of double bonds are not known).

In summary, about 30% of the activity in sample B-SLME (the methyl esters derived from the saponifiable material extracted from incubation flask B) was due to material having the chromatographic properties of the all-trans isomer of methyl farnesoate (representing at least 98% of the C_{15} isoprenoid acids extracted from the incubation mixture). About 43% of the total saponifiable activity was due to a species having the chromatographic properties of all-trans methyl geranylgeranoate, while about 26% of the activity was apparently due to one or more related C_{20} isoprenoids having at least one cis double bond.
Analysis of Nonsaponifiable Lipid Extracts

The nonsaponifiable material recovered from incubation mixtures B and C (i.e. B-NSL and C-NSL, respectively) was analyzed by chromatography on columns of silicic acid-Super Cel (2:1 w/w; 50 x 1 cm) as previously described. The columns were equilibrated and eluted with toluene; the solvent was changed to toluene-diethyl ether, 9:1, at fraction 125 and then to toluene-diethyl ether, 4:1, at fraction 212. Sample B-NSL ($8.78 \times 10^6$ dpm) was supplemented with 10 mg each of carrier cholesterol and commercial lanosterol (a 3:2 mixture of lanosterol and 24,25-dihydrolanosterol); sample C-NSL was not supplemented with carrier sterols. Fractions were collected automatically, evaporated to dryness under nitrogen, redissolved in 2.0 ml of benzene and aliquots (0.2 ml each) were assayed for radioactivity and (where appropriate) carrier sterols as previously described.

The elution profile obtained from sample B-NSL is displayed in Figure VII-16 (applied activity, $8.63 \times 10^6$ dpm); fraction volume, 3.8 ml; flow rate 0.11 ml/min). The carrier sterols eluted in fractions 20-38 ($C_{30}$ sterols) and fractions 45-70 ($C_{27}$ sterol); no resolution of lanosterol and dihydrolanosterol was effected. Although radioactivity eluted in the regions of the carrier sterols, the peak fractions of radioactivity and sterol mass were not coincident (i.e. the colorimetric peak fraction for the $C_{30}$ sterol standard was fraction 26, whereas the peak fraction of radioactivity in that region was fraction 23; similarly, the cholesterol colorimetric peak was centered at fraction 50, whereas the radioactivity in that region was maximal on either side of the colorimetric peak at fractions 47 and 57). Only about 23% of the
FIGURE VII-16

Silicic acid-Super Cel column chromatography of sample B-NSL (non-saponifiable lipids obtained by incubation of a bovine retina S₁₀ homogenate for 24 hours with [2⁻³H]-mevalonic acid). Open circles (o—o), radioactivity; closed circles (●—●), sterols determined colorimetrically. The first colorimetric peak is due to lanosterol and 24,25-dihydrolanosterol (absorbance at 460 nm); the second colorimetric peak is due to cholesterol (absorbance at 620 nm). Arrows denote solvent changes to toluene-diethyl ether, 9:1 (first) and to toluene-diethyl ether, 4:1 (second).
ABSORBANCE

FRACTION NUMBER

72 16 12 8 4 0

RADIOACTIVITY (DPM \times 10^{4})
recovered activity eluted with toluene; the great majority of labelled material was much more polar than typical $C_{27}$ monohydroxy sterols. The overall recovery of applied activity was about 83%. The distribution of radioactivity in each peak of the chromatogram was as follows:

<table>
<thead>
<tr>
<th>B-NSL FRACTION</th>
<th>% RECOVERED ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-9</td>
<td>5.1</td>
</tr>
<tr>
<td>10-19</td>
<td>1.5</td>
</tr>
<tr>
<td>20-23</td>
<td>3.9</td>
</tr>
<tr>
<td>34-76</td>
<td>9.4</td>
</tr>
<tr>
<td>77-98</td>
<td>2.1</td>
</tr>
<tr>
<td>99-110</td>
<td>0.4</td>
</tr>
<tr>
<td>130-280</td>
<td>77.5</td>
</tr>
</tbody>
</table>

The elution profile obtained from sample C-NSL is shown in Figure VII-17 (applied activity, $1.10 \times 10^7$ dpm; fraction volume, 4.6 ml; flow rate, 0.13 ml/min.). The resolution of individual components was apparently more complete than for sample B-NSL, and there was a reasonable degree of correspondence between the individual peaks in the chromatograms shown in Figures VII-16 and VII-17. Again, about 24% of the recovered activity eluted with toluene; the overall recovery of applied activity was about 80%. The distribution of the radioactivity in the individual components was as follows:
FIGURE VII-17
Silicic acid-Super Gel column chromatography of sample C-NSL (non-saponifiable lipids obtained by incubation of a bovine retina S₁₀ homogenate for 24 hours with [2-¹H]-mevalonic acid). Open circles (○—○), radioactivity. No carrier sterols were employed. Arrows denote solvent changes to toluene-diethyl ether, 9:1 (first) and to toluene-diethyl ether, 4:1 (second).
<table>
<thead>
<tr>
<th>C-NSL FRACTIONS</th>
<th>% RECOVERED ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-8</td>
<td>4.2</td>
</tr>
<tr>
<td>9-18</td>
<td>1.2</td>
</tr>
<tr>
<td>19-28</td>
<td>6.5</td>
</tr>
<tr>
<td>29-43</td>
<td>2.7</td>
</tr>
<tr>
<td>44-51</td>
<td>2.5</td>
</tr>
<tr>
<td>52-73</td>
<td>4.9</td>
</tr>
<tr>
<td>74-86</td>
<td>1.7</td>
</tr>
<tr>
<td>140-280</td>
<td>76.3</td>
</tr>
</tbody>
</table>

Considering the fact that the constituents of the incubation mixtures were the same and that the incubation conditions and subsequent work-up of the extracts were essentially identical in both cases, it is most likely that the constituents of a given set of fractions from one column are the same as (or structurally related to) those of the corresponding set of fractions from the alternate column. Assuming this to be true, it was decided to use the material obtained by fractionation of sample B-NSL mostly for gross analyses (TLC analysis and digitonin precipitation) and to use the material obtained from sample C-NSL (free of carrier sterols) primarily for radio-GLC analysis. The fractions listed above were pooled, concentrated under reduced pressure, transferred to individual vials, evaporated to dryness under nitrogen, redissolved in 5.0 ml of benzene and stored at -20°C under argon until ready for further use.
Analysis of Material Obtained by Column Chromatography of Sample B-NSL

A portion of the material obtained from fractions 4–9 (ca. 800 cpm) was analyzed by radio-GLC on a 3% OV-17 column (isothermal column temperature, 230°C; carrier flow rate, 66 ml/min.), along with an authentic standard of squalene. The radiochromatogram is shown in Figure VII-18. Approximately 80% of the recovered activity co-chromatographed with the squalene standard; about 11% of the activity eluted after the squalene standard, while about 9% of the activity eluted with the solvent front. The overall recovery of applied activity was quantitative. The chromatographic properties of the major portion of this sample were therefore consistent with those of squalene.

The material from pooled fractions 10-19 (ca. 4.16 x 10^4 cpm) was divided into three equal portions and chromatographed on Silica Gel G TLC plates with the following three systems: (A) chloroform; (B) benzene-diethyl ether-petroleum ether, 7:3:2; (C) hexane-diethyl ether-glacial acetic acid, 65:35:1. Authentic standards of commercial lanosterol, dolichol and squalene were used as chromatographic standards in a lane adjacent to the labelled material. The plates were sectioned, scraped into vials and radioactivity measured as previously described; the chromatographic standards were visualized by exposure to iodine vapors. The radiochromatograms are shown in Figure VII-19. It was immediately evident that the sample was not homogeneous. The major component was slightly less polar than dolichol but more polar than squalene. The radioactivity clearly did not correspond to either monohydroxy sterols or dolichol. On the TLC systems employed, Coenzyme Q₁₀ would have migrated immediately behind squalene and would have been
FIGURE VII-18

Radio-GLC of pooled fractions 3-9 obtained by silicic acid-Super Cel column chromatography of sample B-NSL (see Figure VII-16). The mass peak is due to squalene. Chromatographic system: 3% OV-17, 230°C isothermal.
well resolved from dolichol; therefore, it is unlikely that the labelled material represented ubiquinone or a closely related type of molecule.

The samples obtained from pooled fractions 20-33 (8.65 x 10^4 cpm; containing carrier C₃₀ sterols) and fractions 34-76 (2.63 x 10^5 cpm; containing carrier cholesterol) were each supplemented so as to contain approximately 10 mg of total carrier cholesterol. Each sample was adjusted to a known volume (10.0 ml) with benzene and duplicate aliquots were assayed for total radioactivity and colorimetrically for cholesterol. The samples were then precipitated with digitonin, as described previously, and the free sterols were recovered from the digitonides and assayed as before for total radioactivity and cholesterol content. As controls, the digitonin precipitation procedure was performed on samples of authentic tritiated cholesterol (obtained biosynthetically by incubating rat liver S₁₀ homogenates with 3RS-[2-³H]-mevalonic acid, followed by purification of the labelled sterol by chromatography on columns of silicic acid-Super Cel and alumina-Super Cel-silver nitrate and further purification by way of the dibromide and recrystallization twice from acetone) and 5α-[2-³H]-lanost-8-en-3β-ol (chemically synthesized as described previously; see Chapter III). A comparison of the specific activity of each sample before and after digitonin precipitation is given in Table VII-2.

The data in Table VII-2 suggested that the great majority of the activity in fractions 20-33 and 34-76 was not due to typical 3β-mono-hydroxy sterols. Since the total radioactivity in both samples represented only 13.3% of the activity recovered from the silicic acid-Super Cel column (and assuming that these fractions represented all
TABLE VII-2

Precipitability of Nonsaponifiable Components with Digitonin

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>SPECIFIC ACTIVITY (dpm/mg sterol)</th>
<th>PER CENT DIGITONIN-PRECIPITABLE MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BEFORE</td>
<td>AFTER</td>
</tr>
<tr>
<td>B-NSL 20-33</td>
<td>2.55 x 10^4</td>
<td>1.17 x 10^3</td>
</tr>
<tr>
<td>B-NSL 34-76</td>
<td>7.50 x 10^4</td>
<td>5 x 10^2</td>
</tr>
<tr>
<td>[^3H]-Cholesterol</td>
<td>6.98 x 10^4</td>
<td>6.65 x 10^4</td>
</tr>
<tr>
<td>[^3H]-Dihydrolanosterol</td>
<td>4.10 x 10^5</td>
<td>1.60 x 10^5</td>
</tr>
</tbody>
</table>

of the likely digitonin-precipitable candidates from sample B-NSL, only about 0.7% of the total activity in sample B-NSL was precipitable with digitonin. Therefore, incubation of this preparation of bovine retina S10 homogenate with mevalonic acid did not produce appreciable amounts of typical sterols (especially cholesterol).

The supernatants obtained during the digitonin precipitation of the material from fractions 20-33 and 34-76 were concentrated and analyzed further by radio-TLC and radio-GLC. Radio-TLC analysis of the material from fractions 20-33 was performed on Silica Gel G plates developed in the following solvent systems (one each): (A) benzene-diethyl ether-petroleum ether, 7:3:2; (B) petroleum ether-diethyl ether, 85:15. The following chromatographic standards were employed: commercial lanosterol, dolichol, Coenzyme Q\textsubscript{10} and squalene. After developing, each plate was analyzed in the usual fashion. The radiochromatograms are shown in Figure VII-20. Using the first solvent system (A), the bulk of the radioactivity co-migrated with the dolichol stan-
FIGURE VII-19

Radio-TLC of pooled fractions 10-19 obtained by silicic acid-Super Cel column chromatography of sample B-NSL (see Figure VII-16). Chromatographic system: Silica Gel G; A, chloroform; B, benzene-diethyl ether-petroleum ether, 7:3:2; C, hexane-diethyl ether-glacial acetic acid, 65:35:1. Chromatographic standards (in order of increasing $R_f$): lanosterol, dolichol and squalene.

FIGURE VII-20

Radio-TLC of the supernatant obtained after digitonin precipitation of pooled fractions 20-33 from silicic acid-Super Cel column chromatography of sample B-NSL (see Figure VII-16). Chromatographic system: Silica Gel G; A, benzene-diethyl ether-petroleum ether, 7:3:2; b, petroleum ether-dimethyl ether, 85:15. Chromatographic standards (in order of increasing $R_f$): lanosterol, dolichol, coenzyme Q$_{10}$ and squalene.
The diagram consists of three graphs labeled A, B, and C, showing migration versus radioactivity (DPM x 10^-3). The x-axis represents migration (cm), and the y-axis represents radioactivity (DPM x 10^-3). The graphs compare the migration of different samples, presumably to analyze their radioactive properties.)
standard; however, chromatography in the second solvent system (B) revealed that the major component of the sample was not dolichol (i.e. it migrated even ahead of the CoQ_{10} standard) and definitely did not correspond to monohydroxy sterols. However, about 20% of the total radioactivity was much more polar than the major component and exhibited the chromatographic behavior consistent with those of typical monohydroxy sterols (although such sterols should have been precipitable with digitonin under the conditions employed).

A similar kind of analysis was performed on aliquots of the sample derived from fractions 34-76. In this case, however, the solvent system was held constant (benzene-diethyl ether-petroleum ether, 7:3:2) while the TLC adsorbant phase was varied as follows: (A) Silica Gel G; (B) Silica Gel G-4% silver nitrate. Cholesterol, lanosterol and squalene were used as standards with the first system, while only cholesterol and squalene were used with the second system. The radiochromatograms are shown in Figure VII-21. On the first TLC system (A), the bulk of the radioactivity co-migrated with the lanosterol standard (which is inconsistent with the column chromatography data); however, about 96% of the radioactivity remained at the origin of the argented plate (system B), while about 2% of the activity migrated in the region of cholesterol. Clearly, the major component of this sample was polyunsaturated and was not a sterol. Considering the nature of the substrate from which this material was derived, the chromatographic properties and the lack of digitonin precipitability, the major constituent of this sample most likely was an open-chain isoprenoid alcohol.
A portion of the sample obtained from fractions 34-76 (3.82 x 10^4 cpm) was analyzed by radio-GLC on a 3% OV-17 column (column temperature, 160°C; carrier flow rate, 66 ml/min.). Authentic standards of farnesol (a mixture of cis, trans- and all-trans isomers) and all-trans geranylgeraniol were employed for comparison. The resulting radiochromatogram is shown in Figure VII-22. About 90% of the recovered radioactivity eluted asymmetrically about the geranylgeraniol peak (retention time, 51.26 min.), suggesting a multiplicity of components. The remaining activity (apparently at least two components) eluted much earlier than the geranylgeraniol standard but later than the farnesol peaks (retention times, 5.02 and 5.76 min.), representing about 7-8% of the recovered radioactivity. Less than 0.5% of the activity eluted with the farnesol isomers. The overall recovery of applied activity was only 67%, suggesting that other more polar (or larger) components failed to elute from the column under the conditions employed. A second injection of the sample (4.39 x 10^4 cpm) was made at a higher column temperature (190°C, isothermal); the radiochromatogram is shown in Figure VII-23. Again, about 88% of the recovered activity was distributed around the geranylgeraniol peak (retention time, 12.60 min.); the radioactivity profile was broad in comparison with the mass standard, again suggesting the presence of more than one component. About 6% of the activity eluted just after the solvent front. No further radioactivity was recovered after the geranylgeraniol peak (with the exception of the "tailing" activity from the major peak of radioactivity) up to 60 minutes post-injection. The overall recovery was about 92%. Therefore, the major labelled component derived from fractions 34-76 exhibited the
FIGURE VII-21
Radio-TLC of the supernatant obtained after digitonin precipitation of pooled fractions 34-76 from silicic acid-Super Cel column chromatography of sample B-NSL (see Figure VII-16). Chromatographic systems: A, Silica Gel G; B, Silica Gel G-5% silver nitrate; each developed once with benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards (in order of increasing R_f): A, cholesterol, lanosterol and squalene; B, cholesterol and squalene.

FIGURE VII-22
Radio-GLC of the supernatant obtained after digitonin precipitation of pooled fractions 34-76 from silicic acid-Super Cel column chromatography of sample B-NSL (see Figure VII-16). Chromatographic system: 3% OV-17, 160°C isothermal. Chromatographic standards (in order of increasing retention time): cis,trans-farnesol, all-trans farnesol and all-trans geranylgeraniol.

FIGURE VII-23
Radio-GLC of the supernatant obtained after digitonin precipitation of pooled fractions 34-76 from silicic acid-Super Cel column chromatography of sample B-NSL (see Figure VII-16). Chromatographic system: 3% OV-17, 190°C isothermal. The mass peak is due to all-trans geranylgeraniol.
behavior of a C\textsubscript{20} isoprenoid alcohol, but did not have the chromatographic properties of all-\textit{trans} geranylgeraniol.

No further analysis of the B-NSL material was performed.

\textbf{Analysis of Material Obtained by Column Chromatography of Sample C-NSL}

An aliquot of the sample from pooled fractions 9-28 (9.55 x 10\textsuperscript{3} cpm) was analyzed by radio-GLC on a 3% OV-17 column (column temperature, 260\textdegreeC; carrier flow rate, 66 ml/min.). The following chromatographic standards were employed (retention times in parentheses, in minutes): squalene (6.30), cholesterol (17.02), 24,25-dihydrolanosterol (25.04) and lanosterol (30.46). The radiochromatogram is shown in Figure VII-24. At least 96% of the recovered radioactivity eluted immediately in the solvent front, while about 2% co-migrated with the lanosterol standard. The recovery of applied activity was 99%. The major labelled component was more polar than squalene (from its chromatographic behavior on the silicic acid-Super Cel column) but apparently much smaller than a C\textsubscript{30} isoprenoid or sterol (as judged from its GLC behavior). It did not exhibit the properties of known sterols, and its chromatographic behavior also suggested that it was not a steryl ketone. The identity of this component remains unknown.

Portions of the sample from pooled fractions 44-51 were analyzed by radio-GLC on the same 3% OV-17 column at three different temperatures. At 256\textdegreeC, the chromatographic properties of the labelled material were compared with those of the following standards (retention times in parentheses, in minutes): squalene (7.10), cholesterol (19.24), 24,25-dihydrolanosterol (28.50) and lanosterol (34.72). The radiochromato-
Radio-GLC of pooled fractions 9-28 obtained by silicic acid-Super Cel column chromatography of sample C-NSL (see Figure VII-17). Chromatographic system: 3% OV-17, 260°C isothermal. Chromatographic standards (in order of increasing retention time): squalene, cholesterol, 24,25-dihydrolanosterol and lanosterol.
gram is shown in Figure VII-25 (applied radioactivity, $9.86 \times 10^3$ cpm). About 98% of the recovered activity eluted with the solvent front; less than 1% of the activity appeared to co-migrate with the dihydrolanosterol standard. The recovery of applied activity was about 90%. The results suggested that the major component of this sample was not a sterol and was apparently smaller than (as well as more polar than) squalene. Since this material was derived from fractions which eluted after those which corresponded to the chromatographic mobility of lano-
sterol on the silicic acid-Super Cel column, it is not possible that the minor component represented dihydrolanosterol. Another portion of the sample ($2.81 \times 10^4$ cpm) was injected onto the column at a lower tempera-
ture ($170^\circ$C) and compared with the chromatographic properties of all-
$trans$ geranylgeraniol (retention time, 30.65 min.). The radiochroma-
togram is shown in Figure VII-26. About 91% of the radioactivity eluted just before the C$_{20}$ isoprenol standard, while at least three other minor components migrated near the solvent front (<5% of the total recovered activity). The overall recovery was about 90%. In order to investigate the minor components further, the column tempera-
ture was lowered to $126^\circ$C and another portion of the sample ($8.3 \times 10^3$ cpm) was analyzed in comparison with the following isoprenoid standards (retention times in parentheses, in minutes): nerol (1.40); geraniol (1.67); nerolidol (8.55); "$cis-trans$" farnesol" (20.76); all-$trans$ farnesol (25.12). Under these conditions, no radioactivity was recovered, whereas all the chromatographic standards eluted normally (within 32 minutes collection time). The labelled components were therefore
FIGURE VII-25

Radio-GLC of pooled fractions 44-51 obtained by silicic acid-Super Cel column chromatography of sample C-NSL (see Figure VII-17). Chromatographic system: 3% OV-17, 256°C isothermal. Chromatographic standards (in order of increasing retention time): squalene, cholesterol, 24,25-dihydrolanosterol and lanosterol.

FIGURE VII-26

Radio-GLC of pooled fractions 44-51 obtained by silicic acid-Super Cel column chromatography of sample C-NSL (see Figure VII-17). Chromatographic system: 3% OV-17, 170°C isothermal. The mass peak is due to all-trans geranylgeraniol.
either larger than the C\textsubscript{15} isoprenol standards or had molecular geometries much different than the normal open-chain isoprenols. The major component of this sample appeared to have chromatographic properties similar to those of the all-trans geranylgeraniol standard, but was apparently either slightly smaller than or slightly less polar than the C\textsubscript{20} isoprenoid standard. The identity of this component was not established.

Portions of the sample from pooled fractions 52-73 were analyzed by radio-GLC at two different column temperatures on the same 3% OV-17 column. At 256\degree\textsubscript{C}, the sample (2.94 x 10\textsuperscript{4} cpm) was compared with the following chromatographic standards (retention times in parentheses, in minutes): squalene (5.83), cholesterol (15.19), 7-dehydrocholesterol (19.09), dihydrolanosterol (23.45) and lanosterol (28.52). The radiochromatogram is shown in Figure VII-27. As previously observed with the other two samples, about 97% of the recovered radioactivity eluted with the solvent front and no activity eluted in the region of the sterol standards. Recovery of applied radioactivity was approximately 80%. At a much lower temperature (126\degree\textsubscript{C}), another portion of the sample (1.64 x 10\textsuperscript{4} cpm) was compared with the following chromatographic standards (retention times in parentheses, in minutes): nerol (1.45); geraniol (1.75); nerolidol (9.12); "cis-trans-" farnesol" (22.20); all-trans farnesol (27.21). The radiochromatogram is shown in Figure VII-28. Only 6% of the injected radioactivity was recovered from the column under the conditions employed (up to 32 minutes). About 95% of the recovered activity appeared to co-chromatograph with the "cis-trans-" isomers of farnesol, while the remaining activity eluted after the C\textsubscript{10}
FIGURE VII-27
Radio-GLC of pooled fractions 52-73 obtained by silicic acid-Super Cel column chromatography of sample C-NSL (see Figure VII-17). Chromatographic system: 3% OV-17, 256°C isothermal. Chromatographic standards (in order of increasing retention time): squalene, cholesterol, Δ⁷-cholestadienol, 24,25-dihydroxolanosterol and lanosterol.

FIGURE VII-28
Radio-GLC of pooled fractions 52-73 obtained by silicic acid-Super Cel column chromatography of sample C-NSL (see Figure VII-17). Chromatographic system: 3% OV-17, 126°C isothermal. Chromatographic standards (in order of increasing retention time): nerol (cis-geraniol), geraniol, nerolidol (the tertiary isomer of all-trans farnesol), cis,trans-farnesol and all-trans farnesol.
isoprenols (nerol and geraniol) but before the tertiary C\textsubscript{15} isoprenol standard (nerolidol). Curiously, the material which had chromatographic properties similar to those of farnesol was apparently composed of at least two components, neither of which seemed to correspond to the all-trans isomer of farnesol.

Further analyses of the other components of samples B-NSL and C-NSL were not performed.

**SUMMARY**

The data presented in this experiment indicated that the bovine retina S\textsubscript{10} preparation was capable of metabolizing mevalonic acid to a variety of lipid products. In striking contrast to results of similar incubations of the given substrate with rat liver S\textsubscript{10} homogenates, the retina preparation converted the majority of the usable mevalonic acid to isoprenoid acids rather than to nonsaponifiable lipids. Incubating an average of about 7.7 x 10\textsuperscript{-4} mmole of mevalonic acid per flask (5.73 nmoles per mg protein) resulted in the conversion of about 75% of the theoretically usable substrate to saponifiable lipids, of which about 80% was represented by isoprenoid acids. Of the total amount of mevalonic acid metabolized, approximately 92% was incorporated into saponifiable material while only about 8% went into nonsaponifiable lipids; the total conversion represented about 80% of the maximum theoretically usable substrate.

Only two classes of isoprenoid acids accumulated: C\textsubscript{15} acids (ca. 20-30%) and C\textsubscript{20} acids (ca. 70-80%). At least 89% of the C\textsubscript{15} class was composed of material having the chromatographic properties of all-trans
farnesoic acid. Since the synthesis of one mole of farnesoic acid (MW 236) requires the use of 3 moles of mevalonic acid, approximately 5 μg of all-trans farnesoic acid were synthesized. Similarly, 4 moles of mevalonic acid are required for the synthesis of one mole of C20 isoprenoid; assuming all-trans geranylgeranoic acid (MW 304) to be representative of this class of isoprenoid acids synthesized by the retina homogenates, about 12 μg of total C20 isoprenoid acids were synthesized. In one of the incubations, about 43% of the total isoprenoid acid fraction exhibited the chromatographic properties of all-trans geranylgeranoic acid, representing about 62% of the total C20 isoprenoid pool. The remainder of the C20 isoprenoid fraction was composed of at least two components, each of which apparently contained at least one cis double bond.

A multiplicity of nonsaponifiable lipids were formed during the course of these incubations, but the great majority (>75%) of the labelled components were much more polar than common isoprenoid alcohols or monohydroxy sterols (cf. Schroepfer and Gore, 1963). Less than 0.7% of the total nonsaponifiable activity was precipitable with digitonin, confirming the lack of 3β-monohydroxy sterols. Considering the nature of the substrate and the chemical and chromatographic properties of the relatively nonpolar components (i.e. those which eluted with toluene from the silicic acid-Super Cel columns), it is likely that most of these components represented isoprenoid alcohols of some sort (with the exception of the material which exhibited the chromatographic properties of long-chains hydrocarbons or ketones). However, these components did not behave chromatographically (by GLC) like isoprenols whose
pyrophosphate derivatives are known intermediates in the biosynthesis of cholesterol (i.e. all-trans farnesyl pyrophosphate, all-trans geranyl pyrophosphate) or related compounds (i.e. all-trans geranylgeranyl pyrophosphate). Although C_{15} and C_{20} isoprenoid acids accumulated in the incubation mixtures, the corresponding all-trans alcohols did not appear in the NSL extracts. Also, there was no evidence to suggest the synthesis of dolichol or related long-chain isoprenoids. The predominant apolar nonsaponifiable isoprenoids appeared to be larger than 10 carbons but less than or equal to 20 carbons in length (based on the GLC data) but did not correspond to known standards of nerol, geraniol, all-trans farnesol or all-trans geranylgeraniol. Material which had the chromatographic properties of squalene represented about 5% of the total nonsaponifiable activity.

**EXPERIMENT VII-2**

In order to confirm the results of the previous experiment (VII-1), the experimental procedures were repeated with a second preparation of bovine retina S_{10} homogenate. The preparation of the incubation buffer, cofactor stock, isotopic substrate stock and the retina homogenate was as previously described (Experiment VII-1). In this case, 30 freshly dissected bovine retinas yielded 68 ml of S_{10} homogenate (6.15 mg protein per ml). Incubations were carried out as previously described; each flask (125 ml screw-top erlenmeyer) contained 12.0 ml of retina homogenate (73.8 mg protein; final concentration, ca. 4.3 mg/ml), 1.0 ml of concentrated cofactor stock (final incubation concentrations: NAD and NADP, 1 mM; G-6-P, 3 mM; ATP, 5 mM) and 1.0 ml of
3RS-[2-\(^3\)H]-mevalonic acid (potassium salt; stock concentration, 2.88 x \(10^8\) dpm/ml), adjusted to a final incubation volume of 17.0 ml with incubation buffer. Each complete incubation mixture was assayed for total initial radioactivity prior to initiation of the incubation. Each flask was flushed with oxygen for 10 seconds, sealed and then incubated at 37°C for the desired amount of time (see table below). In addition, 12.0 ml of retina homogenate was placed in a boiling water bath for 10 minutes (in a sealed incubation flask) prior to addition of the other incubation mixture constituents ("boiled enzyme" control, flask D) and then incubated as usual.

<table>
<thead>
<tr>
<th>FLASK</th>
<th>INCUBATION TIME (hr.)</th>
<th>INITIAL ACTIVITY (dpm x 10^-8)</th>
<th>3RS-[2-(^3)H]-MEVALONIC ACID CONCENTRATION (umolar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>2.69</td>
<td>40.9</td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td>2.67</td>
<td>40.6</td>
</tr>
<tr>
<td>C</td>
<td>24</td>
<td>2.76</td>
<td>41.9</td>
</tr>
<tr>
<td>D</td>
<td>24</td>
<td>2.88</td>
<td>43.8</td>
</tr>
</tbody>
</table>

The incubations were terminated by the addition of 18 ml of 15% ethanolic potassium hydroxide; the flasks were then flushed with nitrogen, lightly sealed and incubated for 3 hours at 80°C (saponification). After allowing the flasks to cool to room temperature, they were stored for 1.5 days at 4°C. Each incubation mixture was then diluted with 45 ml of water and extracted three times with 100 ml portions of petroleum ether. The combined nonsaponifiable lipid (NSL) extracts were washed once with 100 ml of water (water wash added back to initial aqueous-
ethanolic phase), dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in 5.0 ml of benzene. Triplicate aliquotes (0.1 ml each) were assayed from each NSL sample for total radioactivity.

The aqueous-ethanolic phase from each incubation (containing the saponifiable lipids) was chilled to -20°C and then immediately acidified by dropwise addition of concentrated HCl (5.55 ml), with continuous stirring. The acidified mixture was then extracted three times with 200 ml portions of dichloromethane; the combined extracts were washed once with 100 ml of water, dried over anhydrous magnesium sulfate, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in 10.0 ml of benzene. Triplicate aliquots (0.1 ml each) were assayed from each saponifiable lipid (SL) extract for total radioactivity. All samples were stored at 4°C prior to further analysis.

RESULTS

The incorporation of mevalonic acid into saponifiable and nonsaponifiable lipids by the bovine retina homogenates is documented in Table VII-3. After 3 hours of incubation (flask A), about 22% of the initially incubated radioactivity was incorporated into NSL components, while about 18% was found in SL components. This represented approximately a 79% conversion of the biologically "active" form of mevalonic acid; the remainder of the activity was not extractable from the aqueous phase (apparently unmetabolized substrate). At the end of 24 hours of


<table>
<thead>
<tr>
<th>FLASK</th>
<th>NONSAPONIFIABLE</th>
<th>SAPONIFIABLE</th>
<th>PER CENT(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(dpm x (10^{-7}))</td>
<td>% Initial Activity</td>
<td>(dpm x (10^{-7}))</td>
</tr>
<tr>
<td>A</td>
<td>5.85</td>
<td>21.7</td>
<td>4.81</td>
</tr>
<tr>
<td>B</td>
<td>5.93</td>
<td>21.2</td>
<td>7.16</td>
</tr>
<tr>
<td>C</td>
<td>5.88</td>
<td>21.3</td>
<td>6.83</td>
</tr>
<tr>
<td>D*</td>
<td>&lt;0.002</td>
<td>&lt;0.006</td>
<td>&lt;0.95</td>
</tr>
</tbody>
</table>

\(^1\) The maximum theoretical conversion of 3RS-[2-\(^3\)H]-mevalonic acid is 50%, since only the 3R- enantiomer is "biologically active"

* Labeled material extracted from the boiled control was due to unconverted substrate and did not represent metabolites of mevalonic acid (see text)

incubation (flasks B and C), there was no appreciable change in the percentage of activity which was extracted as NSL material, relative to the 3-hour incubation; however, approximately 26% of the initial activity was converted to SL components with a concomitant decrease in the amount of residual aqueous activity. This represented approximately a 94% overall conversion of the usable mevalonic acid. As expected, over 96% of the initially incubated activity remained in the aqueous phase after saponification, acidification and extraction of the incubation mixture of the boiled control (flask D). The small amount of activity which was extracted was found in the saponifiable phase and was found to contain only unconverted substrate (see next section, Figure
VII-29). Increasing the incubation time from 3 hours to 24 hours, therefore, caused a selective increase in the conversion of mevalonic acid to saponifiable components, but the distribution of activity into NSL and SL material (roughly 1:1) was quite different from that found in the previous experiment (where the NSL/SL ratio was about 1:3).

**Analysis of Saponifiable Lipids**

Aliquots of the saponifiable material derived from the 3-hour (A-SL) and 24-hour (C-SL) incubations were analyzed by radio-TLC as described previously. Authentic standards of mevalonic acid (prepared from the lactone by treatment with 5 N potassium hydroxide), mevalonic acid lactone and farnesolic acid (a mixture of isomers) were chromatographed in an adjacent lane on each plate for purposes of comparison. The radiochromatograms are shown in Figure VII-29. Using the hexane-diethyl ether-glacial acetic acid solvent system (80:20:1, v/v), both the free mevalonic acid and its lactone remained at the origin of the plates, whereas the farnesolic acid isomers migrated about one-third of the way up the plates (being resolved into two readily-distinguishable components). The chromatograms of both sample A-SL and C-SL (Figures VII-29A and VII-29B, respectively) exhibited a major component of radioactivity co-migrating with the farnesolic acid standard (ca. 72% of the total activity); about 20% of the activity was more polar, trailing to the origin, while roughly 8-10% of the activity migrated near the solvent front (possibly due to a cyclization product or cross-contamination from the NSL material). There was about 13% less activity at the origin of the plate of sample C-SL than for sample A-SL, consistent
FIGURE VII-29

Radio-TLC of the saponifiable material obtained after incubation of bovine retina $S_{10}$ homogenates with [2-\textsuperscript{3}H]-mevalonic acid for 3 hours (A, sample A-SL) and 24 hours (B, sample C-SL). C and D, boiled control. Chromatographic system: Silica Gel G; A, B and C, hexane-diethyl ether-glacial acetic acid, 80:20:1; D, benzene-acetone, 1:1. Chromatographic standards: mevalonic acid (stippled), mevalonic acid lactone (solid) and farnesolic acid isomers (cross-hatched).
with the finding that about 13% more mevalonic acid was converted to saponifiable lipids after 24 hours of incubation as compared with the 3-hour sample (see Table VII-3). In contrast, the sample obtained from the boiled control (Figure VII-29C) exhibited a chromatogram with at least 99% of the activity at the origin of the plate, co-chromatographing with unmetabolized substrate. Using a solvent system which can resolve the free acid form of mevalonic acid from the corresponding lactone (i.e. benzene-acetone, 1:1), more than 95% of the activity of sample D-SL was observed to co-migrate with the mevalonic acid lactone standard (Figure VII-29D), while the remainder of the activity stayed at the origin with the free acid. Clearly, there was no conversion of mevalonic acid to "fatty acid-like" material in the case of the incubation involving the boiled control. These results were qualitatively quite similar to those of the previous experiment, confirming the conversion of mevalonic acid to "fatty acid-like" material by the retina homogenates.

Samples A-SL and C-SL were esterified with diazomethane and the resulting methyl esters were purified by Unisil column chromatography as described in the previous experiment (recovered activity: A-SLME, 3.45 x 10^7 dpm; C-SLME, 5.69 x 10^7 dpm). Portions of each sample were applied to Silica Gel G TLC plates and developed once with hexane-diethyl ether-glacial acetic acid (80:20:1); standards of all-\textit{trans} methyl farnesoate and squalene were chromatographed in lanes adjacent to the labelled samples. The radiochromatograms (Figures VII-30A and VII-30C, respectively) exhibited a single band of activity which co-migrated with the methyl farnesoate standard; no trace of the previously
FIGURE VII-30

Radio-TLC of the methyl esters of the saponifiable lipids obtained by incubation of bovine retina S₁₀ homogenates with [2⁻³H]-mevalonic acid for 3 hours (A and B, sample A-SLME) and 24 hours (C and D, sample C-SLME). Chromatographic system: A and C, Silica Gel G (developed once with hexane-diethyl ether-glacial acetic acid, 80:20:1); B and D, Silica Gel G-5% silver nitrate (developed twice with hexane-diethyl ether-glacial acetic acid, 94:4:2). Chromatographic standards: A and C, all-trans methyl farnesoate (solid) and squalene (stippled); B and D, FAME standards C₂₂:₆, C₂₀:₃, C₂₀:₂, C₂₀:₁ and C₂₀:₀ (cross-hatched) and all-trans methyl farnesoate (solid).
detected nonpolar component was observed (i.e. the component which migrated near the solvent front). Another portion of each sample was analyzed by radio-TLC on an argentated plate (Silica Gel G - 5% silver nitrate; developed twice with hexane-diethyl ether-glacial acetic acid, 94:4:2), along with standards of all-trans methyl farnesolate and a mixed FAME standard (containing C\textsubscript{20}:0, C\textsubscript{20}:1, C\textsubscript{20}:2, C\textsubscript{20}:3 and C\textsubscript{22}:6 species). The radiochromatograms are shown in Figure VII-30B and VII-30D, respectively. In each case, roughly 93% of the activity co-migrated with the C\textsubscript{20}:3 FAME standard, while about 7% of the activity migrated just ahead of the C\textsubscript{20}:2 standard, coincident with the all-trans methyl farnesolate standard.

Radio-GLC analysis of portions of sample A-SLME (4.67 x 10\textsuperscript{5} cpm) and C-SLME (6.8 x 10\textsuperscript{5} cpm) was performed as previously described, using a 3% OV-17 column (isothermal column temperature, 164\textdegreeC; carrier flow rate, 66 ml/min.), collecting and analyzing one-minute effluent samples as usual. The following chromatographic standards were employed: all-trans methyl farnesolate, methyl phytanate, trans- Δ\textsuperscript{2}-methyl phytanate, and the methyl and ethyl esters of all-trans geranylgeranoate. The radiochromatograms are shown in Figures VII-31 and VII-32, respectively. In each case, the activity was distributed into three major components: (1) co-migrating with the all-trans methyl farnesolate standard; (2) eluting just after the trans- Δ\textsuperscript{2}-methyl phytanate standard, and (3) co-migrating with the all-trans methyl geranylgeranoate standard. The ratio of the percentage of recovered activity in these three components in sample A-SLME was about 8:76:15, respectively, while the ratio was about 7:88:2 for sample C-SLME. Thus, while there was little or no
FIGURE VII-31

Figure VII-32
Radio-GLC of sample C-SLME (from the 24-hour incubation). Chromatographic system: 3% OV-17, 164°C isothermal. Chromatographic standards (in order of increasing retention time): all-trans methyl farnesoate, methyl phytanate, trans-Δ⁴-methyl phytenate and all-trans methyl geranylgeranoate.
difference in the relative amount of activity distributed into the C_{15}
isoprenoid species (comparing 3 hours vs. 24 hours of incubation), the
population of C_{20} isoprenoid acids shifted markedly between 3 hours and
24 hours of incubation such that, at 24 hours, nearly all of the all-
trans component was lost with a concomitant increase in the component
which eluted just after the trans- Δ^2-methyl phytenate standard (re-
ferred to hereafter as "component X"). The overall recovery of applied
activity was about 88% in each case.

Portions of the methyl ester samples were hydrogenated over PtO_2
as described previously and analyzed by radio-TLC on argentated plates,
in comparison with the FAME mixture previously employed. The radiochro-
matograms (Figures VII-33A and VII-33B) indicated that the hydrogenation
was quantitative; greater than 99% of the activity co-migrated with the
saturated FAME standard. Portions of each hydrogenated sample were an-
alyzed by radio-GLC (3% OV-17; isothermal column temperature, 150°C),
along with standards of methyl perhydrofarnesoate (methyl-3,7,11-tri-
methyldodecanoate), methyl phytenate, methyl palmitate and methyl
stearate. The radiochromatogram obtained from hydrogenated sample A-
SLME is shown in Figure VII-34 (applied activity, 2.43 x 10^5 cpm).
The radioactivity was distributed into only two components: 7% co-
chromatographed with the saturated C_{15} isoprenoid standard and about
92% co-chromatographed with the saturated C_{20} isoprenoid standard. No
activity co-chromatographed with the saturated fatty acid methyl ester
standards. The overall recovery of applied radioactivity was about 95%.
A nearly identical radiochromatogram was obtained for hydrogenated sam-
ple C-LSME. These results were consistent with those shown in Figures
FIGURE VII-33

Radio-TLC of hydrogenated methyl esters of the saponifiable lipids obtained from incubations of bovine retina $S_{10}$ homogenates with $[2-^{3}H]$-mevalonic acid for 3 hours (A, sample A-SLME) and 24 hours (B, sample C-SLME). Chromatographic system: Silica Gel G-5% silver nitrate, developed twice with hexane-diethyl ether-petroleum ether, 94:4:2. Chromatographic standards (in order of increasing $R_{f}$): FAME standards $C_{22:6}$, $C_{20:3}$, $C_{20:2}$, $C_{20:1}$ and $C_{20:0}$.

FIGURE VII-34

VII-31 and VII-32, and confirmed that only two classes of isoprenoid acids (the "fatty acid-like" material) accumulated in these incubations, as observed in the previous experiment, with the major components belonging to the C_{20} class. The apparent absence of fatty acid biosynthesis from mevalonic acid, a process which requires the presence of mitochondria (Edmond and Popjak, 1974), is consistent with the assumption that the retina S_{10} homogenates did not contain mitochondria. In contrast to the results of Experiment VII-1, the predominant isoprenoid acid species in this set of incubations was represented by "component X".

The methyl ester samples were fractionated on columns of Silica Gel G-Super Cel-silver nitrate (1:1, w/w; 50 x 1 cm) essentially as described in the previous experiment (applied activity: A-SLME, 2.31 x 10^{7} dpm; C-LSME, 4.35 x 10^{7} dpm). The columns were equilibrated and eluted with benzene (fraction volume: A-SLME, 4.9 ml; C-SLME, 5.0 ml; flow rate: 0.12-0.13 ml/min); the solvent was changed to benzene-chloroform, 93.7, at fraction 136. The elution profiles for samples A-SLME and C-SLME are shown in Figures VII-35 and VII-36, respectively. In each case, ≤ 10% of the recovered radioactivity eluted before the solvent change. Four distinct components were readily observable in each chromatogram; the distribution of activity in these components is given in the table below.

From these results and the radio-GLC data, it was apparent that both the C_{15} and the C_{20} isoprenoid acid classes were heterogeneous, each containing at least two components (note the close qualitative and quantitative agreement between the results obtained by column
FIGURE VII-35
Silica Gel G-Super Cel-silver nitrate column chromatography of sample A-SLME. Solvent system: benzene; arrow denotes a change to benzene-chloroform, 93:7.

FIGURE VII-36
Silica Gel G-Super Cel-silver nitrate column chromatography of sample C-SLME. Solvent system: benzene; arrow denotes a change to benzene-chloroform, 93:7.
TABLE VII-4

Recovery of Labelled Components after Fractionation of the Methyl Esters of Saponifiable Lipids by Silica Gel G-Super Cel-Silver Nitrate Column Chromatography¹

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PEAK</th>
<th>FRACTIONS</th>
<th>PER CENT of TOTAL RECOVERED Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-SLME</td>
<td>I</td>
<td>24 - 38</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>42 - 64</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>160 - 193</td>
<td>90.1</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>196 - 280</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90.1</td>
</tr>
<tr>
<td>C-SLME</td>
<td>I</td>
<td>24 - 38</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>42 - 65</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>162 - 193</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>194 - 280</td>
<td>88.1</td>
</tr>
</tbody>
</table>

¹ Recovery of applied radioactivity: A-SLME, $2.20 \times 10^7$ dpm (95.2%); C-SLME, $4.22 \times 10^7$ dpm (97.0%).

chromatography and radio-GLC). Fractionation of the C₁₅ class was not apparent under the TLC and GLC conditions employed and had not been observed in the previous experiment. The material from each peak was analyzed further by radio-GLC on two different column supports (3% OV-17 and 12% DEGS); in addition, a portion of the material from each peak was hydrogenated as described previously and analyzed on a 3% OV-17 column. The remainder of this section describes the results obtained from the analysis of the components of sample C-SLME; the results obtained from the components of sample A-SLME were in close agreement.
with the results presented below.

A portion of sample C-SLME-I (3.63 x 10⁴ cpm; pooled fractions 24-38) was compared chromatographically with a mixture of methyl farnesoate isomers on a 3% OV-17 GLC column (column temperature, 134°C; carrier flow rate, 66 ml/min.). The elution profile is shown in Figure VII-37. Greater than 98% of the radioactivity co-chromatographed with the all-trans isomer of methyl farnesoate (retention time, 22.68 min.). Under these conditions, the other three isomers of methyl farnesoate eluted with the following relative retention times (relative to the all-trans isomer): 0.660, 0.792 and 0.868. The recovery of applied radioactivity was essentially quantitative. Another aliquot of this sample (5.22 x 10⁴ cpm) was chromatographed on a 12% DEGS column (column temperature, 134°C) along with the methyl farnesoate isomers. The radiochromatogram (Figure VII-38) showed the major radioactive component (>98% of the activity) co-migrating with the all-trans isomer of methyl farnesoate (retention time, 36.35 min.). The other three isomers eluted with the following relative retention times (relative to the all-trans isomer): 0.633, 0.749 and 0.913. About 2% of the recovered radioactivity co-eluted with the less polar of the two "cis-trans-" isomers (relative retention time, 0.749). Recovery of applied activity was almost quantitative (ca. 99%). A portion of sample C-SLME-I (1.48 x 10⁴ cpm) was hydrogenated and applied to a 3% OV-17 GLC column (column temperature, 108°C) along with an authentic standard of methyl-3,7,11-trimethyldecaneoate. The radiochromatogram (Figure VII-39) showed that greater than 98% of the recovered radioactivity co-migrated with the C₁₅ isoprenoid standard; overall recovery was about 87%. The com-
FIGURE VII-37
Radio-GLC of peak I (fractions 24-38) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample C-SLME (see Figure VII-36). Chromatographic system: 3% OV-17, 134°C. Chromatographic standards (in order of increasing retention time): cis,cis-methyl farnesoate, "cis-trans" isomers of methyl farnesoate (second and third peaks) and all-trans methyl farnesoate.

FIGURE VII-38
Radio-GLC of peak I (fractions 24-38) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample C-SLME (see Figure VII-36). Chromatographic system: 12% DEGS, 134°C isothermal. Chromatographic standards (in order of increasing retention time): cis,cis-methyl farnesoate, "cis-trans" isomers of methyl farnesoate (second and third peaks) and all-trans methyl farnesoate.

FIGURE VII-39
Radio-GLC of hydrogenated peak I (fractions 24-38) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample C-SLME (see Figure VII-36). Chromatographic system: 3% OV-17, 108°C isothermal. The mass peak is due to methyl-3,7,11-trimethyldodecanoate.
bined column chromatographic data and radio-GLC results were consis-
tent with the known properties of all-trans methyl farnesoate, which
represented at least 98% of the radioactivity in sample C-SLME-I.

A similar set of analyses were performed on sample C-SLME-II
(pooled fractions 42-65). The radiochromatogram obtained by chromato-
graphing a portion of the sample (7.88 x 10^3 cpm) on a 3% OV-17 column
(column temperature, 132°C) is shown in Figure VII-40. About 98% of
the recovered radioactivity co-migrated with the all-cis isomer of
methyl farnesoate. Under the conditions employed, the following rela-
tive retention times were obtained for the standards of methyl-3,7,11-
trimethyldodecanoate and the four isomers of methyl farnesoate (rela-
tive to the all-trans isomer; retention time, 22.65 min.): 0.368,
0.661, 0.794, 0.871 and 1.000. The recovery of applied radioactivity
was approximately 88%. Another portion of the sample (1.76 x 10^4 cpm)
was analyzed on a 12% DEGS column (column temperature, 134°C) along
with the isomers of methyl farnesoate (Figure VII-41). Again, about
98% of the recovered radioactivity co-chromatographed with the all-cis
isomer. The overall recovery was about 93%. Hydrogenation and subse-
quent chromatography of a portion of the sample (9.6 x 10^3 cpm) on a
3% OV-17 column (column temperature, 108°C) demonstrated that greater
than 99% of the recovered activity co-migrated with the C_{15} isoprenoid
standard (methyl-3,7,11-trimethyldodecanoate) (Figure VII-42). The
recovery was approximately 91%. These data were consistent with the
properties of the all-cis isomer of methyl farnesoate and suggested
that at least 98% of the radioactivity of sample C-SLME-II was due to
this isoprenoid.
FIGURE VII-40
Radio-GLC of peak II (fractions 42-65) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample C-SLME (see Figure VII-36). Chromatographic system: 3% OV-17, 132°C isothermal. Chromatographic standards (in order of increasing retention time): methyl-3,7,11-trimethyldodecanoate, cis,cis-methyl farnesoate, "cis-trans" isomers of methyl farnesoate (third and fourth peaks) and all-trans methyl farnesoate.

FIGURE VII-41
Radio-GLC of peak II (fractions 42-65) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample C-SLME (see Figure VII-36). Chromatographic system: 12% DEGS, 134°C isothermal. Chromatographic standards (in order of increasing retention time): cis,cis-methyl farnesoate, "cis-trans" isomers of methyl farnesoate (second and third peaks) and all-trans methyl farnesoate.

FIGURE VII-42
Radio-GLC of hydrogenated peak II (fractions 42-65) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of sample C-SLME (see Figure VII-36). Chromatographic system: 3% OV-17, 108°C isothermal. The mass peak is due to methyl-3,7,11-trimethyldodecanoate.
An aliquot of sample C-SLME-III (2.12 x 10^4 cpm; pooled fractions 162-193) was chromatographed with standards of methyl phytanate, trans-\Delta^2\text{-methyl phytanate} and the methyl and ethyl esters of all-trans geranylgeranoate on a 3% OV-17 column (column temperature, 174°C). The radiochromatogram is shown in Figure VII-43. Approximately 97% of the recovered radioactivity co-migrated with the all-trans methyl geranylgeranoate standard; the remainder of the activity eluted between the trans-\Delta^2\text{-methyl phytanate} standard and all-trans methyl geranylgeranoate.

The recovery of applied radioactivity from this column was about 85%. A second portion of the sample (2.47 x 10^4 cpm) was analyzed on a 12% DEGS column (column temperature, 176°C) along with the same set of chromatographic standards (Figure VII-44). Again, about 97% of the recovered radioactivity co-migrated with the all-trans methyl geranylgeranoate standard. As observed previously, the methyl and ethyl esters of all-trans geranylgeranoic acid did not completely separate on the DEGS column (retention times, 36.94 and 40.31 minutes, respectively). Recovery of applied activity was 93%. The radiochromatogram of a portion of the hydrogenated sample (2.49 x 10^4 cpm; Figure VII-45) obtained by chromatography on a 3% OV-17 column (column temperature, 160°C) exhibited a single peak of radioactivity which co-chromatographed with the methyl phytanate standard (a C_{20} saturated isoprenoid).

No radioactivity was coincident with the standards of methyl-3,7,11-trimethyldodecanoate, methyl palmitate or methyl stearate. The recovery of applied radioactivity was quantitative. These data were consistent with the properties of all-trans methyl geranylgeranoate, representing about 97% of the activity in sample C-SLME-III.
FIGURE VII-43
Radio-GLC of peak III (fractions 162-193) obtained by Silica Gel G-
Super Cel-silver nitrate column chromatography of sample C-SLME (see
Figure VII-36). Chromatographic system: 3% OV-17, 174°C isothermal.
Chromatographic standards (in order of increasing retention time):
methyl phytanate, trans-\(\Delta^2\)-methyl phytenate, all-trans methyl geranyl-
geranoate and all-trans ethyl geranylgeranoate.

FIGURE VII-44
Radio-GLC of peak III (fractions 162-193) obtained by Silica Gel G-
Super Cel-silver nitrate column chromatography of sample C-SLME (see
Figure VII-36). Chromatographic system: 12% DEGS, 176°C isothermal.
Chromatographic standards (in order of increasing retention time):
methyl phytanate, trans-\(\Delta^2\)-methyl phytenate, all-trans methyl geranyl-
geranoate and all-trans ethyl geranylgeranoate.

FIGURE VII-45
Radio-GLC of hydrogenated peak III (fractions 162-193) obtained by
Silica Gel G-Super Cel-silver nitrate column chromatography of sample
C-SLME (see Figure VII-36). Chromatographic system: 3% OV-17, 160°C
isothermal. Chromatographic standards (in order of increasing reten-
tion time): methyl-3,7,11-trimethyldodecanoate, methyl palmitate,
methyl phytanate and methyl stearate.
Standards of methyl phytanate, trans- Δ²-methyl phytanate and the methyl and ethyl esters of all-trans geranylgeranoate were co-injected with a portion (3.96 x 10⁴ dpm) of sample C-SLME-IV (pooled fractions 194-280) on a 3% OV-17 column (column temperature, 164°C). In addition, an authentic standard of [2-¹⁴C]-Δ²-cis-methyl geranylgeranoate (2.17 x 10⁴ dpm) was also co-injected with the sample mixture. The ¹⁴C-labelled compound was prepared by saponification, acidification and diazomethane treatment of a mixture of the methyl and ethyl esters of [2-¹⁴C]-Δ²-cis-geranylgeranoic acid, followed by purification of the methyl ester by medium pressure liquid chromatography on an alumina-silver nitrate column. The radiochromatogram is shown in Figure VII-46. Greater than 98% of the tritium radioactivity eluted just after the methyl phytanate standard, exhibiting the chromatographic behavior of "compound X" previously observed. The ¹⁴C-labelled standard eluted just before the all-trans methyl geranylgeranoate standard. Under these conditions, there was less than a 2% overlap of the tritium and carbon-14 radioactivity; the overall recovery of applied activity was approximately 85%. A similar analysis (without the ¹⁴C-labelled isoprenoid) was performed on another portion of sample C-SLME-IV (1.39 x 10⁵ cpm) on a 12% DEGS column (column temperature, 176°C). The radiochromatogram is shown in Figure VII-47. Approximately 98% of the radioactivity eluted just after the trans- Δ²-methyl phytanate standard; recovery of applied radioactivity was about 98%. A portion of this sample (1.53 x 10⁵ cpm) was hydrogenated and chromatographed on a 3% OV-17 column (column temperature, 160°C) along with authentic standards of methyl-3,7,11-trimethyldecanoate, methyl phytanate, methyl palmi-
FIGURE VII-46

Radio-GLC of peak IV (fractions 194-280) obtained by Silica Gel G-
Super Cel-silver nitrate column chromatography of sample C-SLME (see
Figure VII-36). Chromatographic system: 3% OV-17, 164°C isothermal.
Chromatographic standards (in order of increasing retention time):
methyl phytanate, trans-Δ²-methyl phytenate and all-trans methyl
geranyleranoate. Dashed line, radioactivity due to [2-¹⁴C]-Δ²-cis-methyl
geranyleranoate.

FIGURE VII-47

Radio-GLC of peak IV (fractions 194-280) obtained by Silica Gel G-
Super Cel-silver nitrate column chromatography of sample C-SLME (see
Figure VII-36). Chromatographic system: 12% DEGS, 176°C isothermal.
Chromatographic standards (in order of increasing retention time):
methyl phytanate, trans-Δ²-methyl phytenate and the methyl and ethyl
esters of all-trans geranyleranoic acid.

FIGURE VII-48

Radio-GLC of hydrogenated peak IV (fractions 194-280) obtained by Sili-
ca Gel G-Super Cel-silver nitrate column chromatography of sample
C-SLME (see Figure VII-36). Chromatographic standards (in order of
increasing retention time): methyl-3,7,11-trimethyldodecanoate, methyl
palmitate, methyl phytanate and methyl stearate.
tate, methyl palmitate and methyl stearate. The radiochromatogram (Figure VII-48) exhibited a single peak of radioactivity which co-migrated with the methyl phytanate standard; the overall recovery of applied radioactivity was quantitative. These data, in conjunction with the chromatographic behavior of component C-SLME-IV on the Silica Gel G-Super Cel-silver nitrate column, were consistent with the properties of a C_{20} isoprenoid methyl ester structurally related to all-trans methyl geranylgeranoate, but apparently containing at least one cis double bond. The data also indicated that the previously observed "component X" and C-SLME-IV were one and the same component.

A summary of the results obtained from the analyses of the saponifiable material from the 3-hour (A-SL) and the 24-hour (C-SL) incubations is given in Table VII-5. It is clear from these results that the C_{15} and C_{20} isoprenoid acids accounted for essentially all of the labelled "fatty acid-like" material at both 3 hours and 24 hours of incubation time, the C_{15}/C_{20} ratio being 1:9 at both time points. What was altered as a function of time, apparently, was the relative proportion of each type of isoprenoid isomer within each class. For instance, at 3 hours, all-trans farnesoic acid represented about 80% of the total C_{15} acid class but declined to about 60% by 24 hours; however, the relative amount of the all-cis isomer of farnesoic acid doubled (i.e. from about 18% to about 39%) over this same time period. Similarly, at 3 hours, the all-trans geranylgeranoic acid isomer accounted for about 13% of the C_{20} acid class, whereas "component X" represented about 75% of that class; by 24 hours, only about 2% of that isoprenoid acid class was accounted for by the all-trans isomer of ger-
TABLE VII-5

Analysis of the Saponifiable Lipid Methyl Esters (SLME) Derived from Incubations of Bovine Retina \textsubscript{10} Homogenates with Mevalonic Acid

<table>
<thead>
<tr>
<th>ISOPRENOID CLASS</th>
<th>A-SLME (3 Hours) % TOTAL SLME</th>
<th>% TOTAL CLASS</th>
<th>% TOTAL CONVERTED ACTIVITY</th>
<th>C-SLME (24 Hours) % TOTAL SLME</th>
<th>% TOTAL CLASS</th>
<th>% TOTAL CONVERTED ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textsubscript{C}_{15}</td>
<td>8.80</td>
<td>100.00</td>
<td>2.81</td>
<td>9.30</td>
<td>100.00</td>
<td>3.60</td>
</tr>
<tr>
<td>all-trans</td>
<td>7.08</td>
<td>80.46</td>
<td>2.26</td>
<td>5.59</td>
<td>60.06</td>
<td>2.16</td>
</tr>
<tr>
<td>&quot;cis-trans&quot;\textsuperscript{a}</td>
<td>0.15</td>
<td>1.70</td>
<td>0.05</td>
<td>0.11</td>
<td>1.24</td>
<td>0.04</td>
</tr>
<tr>
<td>all-cis</td>
<td>1.57</td>
<td>17.84</td>
<td>0.50</td>
<td>3.60</td>
<td>38.70</td>
<td>1.39</td>
</tr>
<tr>
<td>\textsubscript{C}_{20}</td>
<td>90.10</td>
<td>100.00</td>
<td>28.83</td>
<td>90.00</td>
<td>100.00</td>
<td>34.86</td>
</tr>
<tr>
<td>all-trans</td>
<td>13.19</td>
<td>14.65</td>
<td>4.22</td>
<td>1.84</td>
<td>2.04</td>
<td>0.71</td>
</tr>
<tr>
<td>&quot;x&quot;\textsuperscript{b}</td>
<td>74.97</td>
<td>83.20</td>
<td>23.99</td>
<td>86.34</td>
<td>95.93</td>
<td>33.44</td>
</tr>
<tr>
<td>other\textsuperscript{c}</td>
<td>1.94</td>
<td>2.15</td>
<td>0.62</td>
<td>1.82</td>
<td>2.03</td>
<td>0.71</td>
</tr>
<tr>
<td>Other\textsuperscript{c}</td>
<td>1.10</td>
<td>--</td>
<td>0.35</td>
<td>8.70</td>
<td>--</td>
<td>0.27</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Represents cis, trans- and/or trans,cis- isomers of methyl farnesoate.

\textsuperscript{b} Component apparently containing at least one cis double bond and structurally related to methyl geranylgeranoate.

\textsuperscript{c} Unidentified labelled components (mixture).

anylgeranoic acid and the "component X" represented almost 96% of the total \textsubscript{C}_{20} isoprenoid acids. It may not be inferred from these results, however, that there was a "precursor-product" relationship between the all-trans isomer of particular isoprenoid acid class and its corresponding all-cis isomer.
Analysis of Nonsaponifiable Lipids

Aliquots of the nonsaponifiable lipid extracts from both the 3-hour (A-NSL) and the 24-hour (C-NSL) incubations were applied to Silica Gel G TLC plates along with authentic standards of cholesterol, lanosterol, ubiquinone (CoQ10) and squalene. The plates were developed once with either hexane-diethyl ether (85:15) or benzene-diethyl ether-petroleum ether (7:3:2). One-cm sections were scraped and assayed for total radioactivity as usual, while the mass standards were visualized by exposure to iodine vapors. The resulting radiochromatograms are shown in Figure VII-49. For the 3-hour sample (Figures VII-49A and VII-49B), about 77% of the radioactivity co-migrated with the squalene standard, while about 8-10% of the activity exhibited the chromatographic behavior of sterols. Approximately 72% of the radioactivity in the 24-hour sample (Figures VII-49C and VII-49D) co-migrated with the squalene standard, while roughly 20% of the activity behaved chromatographically like sterols (of which about 70% co-migrated with the C30 sterol standard while about 30% co-migrated with the cholesterol standard). The chromatograms were dramatically different from those obtained previously from incubations of retina homogenates with mevalonic acid (see Chapter VI), notably with respect to the almost complete absence of the "very polar component" and the large amount of squalene-like material.

Samples A-NSL (5.24 x 10^7 dpm) and C-NSL (5.28 x 10^7 dpm) were further analyzed by chromatography on silicic acid-Super Cel columns (100 x 1 cm; equilibrated and eluted with toluene) as previously described. Carrier standards of cholesterol and commercial lanosterol
FIGURE VII-49

Radio-TLC of the nonsaponifiable lipids obtained by incubation of bovine retina S10 homogenates with [2-3H]-mevalonic acid for 3 hours (A and B, sample A-NSL) and 24 hours (C and D, sample C-NSL). Chromatographic system: Silica Gel G; A and C, hexane-diethyl ether, 85:15; B and D, benzene-diethyl ether-petroleum ether, 7:3:2. Chromatographic standards (in order of increasing $R_f$): cholesterol, lanosterol, coenzyme Q10 and squalene.
(ca. 10 mg. each) were applied with each sample to the columns. The solvent was changed to toluene-diethyl ether (3:1) at fraction 213. The elution profile obtained from sample A-NSL is shown in Figure VII-50 (fraction volume, 9.0 ml; flow rate, 0.45 ml/min.). Approximately 87% of the recovered activity eluted from the column before the solvent change, in contrast to the results obtained in the previous experiment (compare with Figures VII-16 and VII-17). About 61% of the eluted activity exhibited the chromatographic behavior of a hydrocarbon, eluting with the solvent front (fractions 4-8). There was no resolution of the lanosterol and 24,25-dihydrolanosterol standards (fractions 30-50), but about 5% of the eluted activity co-migrated with the C₃₀ standards. The cholesterol standard eluted in fractions 64-90, but only about 1.5% of the recovered activity eluted within the region expected for C₂₇ monohydroxy sterols. About 4-5% of the activity recovered from the column exhibited the chromatographic behavior consistent with that of C₂₉ and/or C₂₈ monohydroxy sterols (fractions 47-70). The percentage of recovered radioactivity in the individual components isolated from the column was as follows:

<table>
<thead>
<tr>
<th>A-NSL FRACTIONS</th>
<th>% RECOVERED ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-8</td>
<td>61.2</td>
</tr>
<tr>
<td>9-12</td>
<td>6.3</td>
</tr>
<tr>
<td>13-30</td>
<td>7.8</td>
</tr>
<tr>
<td>31-46</td>
<td>5.1</td>
</tr>
<tr>
<td>47-70</td>
<td>4.5</td>
</tr>
<tr>
<td>71-120</td>
<td>1.5</td>
</tr>
<tr>
<td>121-220</td>
<td>0.9</td>
</tr>
<tr>
<td>221-270</td>
<td>12.6</td>
</tr>
</tbody>
</table>
FIGURE VII-50
Silicic acid-Super Cel column chromatography of sample A-NSL. Open circles (○—○), radioactivity; closed circles (●—●), sterols determined colorimetrically. The first colorimetric peak is due to lanosterol and 24,25-dihydrolanosterol (absorbance measured at 460 nm); the second colorimetric peak is due to cholesterol (absorbance measured at 620 nm). Arrow denotes the solvent change to toluene-diethyl ether, 3:1.

FIGURE VII-51
Silicic acid-Super Cel column chromatography of sample C-NSL. Open circles (○—○), radioactivity; closed circles (●—●), sterols determined colorimetrically. The first colorimetric peak is due to lanosterol and 24,25-dihydrolanosterol (absorbance measured at 460 nm); the second colorimetric peak is due to cholesterol (absorbance measured at 620 nm). Arrow denotes the solvent change to toluene-diethyl ether, 3:1. The scale of radioactivity in the inset is DPM x 10^{-4}.
The overall recovery of applied radioactivity was about 73% (3.83 x 10^7 dpm). A similar elution profile was obtained for the NSL sample from the 24-hour incubation (C-NSL; Figure VII-51) although the relative distribution of radioactivity was different (fraction volume 5.8 ml; flow rate, 0.29 ml/min.). Again, approximately 91% of the recovered radioactivity eluted before the solvent change, with about 58% of the recovered activity exhibiting the chromatographic behavior consistent with that of hydrocarbon (fractions 4-8). The C_{30} sterol standards (fractions 51-74) again did not resolve from one another, but considerable radioactivity (fractions 43-76; ca. 16-17% of the recovered activity) co-eluted with these sterols. Although about 5% of the eluted radioactivity exhibited the chromatographic behavior of C_{27} monohydroxy sterols (fractions 103-200), the majority of that activity did not co-migrate with the cholesterol standard (fractions 103-140). A minor portion of the recovered activity behaved chromatographically like C_{29} and/or C_{28} monohydroxy sterols (fractions 77-102; ca. 1.6% of the recovered radioactivity). The percentage of recovered activity in each of the chromatographic components was as follows:

<table>
<thead>
<tr>
<th>C-NSL FRACTIONS</th>
<th>% RECOVERED ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-11</td>
<td>61.0</td>
</tr>
<tr>
<td>12-20</td>
<td>3.7</td>
</tr>
<tr>
<td>21-42</td>
<td>3.0</td>
</tr>
<tr>
<td>43-76</td>
<td>16.5</td>
</tr>
<tr>
<td>77-102</td>
<td>1.6</td>
</tr>
<tr>
<td>103-224</td>
<td>5.2</td>
</tr>
<tr>
<td>225-270</td>
<td>8.9</td>
</tr>
</tbody>
</table>
About 85% of the applied radioactivity was recovered from the column (4.49 x 10^7 dpm).

The material which exhibited the chromatographic behavior of a hydrocarbon was analyzed by radio-GLC on a 3% OV-17 column (column temperature, 230°C; carrier flow rate, 66 ml/min.) in comparison with an authentic standard of squalene. One-minute fractions of effluent gas were collected and assayed for radioactivity as usual. The radiochromatogram of the material obtained from sample A-NSL (fractions 4-8; 1.29 x 10^6 cpm) is shown in Figure VII-52. At least 94% of the recovered radioactivity co-migrated with the squalene standard under the conditions employed (overall recovery of applied radioactivity, ca. 92%). A similar radiochromatogram was obtained for the hydrocarbon-like material from sample C-NSL. Thus, the most prominent labelled component of the nonsaponifiable lipids extracted from the incubation mixtures had the chromatographic properties consistent with those of squalene.

The material from each NSL extract which exhibited the chromatographic behavior of monohydroxy sterols (A-NSL, fractions 32-46, 47-70, 71-120; C-NSL, fractions 45-100, 110-180) was adjusted so as to contain approximately 10 mg of carrier cholesterol; precipitation of sterols with digitonin was then carried out on each sample as previously described. As controls, authentic samples of [1,2-^3H]-cholesterol and [2-^3H]-lanost-8-en-3β-ol were each diluted with about 10 mg of carrier cholesterol and precipitated with digitonin using the identical procedures. The resulting digitonides were "split", and the free sterols (digitonin-precipitable material) were recovered, as previously described. The results are given in Table VII-6.
FIGURE VII-52

Radio-GLC of pooled fractions 4-8 obtained by silicic acid-Super Cel column chromatography of sample A-NSL (see Figure VII-50). Chromatographic system: 3% OV-17, 230°C isothermal. The mass peak is due to squalene.
TABLE VII-6

Digitonin Precipitation of "Sterol-Like" Material Obtained by Incubation of Bovine Retina Homogenates with 3RS-[2-\(^3\)H]-Mevalonic Acid

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>FRACTIONS</th>
<th>SPECIFIC ACTIVITY (dpm/ml Sterol x 10(^{-4}))</th>
<th>PER CENT DIGITONIN PRECIPITABLE MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-NSL</td>
<td>32-46</td>
<td>9.98 3.83</td>
<td>38.4</td>
</tr>
<tr>
<td></td>
<td>47-70</td>
<td>15.4 1.04</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>71-120</td>
<td>6.25 3.08</td>
<td>49.3</td>
</tr>
<tr>
<td>C-NSL</td>
<td>45-100</td>
<td>44.6 24.1</td>
<td>54.0</td>
</tr>
<tr>
<td></td>
<td>110-180</td>
<td>17.7 14.5</td>
<td>81.9</td>
</tr>
<tr>
<td>(^3)H-C(_{30})*</td>
<td>---</td>
<td>17.9 7.87</td>
<td>43.9</td>
</tr>
<tr>
<td>(^3)H-C(_{27})**</td>
<td>---</td>
<td>22.0 19.0</td>
<td>86.4</td>
</tr>
</tbody>
</table>

* \([2-\(^3\)H]-Lanost-8-en-3\(\beta\)-ol

** \([1,2-\(^3\)H]-Cholesterol

These results indicated that the susceptibility of the biologically derived nonsaponifiable lipids to precipitation with digitonin was comparable to that of the 3\(\beta\)-monohydroxy sterols with which they shared certain chromatographic properties. It was apparent that a larger percentage of the C\(_{27}\) sterol-like material from sample C-NSL (fractions 110-180) was precipitable with digitonin than was the corresponding material from sample A-NSL (fractions 71-120). These results suggested that appreciable amounts of sterols were synthesized by the retina homo-
measuring the absorbance at 460 nm after 15 minutes of developing time). 
The results are shown in Table VII-7.

**TABLE VII-7**

Co-Crystallization of the "C₃₀ Sterol-Like" Material of Hydrogenated 
Samples A-NSL and C-NSL with Authentic Lanost-8-en-3β-ol

<table>
<thead>
<tr>
<th>RECRYSTALLIZATION</th>
<th>A-NSL 32-46</th>
<th>C-NSL 45-100</th>
<th>CONTROL**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>6,060 ± 340</td>
<td>33,100 ± 1,000</td>
<td>12,200 ± 700</td>
</tr>
<tr>
<td>Once from Methanol</td>
<td>5,270 ± 320</td>
<td>21,700 ± 1,300</td>
<td>11,500 ± 600</td>
</tr>
<tr>
<td>Twice from Methanol</td>
<td>4,830 ± 160</td>
<td>18,500 ± 600</td>
<td>11,100 ± 200</td>
</tr>
<tr>
<td>Once from Acetone-Water</td>
<td>4,580 ± 370</td>
<td>18,400 ± 800</td>
<td>11,900 ± 400</td>
</tr>
<tr>
<td>Twice from Acetone-Water</td>
<td>4,520 ± 290</td>
<td>18,300 ± 300</td>
<td>11,600 ± 300</td>
</tr>
<tr>
<td>Once from Methanol</td>
<td>4,690 ± 200</td>
<td>18,500 ± 500</td>
<td>11,700 ± 600</td>
</tr>
</tbody>
</table>

* S.D., Standard Deviation (n = 3)
** [2-³H]-Lanost-8-en-3β-ol

After an initial decline in specific activity upon recrystallization from methanol, the specific activity of the samples remained essentially constant (within the limits of the given standard deviations). The relatively substantial drop in the specific activity of the hydrogenated material derived from sample C-NSL could be explained by the presence of material in this sample which did not co-chromatograph with the C₃₀ sterol standard on the silicic acid-Super Cel column (i.e. material which behaved chromatographically like 4-desmethyl sterols).
These results, in conjunction with the information obtained by chromatographic methods, indicated that C_{30} monohydroxy sterols were biosynthesized by the retina S_{10} homogenates from mevalonic acid, although it was impossible to ascertain from the results the exact identities of these products. These findings were in striking contrast to the results obtained in previous incubations of the retina S_{10} homogenate preparation (where there were no indications of appreciable sterol synthesis), but were in agreement with the results obtained by incubation of intact bovine retinas with mevalonic acid.

The material from sample C-NSL which behaved chromatographically and chemically like C_{27} 3β-monohydroxy sterols (i.e. fractions 110-180) and which was recovered from the split digitonides was desiccated for several hours in vacuo over phosphorous pentoxide and then acetylated (in 10 ml of acetic anhydride-pyridine, 3:2, overnight at 40°C). The acetylation was terminated by the addition of five volumes of ice water, followed by extraction of the acetates (three times with two volumes of hexane). The combined organic extracts were washed twice with water, once with 10% aqueous tartaric acid, twice with a saturated sodium bicarbonate solution and twice more with water. The organic phase was then dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in 2.0 ml of benzene. An aliquot (ca. 15-20 μl) was analyzed by radio-TLC (Silica Gel G; hexane-diethyl ether, 85:15) to determine the extent of the acetylation. The radiochromatogram (Figure VII-53) indicated that approximately 82% of the radioactivity behaved chromatographically like cholesteryl acetate,
FIGURE VII-53
Radio-TLC of acetylated material from pooled fractions 110-180 obtained by silicic acid-Super Cel column chromatography of sample C-NSL (see Figure VII-51). Chromatographic system: Silica Gel G, developed once with hexane-diethyl ether, 85:15. Chromatographic standards (in order of increasing $R_f$): cholesterol and 3β-acetoxy-cholesterol.

FIGURE VII-54
Alumina-silver nitrate medium-pressure liquid chromatography (MPLC) of the acetylated material from pooled fractions 110-180 obtained by silicic acid-Super Cel column chromatography of sample C-NSL (see Figure VII-51). Solvent system: hexane-toluene, 91:9; arrow denotes solvent change to hexane-toluene, 3:2. Open circles (o—o), radioactivity; closed circles (•—•), monoene steryl acetates determined colorimetrically; closed triangles (▲—▲), diene sterols determined colorimetrically. Monoene steryl acetates (in order of elution from column): $\Delta^8(14)$, $\Delta^7$ and $\Delta^5$; diene steryl acetates (in order of elution from column): $\Delta^8,14$ and $\Delta^5,7$. Inset: peak of radioactivity coincident with the 3β-acetoxy-$\Delta^8(14)$-cholestene standard.
while about 10% migrated just behind the cholesteryl acetate standard but far ahead of the cholesterol standard. The remainder of the radioactivity trailed diffusely to the origin of the plate.

An alumina-silver nitrate medium pressure liquid chromatography (MPLC) system was prepared and operated as described by Pascal et al. (1979). Neutral aluminum oxide (Woelm; 50-200 mesh; 90 g) was slurried with an aqueous solution of silver nitrate (18 g, in 70 ml of water) and then frozen (dry ice-acetone) and lyophilized for 48 hours (pressure, 50 μg Hg). About 80 g of the lyophilized material was poured into a glass column (100 x 0.9 cm), the air was displaced with toluene and the column was then equilibrated with hexane-toluene, 91:9. The acetylated material (7.78 x 10^5 dpm, in 2.0 ml of hexane-toluene, 91:9) was applied to the column (injection column, 4.5 ml; void volume, 30 ml) along with the following authentic C_{27} sterol acetate standards (superscript numbers refer to positions of the nuclear double bonds): Δ^{8(14)} (3 mg), Δ^{7} (3 mg), Δ^{5} (8 mg), Δ^{8,14} (6 mg) and Δ^{5,7} (8 mg). Fractions 1-140 were eluted with hexane-toluene, 91:9; fractions 141-340 were eluted with hexane-toluene, 60:40 (flow rate, ca. 2.3 ml/min.; fraction volume: 1-220, 7.4 ml; 221-340, 14.0 ml). Fractions were collected automatically on a Gilson fraction collector and aliquots (1.0 ml each from even-numbered fractions) were removed for assay of radioactivity and for colorimetric assay of sterol acetates. The elution profile is shown in Figure VII-54. Approximately 45% of the recovered radioactivity corresponded to monoene sterol acetates. Roughly 0.1% of the activity co-chromatographed with the Δ^{8(14)} standard (fractions 13-30), while 41.1% exactly co-migrated with the Δ^{7} sterol acetate standard.
(fractions 34–74) and 4.0% co-migrated with the cholesteryl acetate standard (fractions 75–140). The remainder of the recovered activity (ca. 55%) exhibited the chromatographic behavior of diene steryl acetates, but there was no correspondence between the radioactivity and the diene steryl acetate standards. Approximately 54.2% of the recovered radioactivity (fractions 150–200) eluted just before the Δ⁸,¹⁴ steryl acetate standard (fractions 170–200) while about 0.6% of the activity eluted in the region of the Δ⁵,⁷ steryl acetate standard (fractions 228–300), but did not form a coherent peak of radioactivity coincident with the Δ⁵,⁷ standard. The overall recovery of applied radioactivity was 96.4%.

The material which behaved chromatographically like cholest-7-en-3β-yl acetate (fractions 34–74) was diluted with about 50 mg of carrier Δ⁷-cholestenyl acetate and was then successively recrystallized twice from methanol and twice from acetone. The results are given in Table VII-8. The material from fractions 34–74 co-crystallized to constant specific activity with the authentic Δ⁷-cholestenyl acetate standard. The chromatographic results and these results suggested that contamination of this material with other related labelled steryl acetate components (i.e. Δ⁸(⁹)-cholestenyl acetate) was negligible. The data were consistent with the known properties of cholest-7-en-3β-yl acetate and indicated that cholest-7-en-3β-ol was synthesized from mevalonic acid by the retina homogenate.

The material which behaved chromatographically like cholesteryl acetate (fractions 76–120) was pooled, diluted with about 100 mg of carrier cholesterol, dried in vacuo over phosphorous pentoxide and then
TABLE VII-8

Co-Crystallization of Fractions 34-74 from the Alumina-Silver Nitrate MPLC Column with Authentic Cholest-7-en-3β-yl Acetate

<table>
<thead>
<tr>
<th>RECRYSTALLIZATION</th>
<th>(dpm per mg Sterol ± S.D.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>3550 ± 430</td>
</tr>
<tr>
<td>Once from Methanol</td>
<td>3580 ± 200</td>
</tr>
<tr>
<td>Twice from Methanol</td>
<td>3350 ± 150</td>
</tr>
<tr>
<td>Once from Acetone</td>
<td>3430 ± 160</td>
</tr>
<tr>
<td>Twice from Acetone</td>
<td>3300 ± 220</td>
</tr>
</tbody>
</table>

* Triplicate aliquots were taken for assay of radioactivity and for colorimetric assay of carrier steryl acetate

redissolved in 10 ml of anhydrous diethyl ether. Lithium aluminum hydride (LiAlH₄; 10 mg) was added to cleave the acetate; the reaction mixture was allowed to stand at room temperature for one hour. Excess hydride reagent was destroyed by cautious addition of ice water, followed by acidification of the mixture with 1 N HCl and extraction of the free sterols with two 20-ml portions of petroleum ether. The organic extracts were combined and washed once with water, then dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under nitrogen in a 25-ml capacity Corex centrifuge tube. The sample was redissolved in 10.0 ml of benzene and aliquots were removed for assay of radioactivity and for colorimetric assay of cholesterol (each in triplicate). The remainder of the sample was evaporated to
dryness under nitrogen and redissolved in 5.0 ml of anhydrous diethyl ether, on ice. The sample was then purified via the dibromide as follows. About 5 drops of bromine were added to the ether solution, after which the sample was vortexed and then allowed to stand for 2 hours on ice. Approximately 2-3 ml of glacial acetic acid (chilled) were added and the sample was vortexed again and allowed to stand for 30 minutes on ice (during which time the dibromide crystals formed). The dibromide crystals were collected by centrifugation (30 min. at 12,000 rpm, Sorvall SS-34 rotor; 0-4°C). The resulting pellet was washed three times with 5-ml portions of chilled glacial acetic acid-methanol (95:5), collecting the crystals each time by centrifugation (as above). The acid-moist dibromide was redissolved in 5 ml of anhydrous diethyl ether and reductively debrominated with 50 mg of zinc dust, with intermittent vortexing for 15 minutes at room temperature. The ether phase was washed once with 5 ml of water, once with 5 ml of 1 N HCl, twice with 5-ml portions of a 10% NaOH solution and three times with 10-ml portions of water. The sample was evaporated to dryness under nitrogen and recrystallized once from methanol. After redissolving the sample in 10.0 ml of benzene, aliquots were removed for assay of radioactivity and for colorimetric assay of cholesterol (each in triplicate). The following results were obtained:

<table>
<thead>
<tr>
<th></th>
<th>SPECIFIC ACTIVITY (dpm/mg Cholesterol ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Dibromide Purification</td>
<td>162 ± 6</td>
</tr>
<tr>
<td>After Dibromide Purification</td>
<td>159 ± 5</td>
</tr>
</tbody>
</table>
The above results and the results obtained by digitonin precipitation and by column chromatography were consistent with the known properties of cholesterol, and indicated that the retina homogenate biosynthesized cholesterol from mevalonic acid. However, labelled cholesterol only represented about 4% of the total labelled C\textsubscript{27} sterol material, or about 0.2% of the total labelled nonsaponifiable material (i.e. \textless 0.1% of the total conversion of substrate).

Fractions 150-200 from the alumina-silver nitrate MPLC column were pooled (4.15 x 10\textsuperscript{5} dpm) and reapplied to the same column, equilibrated and eluted with hexane-toluene, 7:3 (injection volume, 5.5 ml; void volume, 30 ml; flow rate, 2.0 ml/min.; fraction volume, 7.4 ml). The sample already contained carrier \Delta\textsuperscript{8,14}-cholestadienyl acetate and was further supplemented with about 15 mg of carrier desmosteryl acetate (i.e. \Delta\textsuperscript{5,24}). As before, aliquots were removed from even-numbered fractions for assay of radioactivity and for colorimetric assay of steryl acetates. The elution profile is shown in Figure VII-55. The majority of the recovered radioactivity (fractions 19-37; ca. 91.2%) eluted just prior to the \Delta\textsuperscript{5,24} steryl acetate standard (fractions 27-65). A minor amount of the total activity (fractions 10-16; ca. 1.3%) preceded the major peak of radioactivity (perhaps due to some residual monoene from the previous column). About 7.4% of the recovered activity (fractions 38-70) co-chromatographed with the desmosteryl acetate standard. The overall recovery of applied radioactivity was nearly quantitative (98.9%). It should be noted that the ratio of radioactivity of the two major labelled components eluted from this column was about 9:1 (the same value as found for the ratio of radioactivity for
FIGURE VII-55
Alumina-silver nitrate MPLC of fractions 150-200 from the previous 
alumina-silver nitrate MPLC column (see Figure VII-54). Open circles 
(o--o), radioactivity; closed circles (•---•), steryl acetates 
determined colorimetrically. The first colorimetric peak is due to 
3β-acetoxy-Δ^5,2'-cholestadiene; the second colorimetric peak is due 
to 3β-acetoxy-Δ^8,14'-cholestadiene. Solvent system: hexane-toluene, 
7:3.
the $\Delta^7:\Delta^5$ steryl acetates isolated from the previous MPLC column).

Fractions 17-38 (the major labelled component) from the MPLC column were pooled and analyzed further by radio-GLC. A portion of the pooled material ($1.41 \times 10^4$ cpm) was co-injected with a standard of $\Delta^7,9(11)$-cholestadienyl acetate (containing $\Delta^7,14$-cholestadienyl acetate as a minor contaminant) onto a 3% OV-17 column (column temperature, 260°C; carrier flow rate, 66 ml/min.). One-minute effluent fractions were collected and assayed for radioactivity as usual. Under the conditions employed, the retention time of the $\Delta^7,9(11)$ compound was 27.73 minutes while that of the $\Delta^7,14$ diene was 24.36 minutes. The radiochromatogram (Figure VII-56) indicated that none of the radioactivity was coincident with the chromatographic standards; essentially all of the radioactivity (apparently representing at least two components, with a ratio of 5:95) eluted from the column after the $\Delta^7,9(11)$ and $\Delta^7,14$ diene standards (recovery, 99%). Another portion of the sample ($9.12 \times 10^3$ cpm) was injected onto the 3% OV-17 column along with a mixture of steryl 3β-acetates containing (in order of increasing mole per cent) $\Delta^8(9)$, $\Delta^7$, $\Delta^7,24$ and $\Delta^8,24$. The order of elution of these standards from a comparable chromatographic support had been determined previously (Clayton et al., 1963); under the conditions employed here (column temperature, 250°C; carrier flow rate, 66 ml/min.), these steryl acetates eluted in the following order (retention time in parentheses): $\Delta^8(9)$ (24.87 min.), $\Delta^7$ (27.81 min.), $\Delta^8,24$ (30.55 min.) and $\Delta^7,24$ (33.84 min.). The radiochromatogram is shown in Figure VII-57. Approximately 95% of the recovered radioactivity exactly corresponded with the $\Delta^7,24$ steryl acetate standard, while about 5% was coincident with the $\Delta^8,24$ steryl ace-
FIGURE VII-56
Radio-GLC of pooled fractions 17-38 from the second alumina-silver nitrate MPLC column (see Figure VII-55). Chromatographic system: 3% OV-17, 260°C isothermal. The major mass peak is due to 3β-acetoxy-Δ7,9(11)-cholestadiene; arrow denotes a minor impurity due to 3β-acetoxy-Δ7,14-cholestadiene.

FIGURE VII-57
Radio-GLC of pooled fractions 17-38 from the second alumina-silver nitrate MPLC column (see Figure VII-55). Chromatographic system: 3% OV-17, 250°C isothermal. The mass peaks are due to the following steryl acetates (in order of increasing retention time): Δ8(9), Δ7, Δ8,24 and Δ7,24.

FIGURE VII-58
Radio-GLC of pooled fractions 17-38 from the second alumina-silver nitrate MPLC column (see Figure VII-55). Chromatographic system: 1% QF-1, 200°C isothermal. The major mass peak is due to both 3β-acetoxy-Δ7-cholestene and 3β-acetoxy-Δ8,24-cholestadiene, with a minor shoulder due to 3β-acetoxy-Δ8(9)-cholestene; the minor mass peak (longest retention time) is due to 3β-acetoxy-Δ7,24-cholestadiene.
tate standard. The overall recovery of applied radioactivity was 98%. Using the same mixture of steryl acetate standards, a third portion of the sample ($1.44 \times 10^4$ cpm) was analyzed on a 1% QF-1 column (column temperature, 200°C; carrier flow rate, 66 ml/min.). The radiochromatogram is shown in Figure VII-58. On this GLC system, the $\Delta^8(9)$ steryl acetate formed a broad shoulder on the leading edge of the major peak, which was an unresolved composite of the $\Delta^7$ and $\Delta^8,24$ steryl acetates; the $\Delta^7,24$ steryl acetate, however, was almost completely separated from the other standards (retention time, 57.70 min.). Again, approximately 95% of the recovered radioactivity co-chromatographed with the $\Delta^7,24$ steryl acetate standard while about 5% of the activity migrated under the major mass peak. Recovery of applied activity from this column was about 95%. These data indicated that the major component of the sample was derived from cholesta-7,24-dien-3β-ol while a minor component was derived from cholesta-8,24-dien-3β-ol; the ratio of the $\Delta^7,24$ and $\Delta^8,24$ species was 95:5.

The material which behaved chromatographically like desmosteryl acetate (i.e. fractions 37-60 from the second alumina-silver nitrate MPLC column) was pooled and the acetate was cleaved with LiAlH$_4$ as described previously. The recovered free sterol was diluted with 200 mg of carrier desmosterol and was repeatedly recrystallized three times from methanol and twice from acetone-water. After each recrystallization, the recovered sterol was dried under a steam of nitrogen and re-dissolved in an appropriate volume of benzene (i.e. 10.0 ml); aliquots were removed for assay of radioactivity and for colorimetric assay of desmosterol (each in triplicate). Using the usual modified Liebermann-
Burchard reagent, 1 mg of desmosterol gave an absorbance of 0.712 units at 620 nm after 25 minutes of developing time. The results are given in Table VII-9.

**TABLE VII-9**

Co-Crystallization of Fractions 37-60 from the Alumina-Silver Nitrate MPLC Column with Authentic Desmosterol after Conversion to the Free Alcohol

<table>
<thead>
<tr>
<th>Recrystallization</th>
<th>Specific Activity (dpm/mg Sterol ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>112 ± 8</td>
</tr>
<tr>
<td>Once from Methanol</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>Twice from Methanol</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>Three times from Methanol</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>Once from Acetone-Water</td>
<td>94 ± 4</td>
</tr>
<tr>
<td>Twice from Acetone-Water</td>
<td>93 ± 5</td>
</tr>
</tbody>
</table>

Within the experimental error, the labelled material co-crystallized to constant specific activity with the desmosterol standard. These results and the results obtained by column chromatography indicated that desmosterol was biosynthesized from mevalonic acid by the retina homogenate (at least in the 24-hour incubation).

**Summary**

A summary of the distribution of labelled components in the total NSL extracts after 3 hours and 24 hours of incubation of bovine retina
TABLE VII-10

Distribution of Radioactivity in Nonsaponifiable Lipid (NSL) Components Derived from Incubations of Bovine Retina Homogenates with 3RS-[2-\(^3\)H]-Mevalonic Acid

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>% TOTAL NSL ACTIVITY</th>
<th>% TOTAL CONVERSION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 Hours</td>
<td>24 Hours</td>
</tr>
<tr>
<td>Squalene</td>
<td>61.2</td>
<td>61.0</td>
</tr>
<tr>
<td>C(_{30}) Sterols</td>
<td>5.1</td>
<td>16.5</td>
</tr>
<tr>
<td>C(_{27}) Sterols</td>
<td>1.5</td>
<td>5.2</td>
</tr>
<tr>
<td>Other</td>
<td>32.2</td>
<td>17.3</td>
</tr>
<tr>
<td>TOTAL</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

homogenates with 3RS-[2-\(^3\)H]-mevalonic acid is given in Table VII-10. The major NSL component after both 3 hours and 24 hours of incubation was squalene. The data suggested that, while the relative proportion of squalene did not change between 3 and 24 hours of incubation, the "other" (i.e. undefined) NSL components diminished with a concomitant increase in the amount of sterols produced. There was a marked accumulation of C\(_{30}\) sterols after 24 hours as compared with 3 hours of incubation. However, the combined C\(_{30}\) and C\(_{27}\) sterols represented only minor fractions of the total conversion of mevalonic acid by the retina homogenates. Although it appeared that a slightly lower proportion of the converted substrate was incorporated into NSL material after 24 hours (as compared with 3 hours) of incubation, the difference (i.e. between 54.9% and 46.2%) may not have been statistically significant.
<table>
<thead>
<tr>
<th>STEROL</th>
<th>% TOTAL C&lt;sub&gt;27&lt;/sub&gt;</th>
<th>% TOTAL NSL</th>
<th>% TOTAL CONVERSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoenes</td>
<td>45.2</td>
<td>2.35</td>
<td>1.09</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;8&lt;/sup&gt;(14)</td>
<td>&lt;0.1</td>
<td>&lt;0.005</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;7&lt;/sup&gt;</td>
<td>41.1</td>
<td>2.14</td>
<td>0.99</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.0</td>
<td>0.21</td>
<td>0.10</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;8&lt;/sup&gt;(9)</td>
<td>N.D.</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Dienes</td>
<td>54.8</td>
<td>2.84</td>
<td>1.31</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;8&lt;/sup&gt;,14</td>
<td>N.D.</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;7&lt;/sup&gt;,14</td>
<td>N.D.</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;7&lt;/sup&gt;,9(11)</td>
<td>N.D.</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;5&lt;/sup&gt;,7</td>
<td>&lt;0.60</td>
<td>&lt;0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;5&lt;/sup&gt;,24</td>
<td>4.06</td>
<td>0.21</td>
<td>0.10</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;7&lt;/sup&gt;,24</td>
<td>47.46</td>
<td>2.47</td>
<td>1.14</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;8&lt;/sup&gt;,24</td>
<td>2.54</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.13</td>
<td>&lt;0.007</td>
<td>&lt;0.003</td>
</tr>
</tbody>
</table>

1 Analysis of products derived from a 24-hour incubation

* N.D., not detected
Whereas squalene represented the major NSL constituent of the incubations, the C_{20} isoprenoid acids represented the major metabolites of mevalonic acid in these incubations (with the unidentified "component X" being the single most prominent species).

Further analyses of the C_{27} sterols from the 24-hour incubation (sample C-NSL) revealed that this fraction of the total NSL material was composed of two major sterol types: monoenes (45.2%) and dienes (54.8%). The distribution of activity in the C_{27} sterols is summarized in Table VII-11. The major monoene sterol was cholest-7-en-3β-ol (41.1% of the total C_{27} sterol activity), whereas cholesterol represented only 4.0% of the C_{27} sterol activity. Both sterols combined only represented about 1% of the total substrate conversion. The major diene sterols were cholest-7,24-dien-3β-ol, cholest-5,24-dien-3β-ol (desmosterol) and cholest-8,24-dien-3β-ol, accounting for about 47.5%, 4.1% and 2.5%, respectively, of the total C_{27} sterol activity. It is curious that the ratios of activity between the Δ^7 and Δ^5 monoene sterols and between the Δ^7,24 and Δ^5,24 diene sterols were essentially identical (ca. 9:1). Although this may be considered preliminary evidence for the speculation that the conversion of Δ^7 sterols to Δ^5 sterols was limiting in these incubations, further experimentation would be required to confirm this conclusion.

**EXPERIMENT VII-3**

The following experiment was performed to investigate the possibility that the saponification and/or acidification procedures could lead to the isomerization and/or cyclization of all-trans isoprenoid
acids, resulting in the artifactual formation of chemically altered and chromatographically distinct forms of the original isoprenoids.

A portion of the material obtained from the previous 3-hour incubation which exhibited the chromatographic properties of all-trans methyl geranylgeranoate (i.e. sample A-SLME-III, 5.5 x 10⁴ cpm) was evaporated to dryness under nitrogen in a flask. A 15% ethanolic potassium hydroxide solution (17 ml, in 90% ethanol) was added to the flask, followed by 17 ml of the previously described incubation buffer, and the flask was flushed with nitrogen, sealed and incubated for 3 hours at 80°C in a water bath. After allowing the flask to cool to room temperature, the flask was stored in a freezer at -20°C for 2 hours. Upon removal of the flask from the freezer, the mixture was immediately acidified by dropwise addition of concentrated HCl (4.12 ml total) with continuous stirring (to pH 1-2). The acidified mixture was then diluted with water (100 ml) and extracted three times with 200 ml portions of dichloromethane. The combined extracts were washed once with 100 ml of water and then dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in 2.0 ml of benzene. An aliquot (0.2 ml) was removed and assayed for radioactivity; 5.4 x 10⁴ cpm was recovered (ca. 98%). The remainder of the sample was evaporated to dryness under nitrogen and redissolved in 2 ml of diethyl ether-methanol (9:1); the methyl ester was prepared by the addition of freshly-generated diazomethane, as previously described. After allowing the reaction to proceed at room temperature for one hour, the solvent was evaporated under nitrogen and the sample was redissolved in
1-2 ml of hexane. The methyl ester was purified on a small (0.4 g) Unisil column, eluted with hexane-diethyl ether, 98:2 (15 ml). Solvent was removed under nitrogen and the sample was redissolved in about 0.5 ml of hexane, transferred to a small vial and concentrated under nitrogen to approximately 10 μl.

The sample was co-injected with authentic standards of the methyl and ethyl esters of all-trans geranylgeranoic acid onto a 3% OV-17 GLC column (column temperature, 170°C; carrier flow rate, 66 ml/min.); the effluent gas was collected and assayed for radioactivity as described previously. Under these conditions, the methyl ester of all-trans geranylgeranoic acid had a retention time of 28.71 minutes.

The residual material in the sample vial was applied to a Silica Gel G plate, developed once with hexane-diethyl ether-glacial acetic acid (80:20:1, v/v) and one-cm sections were scraped and assayed for radioactivity as usual. A standard of methyl oleate was chromatographed in an adjacent lane and the mass was visualized by exposure to iodine vapors.

RESULTS AND DISCUSSION

The radiochromatogram obtained by radio-TLC of the methyl ester after saponification, acidification and re-esterification is shown in Figure VII-59. Greater than 99% of the radioactivity co-migrated with the methyl oleate standard, indicating that no free acid, polar degradation products of apolar cyclization products were present in the sample.

The results of the radio-GLC analysis are shown in Figure VII-60. At least 98% of the recovered radioactivity co-migrated with the methyl
FIGURE VII-59

Radio-TLC of the methyl ester of tritiated all-trans geranylgeranoic acid (derived biosynthetically from an incubation of a bovine retina S10 homogenate with [2-3H]-mevalonic acid) after saponification, acidification and re-esterification (with diazomethane). Chromatographic system: Silica Gel G, developed once with hexane-diethyl ether-glacial acetic acid (80:20:1). The chromatographic standard is methyl oleate.
FIGURE VII-60

Radio-GLC of the methyl ester of tritiated all-trans geranylgeranoic acid after saponification, acidification and re-esterification (with diazomethane). Chromatographic system: 3% OV-17, 170°C isothermal. The first mass peak is due to all-trans methyl geranylgeranoate; the second mass peak is due to all-trans ethyl geranylgeranoate.
ester of all-trans geranylgeranoic acid. There was no activity present in the region of the chromatogram where "component X" would have been found. The chromatogram was virtually identical to that obtained for the original sample prior to the chemical treatment discussed above (compare with Figure VII-43).

It has been reported (Christophe and Popjak, 1961) that the procedures of saponification and/or subsequent acidification can result in the cyclization of isoprenoid acids, yielding artifacts in the analysis of this class of compounds. It has also been reported (Schroepfer and Gore, 1963) that trans,trans-farnesol can be converted to a multitude of products upon exposure to 5 N HCl (90 minutes at 70°C), most likely due to elimination, cyclization and/or isomerization of the primary isoprenol. Since the incubation mixtures from Experiments VII-1 and VII-2 were saponified (3-4 hours at 80°C) and acidified (by dropwise addition of concentrated HCl, at ~20°C) prior to extraction and analysis of the acidic constituents, it was important to know whether or not such artifacts had been produced during the work-up of these incubation mixtures. The results of this experiment suggest that cyclization and isomerization of the all-trans isoprenoid acids did not occur under the conditions employed. This finding is especially important in view of the fact that a unique and major component of the labelled incubation products (i.e. "component X") did not correspond to the all-trans isomer of an isoprenoid acid and apparently contained at least one cis double bond.

EXPERIMENT VII-4

The following experiment was performed to ascertain whether or not
isoprenoid acids are normal constituents of the bovine retina. Ten freshly dissected bovine retinas were briefly rinsed with water to remove adhering debris and were then transferred to a lyophilization flask, frozen (dry ice-acetone bath) and lyophilized (ca. 30 μm Hg pressure) for 6 days. The retinas were protected from exposure to light during lyophilization by wrapping the flask with aluminum foil. After weighing (689.3 mg, ca. 69 mg/retina), the dried retinas were saponified in 50 ml of 15% ethanolic potassium hydroxide (in 90% ethanol; at reflux for 2 hours, under nitrogen) to which a trace of [9,10-3H]-palmitic acid had been added (New England Nuclear; 4.4 x 10^7 dpm, 291 ng, 1.14 nmoles). The mixture was allowed to cool to room temperature and then 50 ml of water was added and the aqueous-ethanolic mixture was extracted three times with 200-ml portions of hexane. The combined organic extracts were washed once with 100 ml of water and then dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in 10.0 ml of benzene. This nonsaponifiable material was assayed for radioactivity (0.5 ml aliquots, in triplicate).

The remaining aqueous-ethanolic phase was chilled to -20°C and then acidified by dropwise addition of concentrated HCl (13.24 ml) with continuous rapid stirring. The acidified mixture was extracted three times with 200-ml portions of dichloromethane and the combined extracts were washed once with 100 ml of water and processed further as described above. The resulting saponifiable material was redissolved in 10.0 ml of benzene and assayed for radioactivity (0.10 ml aliquots, in triplic-
cate). The remaining saponifiable material was evaporated to dryness under nitrogen, redissolved in 2 ml of diethyl ether-methanol (9:1, v/v) and esterified with excess freshly-generated diazomethane for one hour at room temperature. The solvent was removed under nitrogen and the residue was redissolved in 2-3 ml of chloroform; a small aliquot (ca. 10 μl) was analyzed by radio-TLC (Silica Gel G, developed once with hexane-diethyl ether-glacial acetic acid, 80:20:1), along with authentic standards of palmitic acid and methyl palmitate. Prior to scraping and counting one-cm sections, the plate was exposed to iodine vapors to visualize the retina lipids and the chromatographic standards. Greater than 98% of the total radioactivity co-migrated with the methyl palmitate standard, as did nearly all the detectable mass of the retinal material (i.e. esterification was quantitative). The methyl esters were further purified on a Unisil column (58 x 2 cm, equilibrated and eluted with chloroform; 4 min./fraction). The fractions were assayed for radioactivity and those corresponding to the methyl esters (fractions 4-17) were pooled, concentrated under reduced pressure, transferred to a vial, evaporated to dryness under nitrogen and redissolved in 10.0 ml of hexane. Assay of radioactivity (0.10 ml aliquots, in triplicate) revealed that the recovery of radioactivity was greater than 96%.

The methyl esters were analyzed by GLC on a Varian Model 3700 gas-liquid chromatograph, using a glass column (6 ft. x 1 mm, i.d.) packed with 3% OV-17 (on Gas-Chrom Q, 100/120 mesh; Applied Science Laboratories, Inc.). Helium was used as the carrier gas (flow rate, 44 ml/min.; column head pressure, 46 p.s.i. at 170°C). The injector
port was maintained at 250°C while the detector was maintained at 270°C. Column temperature was programmed at an increment of 2°C/minute (range, 170°C to 250°C) after an initial isothermal setting of 170°C for the first minute after injection. A Supergrator-3 Programmable Computing Integrator (Columbia Scientific Industries, Austin, TX.) was used to integrate peak areas and record retention times; chart speed was 0.166 in./min. A portion of the methyl ester sample (1.10 ml) was combined with a known amount (0.10 ml) of authentic nonadecanoic acid methyl ester (C₁₉:₀; 64.22 nmol/0.10 ml) as an internal standard. The mixture was evaporated under nitrogen and redissolved in 0.10 ml of carbon disulfide. One microliter of this sample was injected onto the GLC column and analyzed as described above. Another portion of the sample (0.05 ml) was evaporated under nitrogen, redissolved in 1 ml of ethanol-hexane (2:1, v/v) and hydrogenated over 2 mg of platinum oxide for 10 minutes at room temperature (1 atmosphere hydrogen). The catalyst was removed vacuum filtration through an extra-fine fritted funnel; the eluant (containing the hydrogenated methyl esters) was evaporated under nitrogen, redissolved in 0.05 ml of carbon disulfide, and an aliquot (1 μl) was analyzed by GLC as described above. The chromatographic behavior of both the hydrogenated and non-hydrogenated methyl ester samples was compared with that of a mixture of authentic fatty acid methyl esters (NHI 'F'; Supelco, Inc.) containing the following species: C₁₄:₀, C₁₆:₀, C₁₈:₀, C₂₀:₀, C₂₂:₀ and C₂₄:₀. Another portion of the hydrogenated sample (1 μl) was analyzed on the 3% OV-17 column under isothermal conditions (150°C) and compared with a mixed standard containing fatty
acid methyl esters ($C_{16:0}$ and $C_{18:0}$) and isoprenoid acid methyl esters ($C_{15:0}$ and $C_{20:0}$).

**RESULTS AND DISCUSSION**

Assaying the radioactivity of the nonsaponifiable and saponifiable lipid extracts revealed that approximately 98% of the labelled palmitic acid was recovered with the saponifiable material while less than 2% partitioned with the nonsaponifiable material. The overall recovery of radioactivity (prior to derivatizing the acids with diazomethane) was quantitative. As previously stated, conversion of the acids to the corresponding methyl esters was nearly quantitative (ca. 99%) and the recovery of methyl esters after purification by Unisil column chromatography was about 96%.

The total fatty acid composition of the bovine retina, expressed in terms of the mole per cent of the individual acyl chain lengths (i.e. without regard to number or positions of double bonds), is given in Table VII-12. It should be noted that the mole per cent values derived from this analysis were calculated from the peak areas, corrected for differences in the detectability of the methyl esters as a function of chain length by using appropriate molar response factors, in comparison with the response of a known amount of methyl nonadecanoate (internal standard). The data calculated from the results of Bartley *et al.* (1962) are given for the purpose of comparison. Their values were based on peak area alone, without regard to differences in detector response; also, their data only accounts for 94.4% of the total fatty acids. The two sets of data are comparable, with respect to the major
**TABLE VII-12**

Fatty Acid Composition of the Bovine Retina

<table>
<thead>
<tr>
<th>CHAIN LENGTH (C Atoms)</th>
<th>MOLE PER CENT</th>
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<td>This Study</td>
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<tr>
<td>15</td>
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<tr>
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<td>0.13</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>

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1 Determined by GLC analysis (3% OV-17) of hydrogenated and non-hydrogenated fatty acid methyl esters obtained by saponification and acidification of whole bovine retinas. Values corrected for variations in detector response as a function of chain length. Methyl nonadecanoate (C₁₉:₀) used as an internal standard.

2 Values obtained as relative peak area percentages (relative to total area of chromatogram peaks).
FIGURE VII-61

GLC profile of the hydrogenated methyl esters derived from bovine reti-
na saponifiable lipids. Chromatographic system: 3% OV-17, 150°C iso-
thermal. Arrows denote the expected positions of C₁₅ (first) and C₂₀
(second) saturated isoprenoid acid methyl esters (in order of increas-
ing retention time). The major mass peaks are due to methyl palmitate
and methyl stearate, respectively (in order of increasing retention
time).
acyl species. These results suggest that there was little, if any, loss of the polyunsaturated fatty acids (especially docosahexaenoic acid) as a result of the saponification and work-up of the retinas. In addition to the acyl species reported by Bartley et al. (1962), components having the chromatographic properties of C_{15}, C_{21}, C_{24} and C_{26} fatty acid methyl esters were also detected as minor constituents of the total acyl pool of the bovine retinas used in this study.

The chromatogram of the hydrogenated retina methyl esters obtained by isothermal GLC analysis on a 3% OV-17 column is shown in Figure VII-61. Even with the C_{16} (retention time, 19.99 min.) and the C_{18} (retention time, 52.23 min.) FAME components grossly off-scale, no isoprenoid acid methyl esters were detected (arrows). Under the same chromatographic conditions, the following standards eluted with the following retention times: methyl palmitate, 19.76 min.; methyl stearate, 51.07 min.; methyl-3,7,11-trimethyldodecanoate, 5.17 min.; methyl phytanate, 38.37 min. Taking into account the detection limits of this instrument under the conditions employed, C_{15} and C_{20} isoprenoid acids represented less than 0.017 and 0.013 mole per cent, respectively, of the total fatty acid composition of the bovine retinas used in this study. Since very respectable recovery of the labile polyunsaturated fatty acids was achieved, it is not likely that the lack of detectable isoprenoid acids was due to losses during saponification and subsequent work-up of the saponifiable material. In addition, it is known that bovine blood contains substantial amounts of phytanic acid (Duncan and Garton, 1963), apparently derived from the isoprenoid side-chain of chlorophyll. The
lack of detectable methyl phytanate in the sample derived from bovine retinas indicated that contamination of the sample with blood lipids was negligible.

The above data suggested that C_{15} and C_{20} isoprenoid acids are not present in appreciable amounts as endogenous retina components.

**GENERAL DISCUSSION**

The results of *in vitro* incubations of bovine retina S_{10} homogenates with 3RS-[2-^{3}H]-mevalonic acid exhibited considerable variability but also showed some striking similarities. In the first set of incubations, the relative conversion of substrate to saponifiable versus nonsaponifiable lipids was 3:1, with about 80% of the saponifiable material being represented by isoprenoid acids. About 75% of the nonsaponifiable material was more polar than typical C_{27} monohydroxy sterols, and less than 1% of the total nonsaponifiable activity behaved chemically and chromatographically like sterols. The isoprenoid acids were represented by C_{15} and C_{20} species; at least 98% of the C_{15} acids was accounted for by all-trans farnesolic acid while about 62% of the C_{20} acids was represented by all-trans geranylgeranoic acid. Another C_{20} isoprenoid acid ("component X"), which apparently contained at least one cis double bond, accounted for about 30% of the total C_{20} isoprenoid acids derived from labelled mevalonic acid. In contrast, the second set of incubations with the same substrate incubated under similar conditions exhibited nearly equal conversion of mevalonic acid to saponifiable and nonsaponifiable lipids. Almost 90% of the total nonsaponifiable material was relatively apolar, with about 61% being
accounted for by squalene. In addition, an appreciable amount of sterols was detected after both 3 hours and 24 hours of incubation; components with the chromatographic properties of lanosterol and monoenic diene \( C_{27} \) monohydroxy sterols were identified in the nonsaponifiable extract obtained from the 24-hour incubation. Again, both \( C_{15} \) and \( C_{20} \) isoprenoid acids were recovered from the incubation mixtures. However, the distribution of radioactivity among these acid components was quite different than in the previous set of incubations. In addition to the formation of all-trans farnesoic acid, components having the chromatographic properties of \( cis,cis- \) and \( cis,trans- \) (or \( trans,cis- \)) isomers of farnesoic acid were observed. Also, the \( C_{20} \) acids accounted for about 90% of the total isoprenoid acids recovered, of which the major species was "component X".

The synthesis of isoprenoid acids upon incubation of rat liver enzyme preparations with mevalonic acid (Popjak, 1959; Popjak et al., 1959) and allyl pyrophosphate intermediates of squalene biosynthesis (Christophe and Popjak, 1961) has been reported. In a preliminary abstract (Popjak et al., 1959), it was reported that four major acids (and at least 13 other minor acid products) were formed from mevalonic acid, the methyl esters having retention times by GLC which did not correspond to those of the normal  \( n \)-fatty acids. The major product had the chromatographic properties of all-trans farnesoic acid. Subsequently, they reported that similar incubation systems yielded acidic products which were identified as trans-geranoic acid, all-trans farnesoic acid and all-trans geranylgeranoic acid (Popjak, 1959). However, the data on which these assignments were based were not presented. In
this regard, it is important to note that a later report from this same laboratory (Christophe and Popjak, 1961) made no mention of the formation of C20 isoprenoid acids under similar incubation conditions. Instead, it was stressed that the conditions used for saponification and/or acidification of the incubation mixtures led to the formation of artifactual compounds having retention times larger than those of the all-trans C10 and C15 isoprenoid acids previously reported, corresponding to various cyclization products and "cis-trans" isomers of these acids. The above-mentioned incubations were performed with a soluble enzyme system (the so-called "Fr60 -enzymes") derived from the 105,000 x g supernatant of rat liver by ammonium sulfate precipitation (30-60% saturation), followed by redissolving the precipitate (in 0.02 M KHCO3) and removing the salts by dialysis. This preparation differs markedly from the S10 homogenates described in this dissertation, and the results derived from these two incubation systems may not be compared directly.

Upon incubation of the Fr60 -enzyme preparation with labelled mevalonic acid (in the presence of ATP, Mg2+ and reduced glutathione), the pyrophosphate derivatives of dimethylallyl alcohol, geraniol, farnesol and geranylgeraniol were reportedly formed (Popjak, 1959; Goodman and Popjak, 1960). These compounds were resistant to alkaline hydrolysis (2 N KOH); however, upon acidification and extraction of the aqueous mixture with petroleum ether, the bulk of the labelled components were found in the organic extract. These components had the chromatographic properties consistent with various isoprenoid alcohols, including dimethylallyl alcohol, linalool (the tertiary isomer of trans-geraniol),
geraniol, nerol (cis-geraniol), trans,trans-farnesol, nerolidol, (the tertiary isomer of trans,trans-farnesol), all-trans geranylgeraniol and geranyllinalool (the tertiary isomer of all-trans geranylgeraniol) (Popjak, 1959). However, the tertiary alcohols and the cis- isomers were found to be due to rearrangements and isomerizations of the all-trans primary isoprenols during the acidification step. Addition of microsomes and pyridine nucleotides after pre-incubation of the $F^{60}_{30}$-enzymes with mevalonic acid caused a decrease in the polyisoprenols and an increase in the production of isoprenoid acids, as well as conversion to squalene and sterols (Popjak, 1959; Popjak et al., 1959). It was demonstrated that the allyl pyrophosphates (primarily of the C$_5$, C$_{10}$ and C$_{15}$ isoprenols) were cleaved primarily by a microsomal pyrophosphatase to the corresponding isoprenols and were then oxidized by two dehydrogenases (i.e. sequentially, by an alcohol and an aldehyde dehydrogenase) to the corresponding isoprenoid acids (Christophe and Popjak, 1961). Although the pyrophosphate derivatives of dimethylallyl alcohol, geraniol and farnesol were recognized as intermediates in the synthesis of squalene in rat liver preparations (Popjak, 1959; Goodman and Popjak, 1960), the corresponding isoprenoid acids could not serve as precursors of squalene or sterols (Popjak, 1959), in contrast to the results reported by Dituri et al. (1957). The lack of conversion of these isoprenoid acids to squalene and sterols had been reported previously as the result of an in vivo experiment with rats (Sandermann and Stockmann, 1956). These investigators found that rats fed $^{14}$C-labelled dimethylacrylic acid, geranoic acid, farnesoic acid and geranylgeranoic acid could convert only the dimethylacrylic acid to squalene, sterols and "fatty
acids" in all major tissues (including liver) whereas the $C_{10}$, $C_{15}$ and $C_{20}$ isoprenoid acids were apparently not further metabolized in this manner.

The results presented in Experiments VII-1 and VII-2 differ markedly from those described in the reports from Popjak's laboratory in several ways. First, bovine retinas were used as the tissue from which the $S_{10}$ homogenates were prepared. Secondly, $S_{10}$ homogenates were used in these experiments (instead of the liver $F_{60}^{30}$-enzymes or other subcellular fractions). Thirdly, isoprenoid acids were the major metabolites derived from mevalonic acid in these incubations, whereas routine incubations of rat liver $S_{10}$ homogenates with mevalonic acid generally produce nonsaponifiable lipids as the major metabolites (predominantly sterols and squalene) and little, if any, isoprenoid acids are formed. Fourthly, the major isoprenoid acids formed in these retina homogenate incubations were $C_{20}$ acids (as opposed to the incubations by Popjak and co-workers, where all-trans farnesic acid was the major isoprenoid acid synthesized). Finally, a substantial proportion of the overall substrate conversion yielded a $C_{20}$ isoprenoid acid which apparently contained at least one cis double bond; this product was shown not to be formed by acid isomerization of the all-trans isomer of geranylgeranoic acid. In one instance, a component having the chromatographic properties of cis,cis-farnesic acid was synthesized; by analogy, it is unlikely that this product was formed by a non-enzymatic isomerization of trans,trans-farnesic acid during the work-up of the incubation mixture. Under the conditions of saponification and acidi-fication employed in this study, cyclization of the acids was not
observed, in contrast to previously reported results (Popjak, 1959; Christophe and Popjak, 1961). In both sets of incubations, only two classes of isoprenoid acids were recovered from the incubation mixtures: C_{15} and C_{20} acids. It is possible that minor amounts of shorter-chain isoprenoid acids (i.e. C_{5} and C_{10} acids) were formed but were lost during the removal of solvent under partial pressure or by evaporation under nitrogen.

In Experiment VII-1, it was apparent that isoprenol-like components were synthesized from mevalonic acid, but these components did not exhibit the chromatographic behavior of the isoprenols derived from the allyl pyrophosphates known to be intermediates in the biosynthesis of squalene. Since this material was extracted directly after saponification of the incubation mixtures, and was never exposed to acidic conditions, it is unlikely that these products represented non-enzymatic rearrangement of isomerization products of the corresponding all-trans primary isoprenoid alcohols. Also, it was noted previously (Schroepfer and Gore, 1963) that alkaline treatment of trans,trans-farnesol did not lead to isomerization or rearrangement; therefore, it is also unlikely that these unidentified isoprenol-like components were produced artifically during the saponification procedure. Allyl pyrophosphates were not observed in these incubations. The most likely explanation for this fact is that sufficient pyrophosphatase activity (perhaps microsomal in origin) was present in the retina S_{10} homogenate to immediately cleave any pyrophosphate compounds derived from mevalonic acid to the corresponding alcohols, in analogy to the rat liver system (Christophe and Popjak, 1961).
A generalized and highly simplified scheme of the metabolism of 3R-[2-^3H]-mevalonic acid is given in Figure VII-62 (for a review, see Popjak and Cornforth, 1960; Bloch, 1965; Clayton, 1965; Frantz and Schroepfer, 1967; Schroepfer et al., 1972; Gaylor, 1974). This scheme assumes no loss of tritium during the equilibration of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMA-PP). From this scheme, it is apparent that at least three major biochemical transformations may take place at the level of farnesyl pyrophosphate (FPP), the C₁₅ allyl pyrophosphate which serves as the penultimate precursor of squalene: (1) two molecules of FPP may condense to form presqualene pyrophosphate (the immediate precursor of squalene) which may then be further metabolized to sterols; (2) FPP may be hydrolized (i.e. by a pyrophosphatase) to form farnesol, which may then be further metabolized oxidatively to the corresponding isoprenoid acid; (3) FPP may condense with more molecules of IPP to form higher conjugates (i.e. geranylgeranyl pyrophosphate, dolichol pyrophosphate, etc.). Thus, the regulation of this "branch point" in terpene biosynthesis may be very critical to sterol biosynthesis, since diversion of the allyl pyrophosphates away from squalene formation would necessarily decrease the production of sterols. Also, farnesolic acid and certain related derivatives are known to inhibit the conversion of mevalonic acid to sterols in rat liver homogenate preparations (Wright and Cleland, 1957; Popjak et al., 1960).

In mammalian tissues, the formation of isoprenoids usually proceeds by trans addition via the so-called "head-to-tail" condensation of IPP and some isoprenoid, resulting in the formation of conjugate of the original isoprenoid which is increased in size by 5 carbons (cf. Beytia
FIGURE VII-62

Metabolism of 3R-[2-^3^H]-mevalonic acid: a hypothetical scheme. Abbreviations: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMA-PP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, trans,trans-farnesyl pyrophosphate. The symbol (★) denotes the position of the tritium label (stereochemistry not assigned).
and Porter, 1976). Almost all known isoprenoid compounds synthesized in mammalian tissues possess trans double bonds; a notable exception is dolichol and its derivatives, which contain predominantly cis isoprene units (Hemming, 1974; cf. Beytia and Porter, 1976). Some general rules have evolved which have been quite useful in predicting the stereochemistry of isoprenoids, based on the stereochemistry of the protons at the C-4 position of mevalonic acid: retention of the "4-pro-R" proton (the "2-pro-S" proton of IPP) leads to trans isoprenoid biosynthesis, while retention of the "4-pro-S" proton (the "2-pro-R" proton of IPP) results in the formation of cis isoprenoids. Such rules have correctly predicted the stereochemistry of a variety of isoprenoid compounds, including intermediates in the biosynthesis of squalene (Cornforth et al., 1966; Popjak and Cornforth, 1966), dolichols (Gough and Hemming, 1970b), betulaprenols (Gough and Hemming, 1970a), cis rubber (Archer et al., 1966) and undecaprenyl pyrophosphate (Gough et al., 1970; Allen et al., 1976; Baba and Allen, 1978). Thus, another locus of regulation exists at the level of the prenyl transferases, the enzymes which catalyze the condensation of isoprene units with stereospecific selectivity, resulting in the biosynthesis of either cis or trans isoprenoids. However, there are known cases which apparently violate these general stereochemistry rules, i.e. where cis isoprenoids are formed with loss of the 4-pro-S proton of mevalonic acid (presumably by initial synthesis of the trans isomer and subsequent isomerization to the cis product). Such is the case in the biosynthesis of 2-cis-6-trans-farnesol and its pyrophosphate derivative (Jacob et al., 1972; Jedlicki et al., 1972), nerol (Francis et al., 1970) and abscisic acid
(Robinson and Ryback, 1969). In all cases, the cis isoprenoids were biosynthesized in plants; such compounds have not been isolated from (or shown to be biosynthesized by) mammalian tissues. Interestingly, the reverse isomerization (i.e. of a cis isoprenoid to the corresponding trans isomer) is not known to occur (George-Nascimento and Cori, 1971; Jacob et al., 1972). In the retina, there is a known precedence for the isomerization of isoprenoids, namely the conversion of all-trans retinaldehyde to the 11-cis isomer (Hubbard, 1956). It is not clear that "retinal isomerase" has a strict requirement in vivo for the aldehyde as opposed to the free alcohol or its corresponding ester (Futterman, 1974; Shichi and Sommers, 1974), and the substrate specificity has not been examined with respect to non-retinoid isoprenoids. Therefore, it is possible that "retinal isomerase" could convert other isoprenoids (i.e. all-trans farnesol, all-trans geranylgeraniol and/or their corresponding aldehydes, esters or acids) to their corresponding cis isomers. Such enzymatic isomerization could explain the accumulation of cis isomers of the C15 and C20 isoprenoid acids extracted from the incubation mixtures.

It has also been known for some time that cattle retinas can regenerate rhodopsin in situ upon incubation of bleached retinas with all-trans retinol (a C20 isoprenol) (Collins et al., 1953, 1954), a process which involves the oxidation and isomerization of the free alcohol to the corresponding 11-cis aldehyde (cf. Wald, 1968). Rat retinas contain two retinol dehydrogenases and an alcohol dehydrogenase; all three enzymes appear to be distinct from the corresponding rat liver enzymes (Koen and Shaw, 1966). The so-called "retinol dehydrogenase" activity
has been reported to be localized in the rod outer segments of frog, cattle and rat retinas (Arden, 1954; Bridges, 1962, 1976, 1977; Futterman and Saslaw, 1961; Futterman, 1963; Koen and Shaw, 1966; Lion et al., 1975). The "retinol dehydrogenase" activity of frog ROS has been reported to be due to two distinct enzymes (on the basis of differential liability to Triton X-100 and requirements for either reduced or oxidized TPNH): a "retinol oxidase" and a "retinaldehyde reductase" (Bridges, 1977). Again, the substrate specificity of these enzymes was not fully examined with regard to non-retinoid isoprenoids. Therefore, it is possible that the isoprenols derived from the allyl pyrophosphates utilized in sterol biosynthesis (and related compounds) could be oxidized (at least up to the aldehyde oxidation level) by known retina enzymes, in a fashion analogous to that employed in the synthesis of isoprenoid acids by rat liver preparations (Christophe and Popjak, 1961). The formation of isoprenoid acids would also require either an aldehyde dehydrogenase or an aldehyde oxidase to complete the oxidation to the acid.

The results of these experiments are novel in at least three ways: (1) isoprenoid acids were major products of the incubations; (2) the C_{20} isoprenoid acids represented the major products of the incubation; (3) cis isoprenoids accumulated in the incubation mixtures, and "component X" (apparently a cis isomer structurally related to all-trans geranylgeranoic acid) was a major incubation product. In addition, a component was identified which had the chromatographic properties of cis,cis-farnesoic acid. The biosynthesis and/or isolation of this isomer of farnesoic acid from a biological source has never been described.
Since such isoprenoid acids were not detected as endogenous constituents of the bovine retina, the biological relevance of the formation of these compounds in vitro is not clear. Since the retina has the ability to synthesize a variety of sterols from mevalonic acid in vitro (see Experiment VII-2) which are putative intermediates in the biosynthesis of cholesterol, the role of "alternate" metabolism of mevalonic acid which diverts isoprenoid intermediates from the sterol biosynthetic pathway is also not understood. One explanation might be that the delicate balance in the regulation at the branch-point involving metabolism of farnesyl pyrophosphate is somehow disrupted by the homogenate preparation procedures or by the in vitro incubation conditions (i.e. loss of subcellular compartmentation, selective damage to the enzymes involved in the formation of squalene and/or sterols, etc.). Alternatively, it is possible that "alternate" metabolism is normal for the retina, and that the accumulation of C_{15} and C_{20} isoprenoids represents the formation of "stranded intermediates" in the biosynthesis of higher isoprenoids. In either case, the allyl pyrophosphates apparently become subject to attack by pyrophosphatases and dehydrogenases (or oxidases), yielding the corresponding isoprenoid acids. As mentioned above, farnesoic acid and certain of its derivatives are known to inhibit the conversion of mevalonic acid to sterols (Wright and Cleland, 1957; Popjak et al., 1960). It has been suggested that the locus of the inhibition is at the level of mevalonate kinase (Popjak et al., 1960). Clearly, the accumulation of labelled isoprenoid acids in these incubations could not have inhibited the synthesis of sterols in this manner since (1) the amounts of such acids were insufficient to affect the enzyme system and
(2) such inhibition of mevalonate kinase would have inhibited the formation of the isoprenoid acids themselves.

Due to the novel aspects of isoprenoid metabolism observed in the retina, further experimentation in this area is warranted but is beyond the scope of this dissertation.
VIII. THE INTERCONVERSION OF STEROL PRECURSORS OF

CHOLESTEROL IN VITRO BY BOVINE RETINA HOMOGENATES

INTRODUCTION

The previous results of incubations of bovine retina 10,000 xg homogenates with a de novo precursor of cholesterol (i.e. mevalonic acid) have indicated that squalene and a variety of sterols which are putative precursors of cholesterol accumulate in vitro, with very minimal synthesis of cholesterol. This is in striking contrast to the steady-state composition of the tissue, where cholesterol is essentially the only sterol present and the various isoprenoid precursors of cholesterol are undetected. The relatively large accumulation of squalene synthesized in vitro suggests that the conversion of squalene to lanosterol in this system is at least partially inhibited, and the accumulation of various sterols further suggests that the conversion of lanosterol to cholesterol may be at least partially blocked at one or more points along the biosynthetic pathway. These observations and conclusions must be considered with the caveat that the in vitro system may not reflect accurately the in vivo physiology and biosynthetic capacities of the retina.

The conversion of lanosterol to cholesterol in animal tissue has been studied extensively for many years (for a review, see Bloch, 1965; Clayton, 1965; Frantz and Schroepfer, 1967; Schroepfer et al., 1972; Gaylor, 1974). The conversion involves three general processes: (1) reduction of the side chain Δ24 double bond; (2) removal of the three "extra" methyl groups; (3) nuclear rearrangements of the Δ8 double bond to the Δ5 position. This is a gross oversimplification of the actual
biosynthetic processes, which may involve an astronomical number of potential intermediates and a very complex enzyme system. Also, the order in which these three general processes occurs is not understood, nor has it been established that there is an absolute, defined order for these processes, physiologically. Furthermore, much of our knowledge concerning the biosynthesis of cholesterol has been derived from experimentation with rat liver preparations; it is not clear that the biosynthesis of cholesterol as performed by rat liver may be generalized to other tissues, especially neural tissue (cf. Davison, 1970; Kabara, 1967, 1973; Ramsey and Nicholas, 1972; Paoletti et al., 1969).

The presence and/or formation of 24,25-dihydrolanosterol (lanost-8-en-3β-ol or 4,4,14α-trimethyl-cholest-8-en-3β-ol) has been reported in animal tissues (Avigan et al., 1963; Gaylor, 1963; Gray et al., 1969; Kandutsch and Russell, 1959) and its convertibility to cholesterol by rat liver homogenates has been demonstrated (Avigan et al., 1963; Kandutsch and Russell, 1960). Similarly, the presence and/or formation of cholesta-8,14-dien-3β-ol has been reported for rat liver (Hunton and Schroepfer, 1971; Lutsky and Schroepfer, 1971; Schroepfer et al., 1971) and its convertibility to cholesterol by rat liver homogenate preparations has been confirmed (Akhtar et al., 1969; Lutsky and Schroepfer, 1968, 1970). The following experiment was designed to examine the convertibility of chemically-synthesized, tritiated 24,25-dihydrolanosterol and cholesta-8,14-dien-3β-ol to cholesterol by bovine retina S₁₀ homogenates, using rat liver S₁₀ homogenates as a control.

**Experiment VIII-1**

[2,4-³H]-Cholesta-8,14-dien-3β-ol (ca. 3.88 x 1₀⁷ dpm/mg) and [2-³H]-
lanost-8-en-3ß-ol (ca. 1.87 x 10^7 dpm/mg) were synthesized as previously described (see Chapter III). Aliquots of each sterol (25 µl, benzene solution) were transferred to 20 ml capacity glass vials, evaporated to dryness under nitrogen and redissolved in propylene glycol (1 ml each) by sonication for one minute at room temperature, under argon (bath-type sonicator; E/MC Corporation). S_10 homogenates were prepared from freshly dissected rat livers (4 livers; 85 ml total homogenate) and bovine retinas (20 retinas; 38 ml total homogenate) as previously described (see Materials and General Methods), except that the incubation buffer (0.10 M potassium phosphate, pH 7.4, containing 5 mM MgCl_2·6H_2O and 30 mM nicotinamide) was supplemented with 50 µg/ml each of penicillin-G and streptomycin sulfate. A concentrated cofactor stock solution (5.3 ml, in incubation buffer) was made, containing the following components; NAD (16.17 mg/ml), NADP (16.83 mg/ml), ATP (60.63 mg/ml) and G-6-P (18.62 mg/ml). The pH of the solution was adjusted to 7.4 by dropwise addition of 6 N potassium hydroxide. Portions (15.0 ml) of either liver homogenate (52.0 mg protein/ml) or retina homogenate (15.3 mg protein/ml) were added to a given vial and the mixture was vortexed for one minute (low speed; 4°C) to insure complete mixing. The homogenate mixtures were then transferred to 125 ml capacity screwtop erlenmeyer flasks containing 0.75 ml of concentrated cofactor stock; the flasks were flushed with oxygen for 10 seconds, sealed and then incubated at 37°C for 3 hours with continuous agitation (Dubnoff Metabolic Incubator; 60 cycles/min.). The final levels of labelled sterols in the incubations were as follows:

2-^3H -lanost-8-en-3ß-ol, 3.84 x 10^6 dpm, 205 µg, 28.6 µM; 2,4-^3H -cholesta-8,14-dien-3ß-ol, 4.16 x 10^6 dpm, 107 µg, 16.6 µM. The final
concentration of cofactors was as follows: NAD, 1 mM; NADP, 1 mM; ATP, 5 mM; G-6-P, 3 mM. Although ATP is not a required cofactor for sterol interconversion reactions, it was included in these incubations in order to be consistent with the previous in vitro conditions.

Incubations were terminated by the addition of 2 ml of an ethanolic cholesterol solution (4 mg/ml) plus 15 ml of 15% ethanolic potassium hydroxide. The mixtures were saponified for 3.5 hours at 80°C, allowed to cool to room temperature and then flushed with nitrogen and stored at 4°C overnight. The mixtures were then diluted with 0.9% NaCl, (1:1, v/v) and extracted three times with 100 ml portions of petroleum ether. The combined organic extracts were washed once with 100 ml of water, dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. The concentrated extracts were transferred to vials, evaporated to dryness under nitrogen and desiccated under vacuum over phosphorous pentoxide for several hours. After the addition of 5 mg of carrier lanosterol/dihydrolanosterol to the extracts obtained from the incubations with tritiated dihydrolanosterol, the residues were re-dissolved in 10.0 ml of benzene. Duplicate aliquots (0.1 ml each) were assayed for total radioactivity.

The homogenate extracts from the labelled C_{27} sterol incubations were evaporated to dryness under nitrogen, desiccated under vacuum over phosphorous pentoxide for two hours and then acetylated with acetic anhydride-pyridine (10 ml total; 1:1, v/v) and worked up as previously described (see Materials and General Methods). The acetylated sterols were then analyzed on columns of Silica Gel G-Super Cel-silver nitrate and alumina-Super Cel-silver nitrate, as previously described.
The homogenate extracts from the labelled C_{30} sterol incubations were evaporated to dryness under nitrogen, redissolved in 0.5 ml of toluene and chromatographed on columns of silicic acid-Super Cel (58 x 2 cm, eluted with toluene); the fractions which co-migrated with the cholesterol standard were acetylated and analyzed further on columns of Silica Gel G-Super Cel-silver nitrate and alumina-Super Cel-silver nitrate, as described previously.

Material which co-chromatographed with authentic cholesteryl acetate on both argentated columns was pooled, concentrated under reduced pressure, evaporated under nitrogen to dryness, diluted with carrier cholesterol (ca. 50-60 mg) and the acetates were cleaved by saponification (12 hours at 37° C, in 10 ml of 15% ethanolic potassium hydroxide, under nitrogen). After dilution with an equal volume of a 5% tartaric acid solution, the free sterols were extracted once with 100 ml of dichloromethane; the organic extracts were washed once with 100 ml of 5% tartaric acid solution and twice with 100 ml portions of water, and then filtered through a bed of anhydrous magnesium sulfate. Solvent was removed under reduced pressure and the residues were transferred to vials with dichloromethane, evaporated to dryness under nitrogen and redissolved in 10.0 ml of benzene. After withdrawing appropriate aliquots (in triplicate) for assaying radioactivity and colorimetric determination of cholesterol, the remaining material was evaporated to dryness under nitrogen and processed via the dibromide, as described previously (see Materials and General Methods). The recovered sterols were then redissolved in benzene and aliquots were assayed, as before, for radioactivity and cholesterol; the specific activities were compared before and after the dibromide
purification.

RESULTS

The recoveries of radioactivity in the nonsaponifiable lipid extracts from incubations of rat liver and bovine retina \( S_{10} \) homogenates are given in Table VIII-1 below.

<table>
<thead>
<tr>
<th>( S_{10} ) HOMOGENATE</th>
<th>SUBSTRATE</th>
<th>RECOVERED ACTIVITY (dpm x 10(^{-6}))</th>
<th>% RECOVERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Liver</td>
<td>( C_{27} )</td>
<td>2.60</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>( C_{30} )</td>
<td>2.87</td>
<td>74.7</td>
</tr>
<tr>
<td>Bovine Retina</td>
<td>( C_{27} )</td>
<td>1.05</td>
<td>25.4</td>
</tr>
<tr>
<td></td>
<td>( C_{30} )</td>
<td>2.94</td>
<td>76.4</td>
</tr>
</tbody>
</table>

* [2,4-\(^3\)H]-Cholesta-8,14-dien-3\(\beta\)-ol (4.16 x 10\(^6\) dpm; 278 nmoles)

**[2-\(^3\)H]-Lanost-8-en-3\(\beta\)-ol (3.84 x 10\(^6\) dpm; 479 nmoles)

Although moderate recoveries of the initially incubated activity were obtained for the liver homogenate incubations, the recovery of labelled \( C_{27} \) sterols from the bovine retina homogenate was unusually low (ca 25% of the initially incubated radioactivity). Comparison with the recovery of radioactivity from the liver homogenate \( C_{27} \) sterol incubation suggests that autoxidation of the labelled products or substrate cannot account for these results. Extraction of the saponifiable phase with more polar
solvents was not performed, therefore this aspect of the incubation results was not pursued further.

Chromatographic Analyses of Nonsaponifiable Lipids Obtained from Incubations of the Labelled C_{30}-Sterol Substrate

The nonsaponifiable extracts obtained from incubations of rat liver (2.81 x 10^6 dpm) and bovine retina (2.87 x 10^6 dpm) homogenates with [2-^3H]-lanost-8-en-3β-ol were applied to columns of silicic acid-Super Cel and eluted with toluene. Fractions (35 minutes each; 6.7 ml for liver extract, 7.1 ml for retina extract) were collected automatically and assayed directly for radioactivity and for carrier sterols, as usual (i.e. since toluene did not evaporate from the fractions appreciably, aliquots were taken without previously evaporating the fractions to dryness and redissolving in solvent to uniform volume). The elution profiles for the liver and retina homogenate extracts are shown in Figures VIII-1 and VIII-2, respectively. The liver extract (which was supposed to serve as an "enzymatic control") showed only minor conversion to material which co-eluted with the cholesterol standard (ca. 7% of the recovered activity); the bulk of the recovered radioactivity co-eluted with the C_{30} sterol standard (fractions 50-105; ca. 83%) while the remaining activity eluted between the C_{30} and C_{27} sterol standards (ca. 10%; C_{28} and C_{29} sterols). Both the radioactivity and the mass peaks were broad, and there was no resolution of the carrier lanosterol from dihydrolanosterol. However, the radioactivity in the C_{30} sterol region was shifted, relative to the C_{30} mass peak, with the peak fraction for radioactivity eluting 3–4 fractions prior to the mass peak fraction; this suggested that the activity in this region of the chromatogram was
FIGURE VIII-1
Silicic acid-Super Cel column chromatography of the nonsaponifiable products obtained by incubation of a rat liver S10 homogenate with [2-3H]-lanost-8-en-3β-ol (solvent system: toluene). Open circles (○—○), radioactivity; closed triangles (▲—▲), cholesterol, determined colorimetrically (absorbance at 620 nm); closed squares (■—■), 24,25-dihydrolanosterol and lanosterol, determined colorimetrically (absorbance at 460 nm).

FIGURE VIII-2
Silicic acid-Super Cel column chromatography of the nonsaponifiable products obtained by incubation of a bovine retina S10 homogenate with [2-3H]-lanost-8-en-3β-ol (solvent system: toluene). Open circles (○—○), radioactivity; closed triangles (▲—▲), cholesterol, determined colorimetrically (absorbance at 620 nm); closed squares (■—■), 24,25-dihydrolanosterol and lanosterol, determined colorimetrically (absorbance at 460 nm).
due to unconverted substrate. The overall recovery of radioactivity from this column was 89%. The elution profile obtained for the retina extract was markedly different. Partial resolution of the C30 carrier sterols was achieved (fractions 44-72) on this column; approximately 98% of the recovered radioactivity co-migrated with the first mass peak (dihydrolanosterol) exclusively, suggesting that this material represented unconverted substrate. The remaining 2% of the recovered radioactivity trailed diffusely from the C30 sterol peak, with less than 0.1% of the activity eluting in the C27 sterol region (fractions 95-125). Overall recovery was about 98%.

The material from the liver extract which co-migrated with the authentic cholesterol standard (fractions 136-200) was pooled, acetylated (as previously described) and applied to a column of Silica Gel G-Super Cel-silver nitrate (50 x 1 cm column, in hexane-toluene, 7:3; applied activity, 1.56 x 10^5 dpm), along with 5 mg each of carrier cholesta-8, 14-dien-3β-yl acetate and 7-dehydrocholesteryl acetate. The column was eluted with hexane-toluene, 7:3, up to fraction 51 (5.2 ml/35-minute fraction), then with hexane-toluene, 6:4, up to fraction 109, and finally with hexane-toluene, 1:1, to fraction 200. The fractions were evaporated to dryness under nitrogen, redissolved in 2.0 ml of benzene, and aliquots (0.2 ml each) were assayed for radioactivity and carrier mass as usual. The elution profile is shown in Figure VIII-2. Greater than 99% of the recovered radioactivity co-eluted with the carrier cholesteryl acetate (fractions 12-33); there was no measurable activity in the region of sterol dienes. The recovery of radioactivity from this column was about 88%. 
FIGURE VIII-3
Silica Gel G-Super Cel-silver nitrate column chromatography of the acetylated material derived from fractions of 136-200 from the previous silicic acid-Super Cel column of products obtained from the rat liver homogenate incubation with [2-3H]-lanost-8-en-3β-ol (see Figure VIII-1). Solvent system: hexane-toluene, 7:3; the first arrow denotes solvent change to hexane-toluene, 3:2; the second arrow denotes solvent change to hexane-toluene, 1:1. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), C27 steryl acetates, determined colorimetrically. The colorimetric peaks (in order of elution from the column) are due to cholesteryl acetate, 3β-acetoxy-Δ8(14)-cholestadiene and 3β-acetoxy-Δ5,7-cholestadiene.

FIGURE VIII-4
Alumina-Super Cel-silver nitrate column chromatography of the monoenes (fractions 12-33) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of products derived from the rat liver homogenate incubation with [2-3H]-lanost-8-en-3β-ol (see Figure VIII-3). Solvent system: hexane-toluene, 9:1; arrow denotes change to hexane-toluene, 7:3. Open circles (o — o), radioactivity; closed triangles (▲ — ▲) C27 steryl acetates, determined colorimetrically. The colorimetric peaks (in order of elution from the column) are due to 3β-acetoxy-Δ8(14)-cholestene, 3β-acetoxy-Δ7-cholestene and cholesteryl acetate.
ABSORBANCE (620 nm)

FRACTION NUMBER

RADIOACTIVITY (DPM × 10³)

566
ABSORBANCE (620 nm)

FRACTION NUMBER

RADIOACTIVITY (DPM x 10^3)
The fractions which co-migrated with cholesteryl acetate were pooled (1.10 x 10^3 dpm) and applied to an alumina-Super Cel-silver nitrate column (100 x 1 cm, in hexane-toluene, 9:1), along with 5 mg each of additional carrier steryl acetates (i.e. $\Delta^5$, $\Delta^7$ and $\Delta^8(14)$). The column was eluted with hexane-toluene, 9:1, up to fraction 173 and then with hexane-toluene, 7:3, up to fraction 210. Fractions (5.6 ml per 30 minutes) were collected and assayed for radioactivity and carrier mass as described previously. The results are shown in Figure VIII-4. The first peak of radioactivity (fractions 12-19) eluted just prior to the $\Delta^8(14)$ steryl acetate mass standard (fractions 16-22), representing about 2% of the recovered radioactivity. The second peak of radioactivity (ca. 14%) exhibited partial resolution into two components (fractions 23-40), with the major species co-migrating with the $\Delta^7$ steryl acetate standard (fractions 28-40). The major radioactive component (fractions 46-75) co-migrated with the cholesteryl acetate standard, representing about 84% of the total activity. The overall recovery of applied radioactivity was 90%. For reasons yet unknown, the steryl acetate standards and all the radioactivity eluted very rapidly from this column (within 80 fractions), in contrast to the normal elution profiles routinely obtained for this type of chromatographic system (cf. Lee et al., 1969).

In summary, the rat liver homogenate converted only about 7% of the incubated C_{30} sterol to C_{27} monohydroxy sterols, of which about 84% had the chromatographic properties of cholesterol. Under similar incubation conditions, the bovine retina homogenate failed to convert the labelled sterol substrate to other monohydroxy sterols. However, it should be noted that the retina homogenate contained only about 30% as
much total protein as the liver homogenate.

Chromatographic Analyses of Nonsaponifiable Lipids Obtained from Incubations of the Labelled C₂₇ Sterol Substrate

The acetylated nonsaponifiable lipids from incubations of rat liver (2.36 x 10⁶ dpm) and bovine retina (9.37 x 10⁵ dpm) homogenates with [2,4-³H]-cholesta-8,14-dien-3β-ol were applied to columns of Silica Gel G-Super Cel-silver nitrate (50 x 1 cm, in hexane-toluene, 7:3), along with 5 mg each of carrier cholesta-8,14-dien-3β-y1 acetate and 7-dehydrocholesteryl acetate. Fractions (4.2 ml/30 min., for liver extract; 4.4 ml/30 min., for retina extract) were eluted with hexane-toluene, 7:3, up to fraction 121, and then with hexane-toluene, 1:1, to fraction 250. Fractions were evaporated to dryness under nitrogen, redissolved in 2.0 ml of benzene and aliquots were assayed for radioactivity and steryl acetate mass as usual. The radiochromatogram for the liver extract is shown in Figure VIII-5. Approximately 95% of the recovered activity co-eluted with the monoene steryl acetate standard (fractions 6-20), while only 4% exhibited the migration of unconverted substrate (fractions 140-160). No activity was detected in the region of the Δ⁵,⁷ steryl acetate standard. Two minor peaks of radioactivity (fractions 43-73 and 74-116) each represented about 0.5% of the total recovered activity; these peaks eluted in the region of the chromatogram which lies between the "monoene" and "diene" regions, and the nature of these components is not known. Overall recovery was about 85%. In striking contrast, only about 47% of the activity recovered by column chromatography of the retina extract co-migrated with the monoene steryl acetate standard (Figure VIII-6; fractions 5-20). Nearly 49% of the activity chromatographed as uncon-
FIGURE VIII-5
Silica Gel G-Super Cel-silver nitrate column chromatography of the acetylated material derived from the nonsaponifiable products obtained by incubation of a rat liver $S_{10}$ homogenate with $[2,4-^3H]$-cholesta-8,14-dien-3β-ol. Solvent system: hexane-toluene, 7:3; arrow denotes change to hexane-toluene, 1:1. Open circles (o – o), radioactivity; closed triangles (▲ – ▲), C$_{27}$ steryl acetates, determined colorimetrically. The colorimetric peaks (in order of elution from the column) are due to cholesteryl acetate, 3β-acetoxy-Δ$^8,14$-cholestadiene and 3β-acetoxy-Δ$^5,7$-cholestadiene.

FIGURE VIII-6
Silica Gel G-Super Cel-silver nitrate column chromatography of the acetylated material derived from the nonsaponifiable products obtained by incubation of a bovine retina $S_{10}$ homogenate with $[2,4-^3H]$-cholesta-8,14-dien-3β-ol. Solvent system: hexane-toluene, 7:3; arrow denotes change to hexane-toluene, 1:1. Open circles (o – o), radioactivity; closed triangles (▲ – ▲), C$_{27}$ steryl acetates, determined colorimetrically. The colorimetric peaks (in order of elution from the column) are due to cholesteryl acetate, 3β-acetoxy-Δ$^8,14$-cholestadiene and 3β-acetoxy-Δ$^5,7$-cholestadiene.
ABSORBANCE (620 nm)

FRACTION NUMBER

0.25  0.20  0.15  0.10  0.05

RADIOACTIVITY (DPM x 10^{-4})

- Insert graph showing data points with linear and non-linear trends.
- Axes labeled with appropriate units.
verted substrate (fractions 142-170). Approximately 4% of the total radioactivity eluted in the region between the monoene and diene steryl acetates (fractions 50-80), this time as a single peak. Less than 1% of the activity trailed after the Δ^8,1^4 diene steryl ester peak, into the region of the Δ^5,7 steryl acetate. The overall recovery of applied activity was 83%.

The fractions derived from the retina extract which co-migrated with the cholesteryl acetate standard (fractions 5-21; 2.20 x 10^4 dpm) were pooled, supplemented with 5 mg each of carrier Δ^8(14), Δ^7 and Δ^5 steryl acetate standards, and applied to an alumina-Super Cel-silver nitrate column (100 x 1 cm; hexane-toluene, 9:1). Fractions were eluted with hexane-toluene, 9:1, up to fraction 159 and then with hexane-toluene, 4:1 to fraction 210; 20-minute fractions (2.0 ml each) were collected up to fraction 76, after which the collection time was increased to 35 minutes per fraction (3.45 ml each). The fractions were evaporated to dryness, redissolved in 2.0 ml of benzene and aliquots (0.4 ml each) were withdrawn for assaying radioactivity and carrier steryl acetate mass. The elution profile is shown in Figure VIII-7. The majority of the recovered radioactivity (ca. 92.5%) eluted as a partially-split peak (fractions 112-162) with the bulk of the activity co-migrating with the Δ^7 steryl acetate standard (ca. 82%); the lesser component had the chromatographic behavior expected of cholest-8(9)-en-3β-yl acetate (cf. Lee et al., 1969). A minor peak of activity (ca. 4.4%; fractions 68-83) eluted just prior to the Δ^8(14) steryl acetate standard, while the remaining 3.1% of the recovered activity co-migrated with the cholesteryl acetate standard (fractions 165-210). The overall recovery was about 92%. Since the
FIGURE VIII-7

Alumina-Super Cel-silver nitrate column chromatography of the monoenois (fraction 5-21) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of retina homogenate incubation products derived from $[2,4^{-3}H]$-cholesta-8,14-dien-3β-ol (see Figure VIII-6). Solvent system: hexane-toluene, 9:1, arrow denotes change to hexane-toluene, 4:1. Open circles (○—○), radioactivity; closed triangles (▲—▲) C$_{27}$ steryl acetates, determined colorimetrically. The colorimetric peak (in order of elution from the column) are due to 3β-acetoxy-Δ$^8$(14)-cholestone, 3β-acetoxy-Δ$^7$-cholestone and cholesteryl acetate.

FIGURE VIII-8

Alumina-Super Cel-silver nitrate column chromatography of the monoenois (fractions 6-20) obtained by Silica Gel G-Super Cel-silver nitrate column chromatography of rat liver incubation products derived from $[2,4^{-3}H]$-cholesta-8,14-dien-3β-ol (see Figure VIII-5). Solvent system: hexane-toluene, 9:1; arrow denotes change to hexane-toluene, 4:1. Open circles (○—○), radioactivity; closed triangles (▲—▲), C$_{27}$ steryl acetates, determined colorimetrically. The colorimetric peaks (in order of elution from the column) are due to 3β-acetoxy-Δ$^8$(14)-cholestone, 3β-acetoxy-Δ$^7$-cholestone and cholesteryl acetate.
material which exhibited the chromatographic properties of cholesteryl acetate represented only 600 dpm, no further analysis of this material was performed.

The material from the liver extract which co-eluted with the mono-ene steryl acetate standard was applied to a similar chromatographic column as described above. However, analysis of the eluted fractions revealed that the column was defective (i.e. yielding a bizarre elution profile, with irregular and overlapping mass and activity peaks). Therefore, the fractions were pooled (recovery, ca. 74%) and applied to a freshly-packed alumina-Super Cel-silver nitrate column, as previously described for the retina sample (applied activity, $1.19 \times 10^6$ dpm). Fractions (4.1 ml per 40 min.) were collected, evaporated to dryness, redissolved in 2.0 ml of benzene and aliquots (0.2 ml each) were taken for measurement of radioactivity and carrier mass. The elution profile is shown in Figure VIII-8. In this case, about 93% of the recovered radioactivity co-migrated with the cholesteryl acetate standard (fractions 78-140). About 6% of the activity co-migrated with the $\Delta^7$ steryl acetate standard (fractions 47-68), while about 0.8% of the activity exhibited the chromatographic behavior characteristic of the $\Delta^8(9)$ steryl acetate. The remaining activity (ca. 0.1%) co-eluted with the $\Delta^8(14)$ steryl acetate standard (fractions 21-33). Overall recovery from this column was about 90%. Fractions 78-140 were pooled, diluted with about 50 mg of carrier cholesterol and the acetate was cleaved by saponification, as previously described. After work-up and extraction of the free sterols (see Materials and Methods), the material was purified via the dibromide, as described previously. The specific activity (dpm/mg
cholesterol) was as follows: before dibromide, 1.12 x 10⁴; after dibromide, 1.24 x 10⁴. Thus, under conditions where the rat liver homogenate converted approximately 88.4% of the initially incubated C₂₇ sterol diene to a product which had the chromatographic and chemical properties of cholesterol, the retina homogenate only metabolized about 47% of the sterol substrate to monoene sterols, of which about 76% (i.e. 35.6% of the initially incubated activity) had the chromatographic properties of cholest-7-en-3β-ol while only 3.1% (ca. 1.5% of the initially incubated activity) exhibited chromatographic properties consistent with cholest-5-ol.

This entire experiment was repeated several weeks later, in a similar fashion, to examine the reproducibility of these results. The following section presents the details of that experiment.

Experiment VIII-2

Incubations of rat liver and bovine retina S₁₀ homogenates were carried out in a similar manner as described for the above experiment, with several modifications. Livers were obtained from male Sprague-Dawley rats which had been maintained on a normal (i.e. not cholesterol-free) lab chow diet. The S₁₀ homogenates were prepared as previously described, except that no antibiotics were included in the incubation buffer. Each incubation mixture contained either liver homogenate (15.0 ml; 29.3 mg protein/ml), retina homogenate (15.0 ml; 7.1 mg protein/ml) or incubation buffer (15.0 ml; nonenzymatic control), plus 1.0 ml of a concentrated cofactor stock (providing the usual amounts of NAD, NADP, ATP and G-6-P) and 1.0 ml of either [2-³H]-lanost-8-en-3β-ol or [2,4-³H]-cholesta-8,14-dien-3β-ol (dissolved in propylene glycol).
Mixing of the homogenate and labelled substrate was effected by adding the homogenate to the vial containing the sterol solution, mixing by repeated aspiration with a Pasteur pipet for 30 seconds and then transferring the mixture to an incubation flask; the concentrated cofactor solution was used to elute residual material from the vial and pipet. The vials were then filled with scintillation cocktail (10 ml each), aspirated several times with the pipets used for mixing and transfer of the homogenate mixtures, and the residual activity was measured and subtracted from the initial activity in each vial. The following levels of activity were incubated:

<table>
<thead>
<tr>
<th>HOMOGENATE</th>
<th>SUBSTRATE</th>
<th>(dpm x 10⁻⁶)</th>
<th>(nmoles)</th>
<th>(µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>C₃₀</td>
<td>3.82</td>
<td>475</td>
<td>28.0</td>
</tr>
<tr>
<td>Retina</td>
<td>&quot;</td>
<td>3.47</td>
<td>433</td>
<td>25.5</td>
</tr>
<tr>
<td>Control</td>
<td>&quot;</td>
<td>3.59</td>
<td>447</td>
<td>26.3</td>
</tr>
<tr>
<td>Liver</td>
<td>C₂₇</td>
<td>3.42</td>
<td>228</td>
<td>13.4</td>
</tr>
<tr>
<td>Retina</td>
<td>&quot;</td>
<td>3.94</td>
<td>263</td>
<td>15.5</td>
</tr>
<tr>
<td>Control</td>
<td>&quot;</td>
<td>2.51</td>
<td>168</td>
<td>9.9</td>
</tr>
</tbody>
</table>

* [2⁻³H]-Lanost-8-en-38-ol

**[2,4⁻³H]-cholesta-8,14-dien-38-ol

The complete incubation mixtures were incubated at 37°C for 4 hours in 125 ml erlenmeyer flasks (loosely sealed with aluminum foil) with continuous gentle agitation, as described previously. Incubations were terminated by the addition of 20 ml of 15% ethanolic potassium hydroxide plus 0.1 ml of an ethanolic cholesterol solution (4 mg/ml);
saponification was carried out as usual (4 hours at 80° C). The mix-
tures were diluted with 10% NaCl (13 ml each) and extracted three times
with 100 ml portions of chloroform; the combined extracts were washed
once with water (100 ml), dried over anhydrous magnesium sulfate, fil-
tered, concentrated under reduced pressure, transferred to vials,
evaporated to dryness under nitrogen and redissolved in 10.0 ml of
benzene. Triplicate aliquots (0.10 ml each) were assayed for radio-
activity.

Chromatographic analyses of the nonsaponifiable lipids recovered
from the incubations were performed in an analogous manner to those de-
scribed previously. The details of the analyses will be presented in the
following section.

RESULTS

The results for the recovery of labelled nonsaponifiable material
from the incubations are presented in Table VIII-2. The liver extracts
exhibited nearly quantitative recovery of incubated activity, whereas
the retina and control extracts contained relatively moderate percentages
of the initially incubated activity. Again, the recovery of total ac-
tivity from the retina C_{27} sterol incubation was considerably lower than
that of the corresponding liver incubation (but essentially comparable
to the buffer control); however, considerably more activity was ex-
ttracted from this retina incubation (using chloroform) than was pre-
viously extracted (using petroleum ether) from the preceding retina
incubation with the C_{27} sterol substrate. For reasons unknown, the re-
covery of activity from the control incubation of the C_{30} sterol was
substantially less than obtained from the retina or liver incubation.
### TABLE VIII-2

Recovery of Radioactivity in Nonsaponifiable Lipids after Incubation of Rat Liver and Bovine Retina Homogenates with Labelled Sterols

<table>
<thead>
<tr>
<th>HOMOGENATE</th>
<th>STEROL SUBSTRATE</th>
<th>RECOVERED ACTIVITY (dpm x 10^-6)</th>
<th>% RECOVERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>$C_{30}$</td>
<td>3.99</td>
<td>104.5</td>
</tr>
<tr>
<td>Retina</td>
<td>&quot;</td>
<td>3.26</td>
<td>93.8</td>
</tr>
<tr>
<td>Control</td>
<td>&quot;</td>
<td>2.56</td>
<td>71.3</td>
</tr>
<tr>
<td>Liver</td>
<td>$C_{27}$</td>
<td>3.34</td>
<td>97.8</td>
</tr>
<tr>
<td>Retina</td>
<td>&quot;</td>
<td>3.02</td>
<td>76.6</td>
</tr>
<tr>
<td>Control</td>
<td>&quot;</td>
<td>2.04</td>
<td>81.1</td>
</tr>
</tbody>
</table>

Analysis of Nonsaponifiable Lipids from the $C_{30}$ Sterol Incubations

Approximately 10 mg each of carrier cholesterol and lanosterol/dihydrolanosterol was added to each nonsaponifiable lipid extract from the incubations of the $C_{30}$ sterol substrate, and each sample was evaporated to dryness under nitrogen and redissolved in 0.5 ml of toluene. Each sample was then applied to a silicic acid-Super Cel column (1:1, w/w; 100 x 1 cm, in toluene) and eluted with toluene-ether, 99:1 (applied activity: liver sample, $3.92 \times 10^6$ dpm; retina sample, $3.21 \times 10^6$ dpm; control sample, $2.52 \times 10^6$ dpm). A solvent system of higher polarity than usual was employed, since this batch of column material had been found previously to be extremely active; in addition, the ratio of silicic acid to Super-Cel was decreased (i.e. from 2:1 to 1:1) in order to achieve better flow rates. Fractions (35 minutes each; liver sample,
3.2 ml; retina sample, 3.6 ml; control sample, 3.1 ml) were collected and assayed directly for radioactivity and carrier sterol mass, as usual.

The resolution of applied sterol standards on these columns was less than satisfactory. The \( C_{30} \) and \( C_{27} \) sterol mass eluted as two relatively symmetrical and narrow, partially-overlapping peaks; no separation of dihydrolanosterol from lanosterol was achieved. The elution profile for the liver sample showed about 97% of the recovered activity co-eluting with the \( C_{30} \) sterol standard (fractions 51-61) while the remaining activity co-migrated with the cholesterol standard (fractions 62-84). Recovery of applied activity was about 92%. In contrast, the elution profiles obtained for the retina and buffer control samples both showed that essentially all of the recovered activity co-migrated with the \( C_{30} \) sterol mass peak, with minor, diffuse trailing of activity into the \( C_{27} \) sterol region. Recovery of activity was 95% and 96%, respectively.

In view of the inadequate resolution of sterol standards on these columns, the total activity recovered from each column was pooled and applied to a second silicic acid-Super Cel column (1:1, w/w; 100 x 1 cm, in toluene). The column support was activated for 7 hours at 145°C prior to use, and the columns were eluted with toluene. Fractions (40 minutes each; liver sample, 4.3 ml; retina sample, 4.9 ml; control sample, 4.7 ml) were collected and assayed for radioactivity and carrier mass as before. In all three cases, these conditions lead to superior separation of the mass standards, with partial resolution of the lanosterol and dihydrolanosterol components. The elution profile for the liver sample (applied activity, \( 3.19 \times 10^6 \) dpm) is shown in Figure
FIGURE VIII-9
Silicic acid-Super Cel column chromatography of the nonsaponifiable products derived from incubation of a rat liver S10 homogenate with [2-\textsuperscript{3}H]-lanost-8-en-3\beta-ol (solvent system: toluene). Open circles (o—o), radioactivity; closed squares (■—■), 24,25-dihydrolanosterol (first peak) and lanosterol (second peak), determined colorimetrically (absorbance at 460 nm). Inset: closed squares (■—■), cholesterol, determined colorimetrically (absorbance at 620 nm).

FIGURE VIII-10
Silicic acid-Super Cel column chromatography of the nonsaponifiable products derived from incubation of a bovine retina S10 homogenate with [2-\textsuperscript{3}H]-lanost-8-en-3\beta-ol (solvent system: toluene). Open circles (o—o), radioactivity; closed squares (■—■), 24,25-dihydrolanosterol (first peak) and lanosterol (second peak), determined colorimetrically (absorbance at 460 nm); closed triangles (▲—▲), cholesterol, determined colorimetrically (absorbance at 620 nm).

FIGURE VIII-11
Silicic acid-Super Cel column chromatography of the nonsaponifiable products derived from the buffer control incubation with [2-\textsuperscript{3}H]-lanost-8-en-3\beta-ol (solvent system: toluene). Open circles (o—o), radioactivity; closed squares (■—■), 24,25-dihydrolanosterol (first peak) and lanosterol (second peak), determined colorimetrically (absorbance at 460 nm); closed triangles (▲—▲), cholesterol, determined colorimetrically (absorbance at 620 nm).
ABSORBANCE

RADIOACTIVITY (DPM x 10^4)
ABSORBANCE

RADIOACTIVITY (DPM x 10^4)

FRACTION NUMBER
ABSORBANCE

RADIOACTIVITY (DPM x 10^4)

FRACTION NUMBER
VIII-9. Greater than 96% of the activity co-migrated with the dihydro-
lanosterol peak, while about 3% of the activity co-migrated with the 
cholesterol standard. Less than 1% of the recovered activity trailed 
from the C_{30} sterol peak, in the region of C_{28} and C_{29} sterols. Re-
covery of applied activity from this column was 97%. As observed 
previously, the elution profiles for the retina sample (Figure VIII-10; 
applied activity, 2.59 \times 10^6 \text{ dpm}) and the control sample (Figure VIII-11; 
applied activity, 1.81 \times 10^6 \text{ dpm}) both showed greater than 99% of the 
recovered activity co-eluting with the dihydrolanosterol peak; the re-
mainder of the activity trailed diffusely from the C_{30} sterol region, 
with no detectable activity migrating with the cholesterol standard. 
Recovery from these columns was also essentially quantitative (ca. 100% 
and 97% respectively).

The results obtained for this set of incubations were consistent 
with those previously obtained for the incubation of the C_{30} sterol sub-
strate. The retina preparation failed to convert the substrate to other 
monohydroxy sterol products, the chromatographic results being essentially 
indistinguishable from the buffer control results. However, even the 
liver preparation (i.e. the enzymatic control) only converted about 3% 
of the initially incubated material to C_{27} monohydroxy sterols, whereas 
the previous incubation showed a conversion of about 7% of the incubated 
activity to C_{27} monohydroxy sterols (of which about 84% was cholesterol). 
It should be noted, however, that the retina homogenate contained only 
about 24% as much total protein as the liver homogenate in these incuba-
tions.
Analysis of Nonsaponifiable Lipids from the C\textsubscript{27} Sterol Incubations

The nonsaponifiable lipids from the incubations of the C\textsubscript{27} sterol substrate were supplemented with 10 mg of carrier cholesterol and then acetylated, worked up and the steryl acetates isolated exactly as described previously (see Materials and General Methods). The acetylated samples (in 0.5 ml of hexane each) were supplemented with 5 mg each of carrier \Delta\textsuperscript{5,7} and \Delta\textsuperscript{8,14} C\textsubscript{27} steryl acetates and applied to Silica Gel G-Super Cel-silver nitrate columns (50 x 1 cm, in hexane-toluene, 7:3). The columns were eluted with hexane-toluene, 7:3, up to fraction 38, and then with hexane-toluene, 1:1, fraction 180. Fractions (40 minutes each; liver sample, 4.85 ml; retina sample, 4.4 ml; control sample, 2.4 ml each) were collected as usual, evaporated to dryness under nitrogen, redissolved in 2.0 ml of benzene and aliquots (0.2 ml each) were assayed for radioactivity and carrier steryl acetate mass as previously described.

The elution profile for the liver sample (Figure VIII-12; applied activity, 3.26 x 10\textsuperscript{6} dpm) showed that only about 23% of the recovered activity co-migrated with the cholesteryl acetate (i.e. monoene) standard (fractions 15-30); this peak was preceded by a very minor (ca. 0.5%) radioactivity peak (fractions 12-14). The majority of the recovered radioactivity (ca. 65%) co-eluted with cholesta-8,14-dien-3\textsubscript{8}-yl acetate (i.e. apparently unchanged substrate). This peak was preceded (just after the solvent change) by a minor peak of activity (ca. 4.9%); the remainder of the recovered radioactivity trailed irregularly after the major peak, extending into the region of the \Delta\textsuperscript{5,7} steryl acetate standard (fractions 114-170). Recovery of the applied activity was
FIGURE VIII-12
Silica Gel G-Super Cel-silver nitrate column chromatography of the acetylated derivatives of the nonsaponifiable products derived from incubation of a rat liver S₁₀ homogenate with [2,4⁻³H]-cholesta-8,14-dien-3β-ol. Solvent system: hexane-toluene, 7:3; arrow denotes change to hexane-toluene, 1:1. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), C₂₇ steryl acetates, determined colorimetrically. The colorimetric peaks (in order of elution from the column) are due to cholesteryl acetate, 3β-acetoxy-Δ⁸,¹⁴-cholestadiene and 3β-acetoxy-Δ⁵,⁷-cholestadiene.

FIGURE VIII-13
Silica Gel G-Super Cel-silver nitrate column chromatography of the acetylated derivatives of the nonsaponifiable products derived from incubation of a bovine retina S₁₀ homogenate with [2,4⁻³H]-cholesta-8,14-dien-3β-ol. Solvent system: hexane-toluene, 7:3; arrow denotes change to hexane-toluene, 1:1. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), C₂₇ steryl acetates, determined colorimetrically. The colorimetric peaks (in order of elution from the column) are due to cholesteryl acetate, 3β-acetoxy-Δ⁸,¹⁴-cholestadiene and 3β-acetoxy-Δ⁵,⁷-cholestadiene.
ABSORBANCE (620 nm)
ABSORBANCE (620 nm)

FRACTION NUMBER

RADIOACTIVITY (DPM x 10^-4)
about 81%. In contrast, the radiochromatogram of the retina sample (Figure VIII-13; applied activity, 2.00 x 10^6 dpm) was notably devoid of activity coincident with the cholesteryl acetate standard; however, a very small split peak of activity (ca. 1.5%) immediately preceded the monoene steryl acetate mass peak (fractions 13-21). Approximately 89% of the recovered radioactivity co-eluted with the carrier Δ^8,14 steryl acetate (fractions 60-90), while about 6.3% of the activity eluted as a sharp peak just after the solvent change (fractions 45-58) but just before the major peak. The remaining radioactivity trailed irregularly after the major peak, with negligible activity in the region of the Δ^5,7 standard. The overall recovery of radioactivity from this column was only about 75%.

It was evident from the elution profile of the control sample that the column was defective; the fractions were therefore pooled and re-applied to another Silica Gel G-Super Cel-silver nitrate column, eluting with hexane-toluene, 7:3, up to fraction 43 and then with hexane-toluene, 1:1, to fraction 180 (applied activity, 9.46 x 10^5 dpm). Fractions (4.2 ml per 30 minutes each) were collected and assayed for radioactivity and carrier mass as before. The elution profile (Figure VIII-14) proved to be almost identical to that obtained for the retina sample. About 90% of the recovered activity co-migrated as unconverted substrate (fractions 51-70); this peak was immediately preceded by a smaller peak (ca. 7.2% of the activity), which eluted immediately after the solvent change. A very minor peak (ca. 0.5%) immediately preceded the cholesteryl acetate mass peak, but no activity was coincident with the monoene standard (fractions 15-23). The remaining activity trailed irregularly after
FIGURE VIII-14

Silica Gel G-Super Cel-silver nitrate column chromatography of the acetylated derivatives of the nonsaponifiable products derived from incubation of the buffer control with [2,4-3H]-cholesta-8,14-dien-3β-ol. Solvent system: hexane-toluene, 7:3; arrow denotes change to hexane-toluene, 1:1. Open circles (o — o), radioactivity; closed triangles (▲ — ▲), C$_{27}$ steryl acetates, determined colorimetrically. The colorimetric peaks (in order of elution from the column) are due to cholesteryl acetate, 3β-acetoxy-Δ$_{8,14}$-cholestadiene and 3β-acetoxy-Δ$_{5,7}$-cholestadiene.
the major peak. Overall recovery was about 88%.

The fractions from the liver sample which co-migrated with the monoene standard (fractions 13-36; 5.09 x 10^5 dpm) were applied with the usual complement of monoene steryl acetate standards to an alumina-Super Cel-silver nitrate column and eluted with hexane-toluene, 9:1 (40 minute fractions, 5.8 ml each). The severe irregularity and broadness of the peaks, as well as the gross overlapping of mass standards and extensive trailing of radioactivity revealed that this column was defective. The only "normal" peak was that of the Δ^8(14) steryl acetate standard (fractions 33-63), which was associated with a small, somewhat skewed peak of radioactivity (ca. 1.2%; fractions 30-60). Fractions 61-190, corresponding to the remainder of the radioactivity and carrier mass, were pooled (ca. 3.53 x 10^5 dpm) and applied with additional carrier Δ^7 and Δ^5 steryl acetate standards (5 mg each) to an alumina-silver nitrate medium-pressure liquid chromatography column. This chromatographic system has been described in detail recently (Pascal et al., 1979).

The column (1000 x 9 mm; packed with a lyophilized mixture of neutral alumina and silver nitrate, 5:1, w/w) was pre-equilibrated with hexane-toluene, 91:9, and eluted with this same solvent system (flow rate, 2.5 ml/min.; fraction volume, 7.0 ml; injection volume, 4.5 ml; void volume, 30 ml). Aliquots were withdrawn directly from the fractions for assay of radioactivity and colorimetric determination of steryl acetates.

The great advantage of this chromatographic system was that it only required 6.5 hours to collect the necessary number of fractions for the analysis, and the recovery of applied radioactivity was nearly quantitative (> 99%). The elution profile is shown in Figure VIII-15. About
FIGURE VIII-15

Alumina-silver nitrate medium pressure liquid chromatography (MPLC) of the monoene steryl acetates obtained from the rat liver homogenate incubation with [2,4-\textsuperscript{3}H]-cholesta-8,14-dien-3\beta-ol. Solvent system: hexane-toluene, 91:9. This material corresponds to fractions 13-36 from a previous Silica Gel G-Super Cel-silver nitrate column (see Figure VIII-12). Open circles (o——o), radioactivity; closed triangles (▲——▲) 3\beta-acetoxy-\textsuperscript{7}cholestan (first peak) and cholesteryl acetate (second peak), determined colorimetrically.
91% of the activity co-migrated with the cholesteryl acetate standard (fractions 61-120). This peak was immediately preceded by the Δ⁷ sterol acetate peak (fractions 35-60), which was attended by a peak of radioactivity (8.5%; fractions 30-60); the lack of exact correspondence between the radioactivity and mass profiles of the peaks suggested the presence of at least one additional radioactive component (most likely the Δ⁸(⁹) isomer). A very minor peak of radioactivity (ca. 0.4%; fractions 11-20) preceded the other two peaks (probably residual Δ⁸(¹⁴) sterol acetate from the previous column).

The fractions corresponding to the cholesteryl acetate peak were pooled, concentrated under reduced pressure, transferred to a vial and evaporated to dryness under nitrogen. Approximately 55 mg of carrier cholesterol was added and the sample was redissolved in 10 ml of anhydrous ether. Cleavage of the acetate was effected by the addition of 15 mg of lithium aluminum hydride; the reaction mixture was allowed to stand at room temperature for 2 hours. Excess hydride reagent was then destroyed by cautious addition of ice, followed by 5 ml of water and then 1 ml of 6 N HCl. The mixture was vortexed and the ether layer was withdrawn; the aqueous phase was extracted twice with 15 ml portions of ether and the combined organic extracts were evaporated under nitrogen (residual water was removed by forming an azeotrope with benzene-methanol, 7:3). The free sterol was redissolved in 10.0 ml of benzene and assayed for radioactivity (0.10 and 0.20 ml aliquots, in duplicate) and cholesterol content (0.02 and 0.04 ml aliquots, in duplicate), as usual. The remaining material was processed via the dibromide, as previously described, and the resulting product was recrystallized once from acetone,
radissolved in 10.0 ml of benzene and assayed for radioactivity (0.20 and 0.50 ml aliquots, in duplicate) and cholesterol content (0.10 and 0.20 ml aliquots, in duplicate) as before. The specific activity (dpm per mg of cholesterol) was as follows: before dibromide, $4.44 \times 10^3$; after dibromide, $4.71 \times 10^3$. These results confirmed that approximately 90% of the activity which was converted to monoene sterols by the liver homogenate (upon incubation with the C$_{27}$ diene substrate) was due to cholesterol, even though the total conversion of the diene substrate to monoene sterols was only about 25%.

Incubation of the C$_{27}$ diene sterol substrate with all three systems (liver homogenate, retina homogenate and the buffer control) lead to the formation of a product which exhibited a chromatographic mobility intermediate between that of the monoene and diene steryl acetate standards on columns of Silica Gel G-Super Cel-silver nitrate. This component represented about 5-7% of the total recovered activity from these columns. A component of similar chromatographic behavior had been observed previously in incubations of the C$_{27}$ sterol substrate with the liver and retina homogenates. In an attempt to investigate the nature of this component further, fractions 45-58 from the retina chromatographic column (see Figure VIII-13) were pooled, evaporated to dryness under nitrogen and redissolved in hexane (10-15 μl). An aliquot (4 μl) was analyzed by radio-GLC (3% OV-17; column temperature, 260° C; carrier flow rate, 66 ml/min.), along with co-injected carrier standards of cholesta-7,14-dien-3β-yl and cholesta-7,9(11)-dien-3β-yl acetate. Mass tracings of the injected steryl acetate standards were recorded before and after injection of the labelled material; effluent gas from the column was
FIGURE VIII-16

Radio-GLC of fractions 45-58 from the Silica Gel G-Super Cel-silver nitrate column chromatography of the bovine retina homogenate incubation products derived from [2,4-^3H]-cholesta-8,14-dien-3β-ol (see Figure VII-13). Chromatographic system: 3% OV-17, 260°C isothermal; carrier flow rate, 66 ml/min. The first mass peak is due to 3β-acetoxy-Δ^7,14-cholestadiene; the second mass peak is due to 3β-acetoxy-Δ^7,9(11)-cholestadiene.
DETECTOR RESPONSE

RETENTION TIME (min)

RADIOACTIVITY (DPM x 10⁻²)
collected and assayed for radioactivity as previously described. The radiochromatogram is shown in Figure VIII-16. Under the conditions employed, distinct (but not baseline) resolution of the chromatographic standards was achieved, with approximately 92% of the recovered activity co-migrating with the $\Delta^7,9^{(11)}$ steryl acetate standard. A minor radioactive component (ca. 6%) preceded the chromatographic standards. The recovery of applied activity was only about 47%; this was most likely due to autoxidation of the labile diene during handling, due to the fact that the fractions pooled from the column contained negligible mass.

The radio-GLC analysis was performed on the corresponding sample from the buffer control (fractions 43-51, see Figure VIII-14), under identical chromatographic conditions. Approximately 91% of the radioactivity corresponded to the $\Delta^7,9^{(11)}$ standard, while about 6% of the activity eluted just prior to the $\Delta^7,14^{(14)}$ mass peak. Again, the recovery of activity was only about 45%. The above results suggest that the minor component which exhibited chromatographic mobility intermediate to the monoene and diene steryl acetate standards on Silica Gel G-Super Cel-silver nitrate columns and which had the chromatographic properties of cholesta-7,9(11)-dien-38-yl acetate on a 3% OV-17 GLC column was derived nonenzymatically from the incubated [2,4-$^3$H]-cholesta-8,14-dien-38-ol. Such a component was not evident in the chemically-synthesized sterol substrate prior to incubation (see Chemical Synthesis, Chapter III).

It is assumed, by analogy, that the material contained in fractions 44-55 derived from the liver sample (see Figure VIII-13) was also largely composed of material having the chromatographic properties of cholesta-7,9(11)-dien-38-yl acetate. However, the mechanism by which such a
product would be formed from the starting substrate is not readily apparent and the identity of this component remains unconfirmed.

**DISCUSSION**

A wide variety of 3β-hydroxy sterols have been shown to be convertible to cholesterol upon incubation with rat liver homogenate preparations, and this criterion has been accepted as preliminary evidence that such sterols are potential intermediates of cholesterol biosynthesis **in vivo** (cf. Schroeper et al., 1972). Pertinent to this study, it has been demonstrated previously that 24,25-dihydrolanosterol (Avignan et al., 1963; Kandutsch and Russell, 1960) and cholesta-8,14-dien-3β-ol (Akhtar et al., 1969; Lutsky and Schroeper, 1968, 1970) are convertible to cholesterol in the rat liver homogenate system. Therefore, using the rat liver S₁₀ preparation as a control, I have investigated the convertibility of [2-³H]-lanost-8-en-3β-ol and [2,4-³H]-cholesta-8,14-dien-3β-ol to cholesterol by bovine retina S₁₀ preparations.

Comparing the results of two different sets of incubations with the above mentioned labelled sterols as substrates, it was noted that marked variability existed in the convertibility of these substrates to other sterols. In the first trial utilizing the C₃₀ sterol substrate, about 7% of the activity recovered from the incubation with the rat liver homogenate exhibited the chromatographic properties of C₂₇ monohydroxy sterols; the majority (ca. 83%) of the remaining activity was due to unconverted substrate, while about 10% behaved chromatographically like C₂₈ and/or C₂₉ monohydroxy sterols. Essentially all of the C₂₇ monohydroxy sterol products were monoenes; approximately 84% was due to cholesterol, while smaller amounts of products having the chromatographic
behavior consistent with cholest-7-en-3β-ol, cholest-8(9)-en-3β-ol and cholesterol were observed. In the second such incubation (which contained about 56% as much total protein as the previous liver homogenate), only about 3% of the incubated substrate was converted to $C_{27}$ monohydroxy sterols; about 96% remained unconverted while less than 1% of the activity chromatographed with the expected behavior of $C_{28}$ and/or $C_{29}$ monohydroxy sterols. In contrast, both incubations of the retina homogenate preparation with the $C_{30}$ sterol substrate failed to exhibit detectable conversion to products other than the initially incubated substrate; essentially identical results were obtained with the buffer control incubation. Incubations utilizing the $C_{27}$ sterol substrate were even more variable. In the first such incubation with the rat liver homogenate, about 95% of the recovered activity was composed of monoene sterols while only 4% of the activity was due to unconverted substrate; about 1% of the recovered activity exhibited "anomalous" chromatographic behavior (i.e. intermediate between that of monoene and diene sterol standards). The monoenes were composed largely of cholesterol (ca. 93%) along with minor components having the chromatographic behavior of cholest-7-en-3β-ol, cholest-8(9)-en-3β-ol and cholest-8(14)-en-3β-ol. However, in the second incubation of the rat liver homogenate with the labelled $Δ^8,14$ sterol, only 25% was converted to monoene sterols; nearly 65% of the activity remained unmetabolized while about 5% exhibited the "anomalous" chromatographic behavior previously mentioned. Even though the conversion to monoene was relatively minor, about 90% of the monoene material was composed of cholesterol, while the remainder was represented by components having the chromatographic properties of cholest-7-en-
38-ol, cholest-8(9)-en-3β-ol and cholest-8(14)-en-3β-ol. The initial incubation of the retina homogenate with the C_{27} sterol diene exhibited about 47% conversion to monoene sterols; about 49% of the recovered activity chromatographed as unmetabolized substrate while about 4% exhibited the "anomalous" behavior previously described. Unlike the liver system, however, the gross majority of the monoene material was due to components having the chromatographic properties of cholest-7-en-3β-ol and cholest-8(9)-en-3β-ol (ca. 92.3%, of which about 82% corresponded to the Δ^7 isomer); only about 3% of the monoene material exhibited the chromatographic behavior of cholesterol while another component having the chromatographic properties of cholestanol was observed (ca. 4.4% of the activity). In the second incubation of this type with the retina homogenate, the metabolism of the diene substrate to monoene sterols was less than 1%, while at least 6% of the recovered activity exhibited the "anomalous" chromatographic behavior observed previously in other samples; the results obtained with the nonenzymatic (buffer) control were very similar.

The components from the retina and buffer control incubations which exhibited "anomalous" chromatographic behavior on columns of Silica Gel G-Super Cel-silver nitrate were analyzed by radio-GLC on 3% OV-17 and found to have chromatographic properties consistent with cholesta-7,9(11)-dien-3β-ol (ca. 94%); at least one other compound (identity unknown) was detected as a constituent of the "anomalous" component. The occurrence of this component in the buffer control incubation indicated that the component was not enzymatically formed from the diene sterol substrate.
The reasons for the marked variability of substrate conversion in the incubations described above are not fully understood. One likely factor, with regard to differences between the liver and retina systems, is protein concentration; the liver homogenates contained between three and four times as much protein as the corresponding retina homogenates. Intrinsic tissue differences precluded the preparation of retina homogenates with the same protein content as the liver homogenates. It is even more likely that intrinsic differences which distinguish retinal tissue from liver resulted in the considerable differences in conversion of the sterol substrates, i.e., the biosynthesis of sterols is a known (and perhaps major) function of liver, whereas the retina may possess only marginal capabilities of this kind. Again, the homogenization and incubation conditions used for the liver preparations may not be suitable for retinal sterol biosynthesis in vitro.

Even more puzzling is the marked variability of metabolism of a given substrate with a given tissue homogenate. For instance, the liver homogenate was supposed to serve as an "enzyme control", metabolizing the known intermediates to cholesterol. However, only marginal conversion of the C\textsubscript{30} sterol substrate to C\textsubscript{27} monohydroxy sterols was observed in the liver homogenate incubations (using about 781 mg and 440 mg of total protein, respectively, and 479 nmoles and 475 nmoles of sterol substrate, respectively, in the first and second incubations). In contrast, Kandutsch and Russell (1960) reported 18.4% conversion of \textsuperscript{14}C-labelled 24,25-dihydrolanosterol to cholesterol, using about 15-fold greater sterol substrate and about 3-fold more rat liver homogenate, under similar in vitro conditions (also, about 45% of the sterol was
unmetabolized). Lutsky and Schroepfer (1971) observed about 75% conversion of \([3\alpha-^3H]\)-cholesta-8,14-dien-3β-ol to cholesterol in aerobic incubations containing 30 ml of rat liver S10 homogenate and 140 μg (ca. 365 nmol) of sterol substrate (i.e. about twice as much homogenate as used in the incubations described above, but just less than twice the amount of C27 sterol substrate), in contrast with the results presented above for incubations of a similar substrate with the liver homogenate. It should be noted that the current results and those presented by other researchers may not be compared directly, since differences did exist in the exact constituents of the incubation mixtures and the method of introduction of the substrates into aqueous solution. These incubations contained 5 mM ATP, a component which is not required for the further metabolism of the sterol precursors employed in these incubations and which is not a routine component of the incubation mixtures for such experiments; it was included in these incubations in order to be consistent with the composition of the incubation mixtures previously used for studies of the conversion of de novo substrates to sterols by the in vitro systems. The presence of ATP may have affected the sterol metabolism in these preparations. Also, propylene glycol was present at the level of about 6%, by volume, of the total incubation mixture in these experiments in order to solubilize the sterol substrates employed. The levels of propylene glycol used by Kandutsch and Russell (1960) (i.e. 2.5%, by volume) and Lutsky and Schroepfer (1970) (i.e. 0.33%, by volume) were much lower than used here. It should be noted, however, that 5% propylene glycol has been found to effect a slight increase in the overall conversion of acetate and mevalonic acid to digitonin-
precipitable material by rat liver homogenates (Raulston, 1979). In the incubations described here, the substrates and homogenates were mixed either by low-speed vortexing or by aspiration with a Pasteur pipet; it is not known whether or not this form of mechanical agitation adversely effects the metabolism of the given substrates by the in vitro systems. It should be realized, however, that the shear forces exerted upon the homogenates during the initial homogenization of the tissues were considerably greater than those created by either low-speed vortexing or aspiration by pipet. Oxygen was probably not limiting in these incubations, since even air-equilibrated buffer contains oxygen at the level of 250 μM. Molecular oxygen is required for the formation of 7-dehydrocholesterol from cholest-7-en-3β-ol, apparently a penultimate reaction in the formation of cholesterol (Dempsey et al., 1964; Frantz et al., 1959; Schroepfer and Frantz, 1961). Although the rat liver homogenate successfully converted substantial amounts of the labelled cholesta-8,14-dien-3β-ol to cholesterol, the accumulation of monoene sterols, the absence of Δ5,7 sterols and the paucity of material having the chromatographic properties of cholesterol in the retina homogenate incubations (using the diene sterol substrate) was strikingly similar to what one would expect for an anaerobic incubation (cf. Lutsky and Schroepfer, 1970).

It was noted that, in addition to the lack of metabolism of the C30 sterol substrate by the retina homogenates and the very minimal conversion of this substrate to C27 monohydroxy sterols by the rat liver homogenates, neither the retina homogenates nor the liver homogenates appeared to convert the labelled dihydrolanosterol to lanosterol, i.e.
reduction of the Δ²⁴ double bond was not reversible. The lack of reversibility of the reduction of the side-chain double bond has been previously reported for rat liver homogenates (Avigan et al., 1969; Kandutsch and Russell, 1960) and the regulation of this reduction reaction is apparently very critical in neural cholesterol biosynthesis (cf. Davison, 1970; Kabara, 1967, 1973; Ramsey and Nicholas, 1972; Paoletti et al., 1969).

The results of these experiments suggest that the liver and retina systems in vitro are quite different with respect to their proficiency in metabolizing the given sterol substrates to cholesterol. It must be emphasized, however, that the results were obtained from a very limited number of experiments; this fact, in addition to the numerous factors mentioned above which may have affected the in vitro systems and the limitations inherent to in vitro systems in general, precludes any conclusions regarding the absolute biochemical capacities of the tissues with respect to sterol biosynthesis. The results obtained so far suggest that bovine retinas have the capability to synthesize cholesterol (as well as other sterols and isoprenoids) although the expression of this capability in vitro is very variable and, in the case of sterol biosynthesis, extremely limited. Is the utilization of mevalonic acid by retinas in vitro an accurate reflection of the in vivo processes, i.e. can the retina synthesize cholesterol, in vivo, from mevalonic acid? This question will be addressed in the following chapter.
IX. IN VIVO UPTAKE AND UTILIZATION OF
MEVALONIC ACID BY
BOVINE OCULAR TISSUES

INTRODUCTION

The results of previous experiments have demonstrated that bovine retina sterol biosynthetic capabilities, in vitro, are extremely limited. Although the results of previous studies with intact retinas and retina homogenates demonstrated the uptake and utilization of mevalonic acid, the gross majority of substrate was converted to alternate (i.e. non-sterol) isoprenoid products. It is not clear whether this represents a physiologically significant property of the retina or whether the treatment of the tissue and the in vitro conditions were simply not optimal for sterol biosynthesis in this tissue. For instance, it has been found that the conditions for the preparation of rat liver S_{10} homogenates are very critical for optimal sterol biosynthetic activity (cf. Raulston, 1979), but the "optimal" conditions for the rat liver homogenate system may not be optimal for retina sterol biosynthesis. It is possible that the enzymes for sterol biosynthesis in the retina are very labile and cannot withstand the homogenate preparative procedures and/or the in vitro incubation conditions. Furthermore, neural tissue in general is known to be exquisitely sensitive to anoxia and ischemia, more so than any other tissue. Thus, the time between enucleation of the eyes and initiation of the incubations may be a terminally stressful period for the retina (i.e. the in vitro system is a "dying" system, and may not reflect accurately the biochemical competence of a "living" system). Indeed, the question of the "physiological relevance" of in vitro
systems has long been a proverbial "thorn in the side" of biochemists; the results of in vitro experiments must be confirmed in vivo in order to evaluate the "physiological relevance" of the results.

Clearly, an in vivo experiment involving a cow presents a technically challenging problem (not to mention the expense and novelty of such a "laboratory experiment"). I report here the first such study on bovine ocular lipid metabolism, using 3RS-[2-14C]-mevalonic acid as the de novo substrate.

MATERIALS AND METHODS

Procedures for the saponification and extraction of lipids, thin-layer chromatography, column chromatography, gas-liquid column chromatography and colorimetric assay of sterols have been described previously (see Materials and General Methods). Carbon-14 counting efficiency was 95.0 ± 0.5%.

3RS-[2-14C]-Mevalonic acid lactone (Amersham; ca. 250 μCi, 10.9 Ci/mol, in 1.0 ml of benzene) was evaporated to dryness under nitrogen at room temperature and redissolved in 0.50 ml of sterile phosphate-buffered saline (0.10 M potassium phosphate, pH 7.4, containing 0.9% NaCl) by sonication for one minute; the solution was stored overnight at 4° C.

Surgical Procedures and Administration of Isotopic Substrate

All surgical procedures were performed at the Baylor College of Medicine Department of Animal Surgery, under the aid and direction of Dr. Louise Cope Moorhead. A female calf, age 1-2 years (weight 255 lbs.), previously in generally healthy condition, was placed under endotracheal general anesthesia (i.e. 2% halothane in nitrous oxide-oxygen, 35:40).
A lateral canthotomy was performed for maximal exposure of the globe; a limbal pyridomy (360°) was performed and the four rectus muscles were isolated, tagged with sutures for traction, and the globe was proptosed. Pupillary dilation was effected by instilling Tropicamide (1%) and phenylephrine hydrochloride (10%) into each eye.

A binocular indirect ophthalmoscope was employed for visualization of the retina. The isotopic substrate solution (0.12 ml/eye; ca. 120 μCi, 2.64 x 10^8 dpm) was injected intravitreally through the sclera overlying the peripheral retina, with a 1.0 cc #25 tuberculin syringe. The needle tip was placed within one mm of the retinal surface (by visual estimation), one disc diameter inferior to the disc (along the inferior vein). During the injection, vitreal turbulence was easily observed. The needle was allowed to sit in place for about 30 seconds after the injection and was then withdrawn slowly from the eye; no leakage of vitreous around the injection locus was observed. One hour later, the contralateral eye was injected with substrate. After an additional hour, both eyes were enucleated and stored on ice. The animal was terminated after enucleation by pentobarbital overdose (i.v.).

Tissue Dissection and Fractionation

Each eye was dissected (within 1 hour after enucleation) into individual components for further study. The following tissues were dissected (numbers refer to the hours of incubation time): lens (L-1, L-2), vitreous humor (V-1, V-2), retina (R-1, R-2), cornea (C-1, C-2), and the remaining eye-cup (E-1, E-2). Tissues other than retinas were transferred to individual tubes filled with liquid nitrogen and stored at -76° C.
Each retina was transferred to a 35 ml capacity ultracentrifuge tube containing 5 "carrier" bovine retinas and homogenized in 10 ml of ice-chilled 42% (w/v) sucrose with a tight-fitting, motor-driven teflon pestle (15 passes, 2 minutes at 1000 rpm). The homogenates were then overlayered with 5 ml portions of decreasingly-dense sucrose solutions (i.e. 1.15, 1.13 and 1.11 g/ml; see Materials and General Methods) and the tubes were centrifuged for 90 minutes at 25,000 rpm (Beckman SW 27 rotor; L5-50 ultra-centrifuge; 0-4°C). Since the majority of ROS material from light-adapted retinas bands at the 1.13/1.15 g/ml interface (cf. Papermaster and Dreyer, 1974), the purity of the ROS material was sacrificed and the yield maximized by pooling the material from both the 1.11/1.13 and 1.13/1.15 g/ml interfaces; this material (designated ROS-1 and ROS-2, respectively) represents a crude, ROS-enriched fraction of the retina and should not be confused with the highly-purified ROS material described previously (see Chapter IV). The crude ROS material was diluted to about 40 ml with ice-cold phosphate-buffered saline and harvested by centrifugation (1 hour at 25,000 rpm, Beckman SW 27 rotor); the resulting pellets were washed twice more in the same fashion with 40 ml portions of buffer.

The remaining material from each sucrose gradient (representing the so-called "rest of retina" fraction) was diluted to about 40 ml with phosphate-buffered saline, centrifuged (2 hours at 25,000 rpm; Beckman SW 27 rotor) and the resulting pellets were washed twice more with buffer as described above. The resulting pelleted material was designated RR-1 and RR-2 respectively.
Saponification and Work-Up of Samples

All samples were saponified for 4 hours at 75-80°C, under nitrogen atmosphere, in 15% ethanolic potassium hydroxide (10 ml each for samples L-1, L-2, C-1, ROS-1, ROS-2, RR-1 and RR-2; 50 ml each for V-1 and V-2; 100 ml each for E-1 and E-2). After saponification, the samples were allowed to cool to room temperature; each sample was diluted with one volume of water, after the addition of carrier cholesterol (1 ml of an ethanolic solution, 4 mg/ml), and was then extracted three times with two volumes of petroleum ether (2.5 volume portions were used for samples E-1 and E-2). Combined organic extracts were washed once with water (20 ml each for samples L-1, L-2, C-1, C-2, ROS-1, ROS-2, RR-1 and RR-2; 100 ml each for samples V-1, V-2, E-1 and E-2), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The concentrated extracts were transferred to vials, evaporated to dryness under nitrogen and redissolved in 5.0 ml of benzene each. Aliquots were assayed in triplicate for radioactivity.

The chromatographic analyses of the nonsaponifiable lipids from the various ocular tissue samples will be discussed in the following sections.

RESULTS

The recovery of radioactivity in the nonsaponifiable lipid extracts of the various ocular tissues is tabulated in Table IX-1.

Recovery of radioactivity in the nonsaponifiable lipids of the total ocular tissue processed from the one-hour (ca. 4.77 x 10^5 dpm) and two-hour (ca. 1.07 x 10^6 dpm) incubations represented 0.18% and 0.41%, res-
TABLE IX-1

Incorporation of RS-[2-\textsuperscript{14}C]-Mevalonic Acid into the Nonsaponifiable Lipids of Bovine Ocular Tissues \textit{In Vivo}

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>RECOVERED ACTIVITY (dpm x 10\textsuperscript{-4})</th>
<th>% TOTAL RECOVERED ACTIVITY</th>
<th>% INJECTED DOSES</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-1, L-2</td>
<td>3.60, 7.75</td>
<td>7.55, 7.24</td>
<td>0.014, 0.029</td>
</tr>
<tr>
<td>C-1, C-2</td>
<td>6.71, 5.75</td>
<td>14.06, 5.37</td>
<td>0.025, 0.022</td>
</tr>
<tr>
<td>V-1, V-2</td>
<td>1.26, 1.25</td>
<td>2.64, 1.17</td>
<td>0.005, 0.005</td>
</tr>
<tr>
<td>E-1, E-2</td>
<td>16.4, 20.0</td>
<td>34.32, 18.72</td>
<td>0.062, 0.076</td>
</tr>
<tr>
<td>RR-1, RR-2</td>
<td>16.4, 60.6</td>
<td>34.37, 56.61</td>
<td>0.062, 0.230</td>
</tr>
<tr>
<td>ROS-1, ROS-2</td>
<td>3.37, 11.7</td>
<td>7.06, 10.89</td>
<td>0.013, 0.044</td>
</tr>
</tbody>
</table>

pectively, of the injected radioactivity. Since the aqueous-soluble fractions of the tissues were not assayed after removal of the nonsaponifiable lipids, it is not known whether the remainder of the radioactivity was simply not taken up and utilized or whether the substrate was predominantly converted to acidic products. Incorporation of radioactivity into retina (ROS and RR fractions) nonsaponifiable lipids represented about 41\% and 67\%, respectively, of the one-hour and two-hour incorporation into total ocular nonsaponifiable lipids. A surprising result was that substantial radioactivity was incorporated into both lens and cornea NSL material; these tissues were quite distal to the locus of the injection of substrate. Since the vitreous is about 99\% water and contains an extremely sparse population of hyalocytes, and the amount of
total radioactivity recovered from the V-1 and V-2 fractions was nearly identical, it is most likely that the NSL activity recovered from vitreous samples represented contamination by other eyecup material (i.e. most probably from the pigmented epithelium) and not endogenous biosynthesis.

**Chromatographic Analyses of Retina Nonsaponifiable Lipids**

The retina samples from the one-hour incubation (ROS-1, 2.82 x 10^4 dpm; RR-1, 1.47 x 10^5 dpm) were each supplemented with 10 mg of additional carrier cholesterol and applied (in 0.5 ml of benzene) to silicic acid-Super Cel columns (100 x 1 cm, in benzene). The samples were eluted with benzene for the first 128 fractions; the solvent was then changed to benzene-ether, 9:1, and 70 fractions more were collected. Fractions (3.2-3.4 ml per 50 minutes) were collected automatically, evaporated to dryness under nitrogen, redissolved in 2.0 ml of benzene and aliquots (0.2 ml each) were removed for assay of radioactivity and for colorimetric determination of cholesterol.

The elution profile for sample RR-1 is shown in Figure IX-1. About 75% of the recovered activity eluted with the solvent front (fractions 13-18). Approximately 14% of the radioactivity exhibited the behavior characteristic of C30 sterols (fractions 39-50), while only minor activity (ca. 4%) eluted in the C27 sterol region (fractions 64-83). The activity which eluted before the solvent change represented about 99.5% of the total recovered radioactivity; the overall recovery was 88%.

The radiochromatogram obtained for sample ROS-1 (Figure IX-2) was quite similar. The major component again eluted with the solvent front (fractions 13-18; ca. 56%), with appreciable activity also eluting in
FIGURE IX-1

Silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from the "rest of retina" fraction of a bovine retina one hour after intraocular injection of [2-\textsuperscript{14}C]-mevalonic acid. Solvent system: benzene; arrow denotes change to benzene-ether, 9:1. Open circles (o—o), radioactivity; closed squares (■—■), cholesterol, determined colorimetrically.

FIGURE IX-2

Silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from the crude ROS fraction of a bovine retina one hour after intraocular injection of [2-\textsuperscript{14}C]-mevalonic acid. Solvent system: benzene; arrow denotes solvent change to benzene-ether, 9:1. Open circles (o—o), radioactivity; closed squares (■—■), cholesterol, determined colorimetrically.
ABSORBANCE (620 nm) vs. FRACTION NUMBER

Radioactivity (DPM x 10^4)
the region expected for \( C_{30} \) sterols (fractions 38-50; ca. 13%). Approximately equal amounts of activity (ca. 5-7% each) eluted just before, just after and almost coincident with the cholesterol standard (fractions 63-80). As observed previously for sample RR-1, the peak fraction of radioactivity which eluted with the cholesterol standard (fraction 63) did not correspond to the peak fraction for the cholesterol mass (fraction 65), determined colorimetrically. Again, about 97% of the radioactivity recovered from the column eluted before the solvent change; the overall recovery of radioactivity was about 88%.

The following fractions were pooled for further analysis: RR-1 13-18, 39-50; ROS-1 12-18. Samples were concentrated under reduced pressure, transferred to vials, evaporated to dryness under nitrogen and redissolved in about 0.10 ml of hexane.

Aliquots (ca. 15-20 µl each) of samples RR-1/13-18 and ROS-1/12-18 were analyzed with carrier squalene on silica gel G TLC plates, developed once in petroleum ether. One-cm sections were scraped into vials and assayed for radioactivity as usual; the standard lane was sprayed with molybdic acid reagent and charred to visualize mass. The radiochromatograms are shown in Figure IX-3. The majority of the radioactivity (ca. 91% for RR-1, ca. 85% for ROS-1) co-migrated with the squalene standard; the remainder of the radioactivity stayed at or near the origin.

Samples RR-1/13-18 and ROS-1/12-18 were analyzed further by radio-GLC using a 3% OV-17 column (column temperature, 230° C; carrier flow rate, 66 ml/min.), as previously described (see Materials and General Methods). Fractions of effluent gas were collected at one-minute intervals, eluted into vials and assayed for radioactivity as usual. Mass
FIGURE IX-3

Radio-TLC of (A) fractions 13-18 and (B) fractions 12-18 obtained by silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from the "rest of retina" and crude ROS fractions, respectively, of a bovine retina one hour after intraocular injection of [2-14C]-mevalonic acid (see Figure IX-1 and IX-2, respectively). Chromatographic system: Silica Gel G, developed once with petroleum ether. The chromatographic standard is squalene.

FIGURE IX-4

Radio-GLC of fractions 13-18 obtained by silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from the "rest of retina" fraction of a bovine retina one hour after intraocular injection of [2-14C]-mevalonic acid. Chromatographic system: 3% OV-17, 230°C isothermal; carrier flow rate, 66 ml/min. The mass peak is due to squalene.

FIGURE IX-5

Radio-GLC of fractions of 12-18 obtained by silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from the crude ROS fraction of a bovine retina one hour after intraocular injection of [2-14C]-mevalonic acid. Chromatographic system: 3% OV-17, 230°C isothermal; carrier flow rate, 66 ml/min. The mass peak is due to squalene.
DETECTOR RESPONSE

RETENTION TIME (min)

(RADIOACTIVITY (DPM x 10^-2))
tracings of authentic squalene were recorded before and after injection and collection of the radioactive samples. Due to the paucity of material for analysis, each sample was concentrated to about 6-8 \( \mu l \) and the entire sample was injected onto the column. The elution profile for sample RR-1/13-18 is shown in Figure IX-4. Approximately 88% of the recovered radioactivity co-migrated with the squalene standard, while about 5% of the activity immediately preceded the major peak of radioactivity. The ROS-1/12-18 sample (Figure IX-5) showed a very similar elution pattern; about 90% of the radioactivity co-eluted with the squalene standard, while about 7% immediately preceded the squalene peak.

Sample RR-1/39-50 was applied to a TLC plate along with carrier standards of cholesterol, 4\( \alpha \)-methyl-cholesterol-7-en-3\( \beta \)-ol, lanosterol/dihydrosterol and squalene; the plate was developed once in benzene-etherpetroleum ether, 7:3:2. After visualizing mass by exposure to iodine vapors, one-cm sections were scraped into vials and radioactivity was measured as usual. The radiochromatogram is shown in Figure IX-6. Approximately 93% of the recovered activity migrated in the region of methylated sterols, with more of the activity co-migrating with the \( C_{28} \) sterol standard than with the \( C_{30} \) sterols. However, the chromatographic properties of the labelled product or products are also quite similar to those of various non-sterol isoprenoid alcohols; thus, no assignment of structure can be made solely on the basis of these data.

A similar series of analyses was performed on the retina samples from the two-hour incubation (RR-2, \( 5.66 \times 10^5 \) dpm; ROS-2, \( 1.02 \times 10^5 \) dpm). Each sample was supplemented with 10 mg of carrier cholesterol and 5 mg of lanosterol/dihydrolanosterol; samples were dissolved in 0.5 ml of
FIGURE IX-6

FIGURE IX-7
Silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from the "rest of retina" fraction of a bovine retina two hours after intraocular injection of [2-\(^{14}\)C]-mevalonic acid. Solvent system: benzene-diethyl ether, 99:1. Open circles (○-○), radioactivity; closed squares (■-■), 24,25-dihydrolanosterol and lanosterol, determined colorimetrically (absorbance at 460 nm); closed triangles (▲-▲), cholesterol, determined colorimetrically (absorbance at 620 nm).

FIGURE IX-8
Silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from the crude ROS fraction of a bovine retina two hours after intraocular injection of [2-\(^{14}\)C]-mevalonic acid. Solvent system: benzene-diethyl ether, 99:1. Open circles (○-○), radioactivity; closed squares (■-■), 24,25-dihydrolanosterol and lanosterol, determined colorimetrically (absorbance at 460 nm); closed triangles (▲-▲), cholesterol, determined colorimetrically (absorbance at 620 nm).
(DPM x 10^{-4})
benzene, applied to silicic acid-Super Cel columns (100 x 1 cm, benzene slurry) and eluted with benzene-ether, 99:1. Fractions were collected and assayed for radioactivity and sterol mass as before.

The elution profile for sample RR-2 (Figure IX-7) was very similar to that of the RR-1 sample (column flow rate, 2.8 ml per 50-minute fraction). The majority of the activity (ca. 72%) eluted with the solvent front (fractions 15-19), while about 16% co-migrated with the C<sub>30</sub> sterol standard (fractions 30-40). As observed for the previous samples from this experiment, very minor activity (ca. 3%) eluted with the cholesterol standard (fractions 50-62); multiple components of radioactivity were observed in the C<sub>27</sub> sterol region of the radiochromatogram. Overall recovery of radioactivity from this column was about 97%.

The ROS-2 sample exhibited a very similar elution profile to that of the ROS-1 sample (Figure IX-8; column flow rate, 2.5 ml per 50 min.). The major component (ca. 61%) eluted with the solvent front (fractions 17-22). A multiplicity of components was again observed in the region of C<sub>27</sub>-C<sub>30</sub> monohydroxy sterols, with the major component (ca. 19%) co-migrating with the lanosterol/dihydrolanosterol standard (fractions 30-38). About 4% of the total recovered activity co-eluted with the cholesterol standard (fractions 47-55). Overall recovery of activity was about 93%.

The fractions obtained from column chromatography of the retina nonsaponifiable material from the two-hour incubation were not analyzed further.

**Chromatographic Analyses of Lens and Eyecup Nonsaponifiable Lipids**

The nonsaponifiable lipids extracted from the lens (L-2) and eyecup
FIGURE IX-9
Silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from bovine lens two hours after intraocular injection of [2-\(^{14}\)C]-mevalonic acid. Solvent system: benzene. Open circles (o—o), radioactivity; closed squares (■—■), 24,25-dihydrolanosterol (first peak) and lanosterol (second peak), determined colorimetrically (absorbance at 460 nm); closed triangles (▲—▲), cholesterol, determined colorimetrically (absorbance at 620 nm).

FIGURE IX-10
Silicic acid-Super Cel column chromatography of the nonsaponifiable lipids derived from the retina-free bovine eyecup two hours after intraocular injection of [2-\(^{14}\)C]-mevalonic acid. Solvent system: benzene. Open circles (o—o), radioactivity; closed squares (■—■), 24,25-dihydrolanosterol (first peak) and lanosterol (second peak), determined colorimetrically (absorbance at 460 nm); closed triangles (▲—▲), cholesterol, determined colorimetrically (absorbance at 620 nm).
ABSORBANCE

FRACTION NUMBER

RADIOACTIVITY (DPM x 10^3)
(E-2) of the eye which had been incubated for two hours were supplemental-
ized with 9 mg of carrier cholesterol and 5 mg of lanosterol/dihydrolanoster-
ol, and were then applied to silicic acid-Super Cel columns (100 x
1 cm, in benzene; applied activity: L-2, 6.45 x 10^4 cpm; E-2, 1.85 x
10^5 cpm). Benzene was used as the eluting solvent through fraction 174; the solvent was then changed to benzene-ether, 85:15, up to fraction 224. Fractions (50 minutes each; 3.0 ml for L-2, 3.25 ml for E-2) were collected automatically, evaporated to dryness under nitrogen, redivolv-
ed in 2.0 ml of benzene, and then aliquots were taken for assay (1.0 ml each for radioactivity, 0.2 ml each for colorimetric sterol determina-
tion). The column packing material was made from a freshly-opened batch of silicic acid which was apparently more active than the previously used material.

The radiochromatogram obtained for sample L-2 is shown in Figure IX-9. The elution profile was highly simplified, relative to previous radiochromatograms. Only two radioactive components were observed; the major one (fractions 14-19; ca. 88%) eluted with the solvent front, while the minor one (fractions 80-100; ca. 12%) eluted with the lanosterol standard. The C_{30} sterols were partially resolved (fractions 70-100), with the dihydrolanosterol eluting just prior to the lanosterol standard; the radioactivity in this region of the chromatogram was clearly coincident with only the lanosterol peak and not with the dihydrolanosterol peak. No activity was observed in the C_{27} sterol region (fractions 147-183) or in the region between the lanosterol and choles-
terol standards. Recovery of activity was about 81%.

The elution profile for the E-2 sample (Figure IX-10) was a bit more
FIGURE IX-11

Radio-TLC of (A) fractions 14-18 and (B) fractions 13-24 obtained by silicic acid-Super Cel column chromatography of the nonsaponifiable lipids from bovine lens and retina-free eyecup, respectively, two hours after intraocular injection of [2-¹⁴C]-mevalonic acid. Chromatographic system: Silica Gel G, developed once with petroleum ether. The chromatographic standard is squalene.
complex. About 69% of the recovered activity eluted with the solvent front (fractions 13-20). Again, the C\textsubscript{30} sterol standards (fractions 62-100) were partially resolved and the radioactivity in this region co-migrated exclusively with the lanosterol peak (fractions 74-100; ca. 16%). A shoulder was observed on the leading edge of the radioactive peak, but no concomitant shoulder was observed on the lanosterol mass peak. Only about 1% of the recovered activity co-eluted with the cholesterol standard (fractions 135-165); the distribution of radioactivity under this peak was diffuse, and a multiplicity of components eluted both prior to and after the cholesterol mass peak, accounting for about 5% of the total recovered activity. Overall recovery was about 83%.

The pooled activity from samples L-2 (fractions 14-18) and E-2 (fractions 13-24) which eluted with the solvent front were applied to TLC plates, along with standards of squalene and Coenzyme Q\textsubscript{10}; the plates had been activated for 30 minutes at 120\degree C and allowed to cool to room temperature in a desiccator prior to use. After developing the plates once in petroleum ether, one-cm sections of the sample lanes were scraped into vials and assayed for radioactivity as usual. The standard lanes were sprayed with molybdic acid reagent and charred for visualization of mass. The radiochromatograms are shown in Figure IX-11. The overwhelming majority of radioactivity from both samples (98% of L-2/14-18; 94% of E-2/13-24) co-migrated with the squalene standard; the CoQ\textsubscript{10} standard did not migrate from the origin.

The other radioactive fractions from these samples were not analyzed further. The nonsaponifiable extracts from the other ocular tissues
DISCUSSION

The results of this study indicate that bovine ocular tissues have the ability to take up exogenously-administered mevalonic acid and metabolize it to form nonsaponifiable lipids in vivo. After one and two hours of incubation, the major nonsaponifiable product formed in retina, lens and the retina-free eyecup exhibited the properties of squalene on silica gel G thin-layer chromatography, silicic acid-Super Cel column chromatography and (in the case of retina) gas-liquid chromatography on a 3% OV-17 column (i.e. between 56% and 88% of the total nonsaponifiable lipid activity, depending on the tissue and incubation duration). The next major component biosynthesized de novo from mevalonic acid had the chromatographic properties of a C30 sterol, but no assignment of structure could be made from these properties; in the case of the lens and eyecup lipids from the two-hour incubation, it was evident that dihydro-lanosterol was not a candidate for this product (i.e. between 12% and 19% of the total nonsaponifiable lipid activity). Less than 5% of the total nonsaponifiable lipid activity of retina exhibited the chromatographic behavior of cholesterol, and it is uncertain that the labelled component(s) was, in fact, a sterol. The formation of a "cholesterol-like" component was even less pronounced in the two-hour eyecup sample (ca. 1% of the total nonsaponifiable lipid activity), and the formation of C27 sterols was notably absent in the two-hour lens sample (this tissue exhibiting the largest accumulation of label in the "squalene-like" component, ca. 88% of the total nonsaponifiable activity).

The retinas were crudely fractionated by homogenization and subse-
quent centrifugation on discontinuous sucrose gradients into a "crude ROS" and "rest of retina" fractions. Although (as expected) more total radioactivity was incorporated into the two-hour retina fractions than the corresponding one-hour retina fractions, the ratio of nonsaponifiable activity for the two fractions at a given incubation time (i.e. ROS-1/RR-1 and ROS-2/RR-2) was essentially constant. Also, the chromatographic profiles for ROS and RR nonsaponifiable lipids at both one and two hours of incubation were qualitatively very similar. Therefore, no precursor-product relationship could be concluded from the data (i.e. it cannot be concluded that nonsaponifiable lipids were synthesized in the neural retina and then transported to the rod outer segments). The problem lies in the fact that the ROS fraction does not represent a highly-purified ROS membrane preparation; in addition to ROS, the fraction was also enriched in other subcellular membrane components (i.e. endoplasmic reticulum, mitochondria, etc.). Thus, the "ROS" fractions reflect the nonsaponifiable lipid composition of the rest of the retina. The predominence of squalene as the major labelled nonsaponifiable lipid in both "ROS" and "RR" fractions further suggests this point, since squalene has neither been detected as an endogenous component of ROS membranes, nor is it a known constituent of plasma membranes, in general (although it is apparently associated intimately with microsomal enzymes during the biosynthesis of sterols and steroids).

The radiochromatograms obtained from the retina samples are very similar to those previously obtained from in vitro incubations of both intact adult bovine retinas and retina S10 homogenates, using labelled mevalonate as the de novo precursor (see Chapters V through VII). The
striking correspondence between these in vivo results and the in vitro data suggests that the endogenous capabilities of the bovine retina to synthesize sterols (especially cholesterol) de novo from mevalonate are extremely limited, i.e. cholesterol biosynthesis in the bovine retina may not be a significant process, at least in animals of this age. The accumulation of labelled products in vivo and in vitro (especially squalene) derived biosynthetically from mevalonate is in striking contrast to the steady-state composition of the retina. Cholesterol is the major nonsaponifiable lipid and essentially the only sterol in bovine retina (see Chapter IV); other sterols (especially C_{30} sterols) and squalene are either absent or barely detectable. Clearly, the retina sterol composition cannot be explained solely as the result of de novo biosynthesis.

The in vivo experiment described here was not strictly physiological on at least four accounts: (1) mevalonate is an intracellular component derived biosynthetically from acetyl-CoA, and the circulating levels of mevalonate are most likely extremely small (i.e. for the rat, 20-40 ng/ml of blood; Hagenfeldt and Hellstrom, 1972); (2) the exchange of nutrients between the retina and the vitreous is extremely limited and physiologically insignificant for the maintenance of the retina (i.e. intravitreal administration of substrate is not physiologically relevant); (3) the buffer in which the mevalonolactone was dissolved did not reflect the physiological composition of the fluid which surrounds the retina (i.e. differences in ionic and osmotic strength and possibly even in pH could have caused a "shock" to the ocular tissues upon administration of the substrate solution); (4) the animal was under continual
sedation with a general anesthetic, respiration being mechanically assisted and general metabolism being at a basal (or near basal) level. With these caveats in mind, conclusions concerning the absolute endogenous biosynthetic capabilities of the retina and surrounding ocular tissues (with regard to cholesterol biosynthesis) must be made with due caution.

All ocular tissues studied (i.e. retina, lens, cornea, vitreous and the retina-free eyecup) appeared to have the capacity to incorporate mevalonic acid into nonsaponifiable lipids, to varying extents. The incorporation into lens was most unusual, since this tissue contains relatively few "metabolically competent" cells (which contain few mitochondria), lacks access to a blood supply and relies almost exclusively on anaerobic glycolysis for its energy requirements and on diffusion and active transport for nutrient supply and metabolite exchange (Kuck, 1970, 1975; van Heyningen, 1969). The lens synthesized material having the chromatographic properties of squalene and a C\textsubscript{30} sterol. It has been known for some time that the bovine lens increases its cholesterol content with age (to about 0.6–0.8 mg per gram wet weight) (Bellows, 1944). In rabbits, feeding cholesterol (1 gram/kg/day for 3 months) has been reported to cause a slight increase in lens cholesterol content (from 0.8 mg/gram to 1.1 mg/gram, wet weight) as well as a slight increase in total phospholipid and free fatty acid content of the tissue (Vass \textit{et al.}, 1966). It was assumed for many years that lens cholesterol was not endogenously synthesized but was transported intraocularly via the aqueous humor (cf. van Heyningen, 1969). Hammar (1965) demonstrated that the rate of incorporation of \textsuperscript{14}C-labelled glucose into the total lipids of rat lens, \textit{in vivo}, was about 220-fold less than the incorporation into
lactic acid in that tissue, indicating that lipid biosynthesis in the lens was very minimal. Subsequently, Culp and co-workers reported that intraocular injection of $^{14}$C-labelled sodium acetate in rabbits resulted in extremely low incorporation of label into cholesterol in lens, cornea and iris (Culp et al., 1970a). The per cent incorporation into cholesterol (based on total incorporation into lipids) in lens was 3.3% and 1.1% in cornea; most of the metabolized label was incorporated into lecithins (54.0% in lens, 56.6% in cornea) and cephalins (29.5% in lens, 22.6% in cornea). A study of the distribution of label in the lens total lipids, on a specific activity basis, revealed the highest incorporation in lens epithelium (1.0) with lower levels in lens cortex (1.0) with lower levels in lens cortex (0.5) and nucleus (0.05); cholesterol exhibited a high specific activity of incorporation relative to other lipids (Culp et al., 1970b). Thus, it appears that the lens has the ability to synthesize lipids, de novo, although lipid biosynthesis in this tissue is extremely limited. Although corneal lipids were not analyzed in the current study, significant uptake and incorporation of mevalonic acid into nonsaponifiable material was exhibited by this tissue. Andrews (1966) reported that bovine corneas, in vitro, could synthesize both fatty acids and cholesterol from $^{14}$C-labelled glucose or sodium acetate. The lipid content of bovine cornea is very low (about 2% by dry weight) and the cholesterol content is even lower (about 0.4% by dry weight) (D'Asaro et al., 1954; Krause, 1934, 1935; Thiele and Denden, 1967). The apparent incorporation of mevalonic acid into nonsaponifiable material in the vitreous probably represents contamination from eyecup debris rather than de novo synthesis endogenous to the
vitreous. This is quite reasonable, considering the nature and documented composition of the vitreous body (Balazs, 1960, 1961, 1968; Berman and Voaden, 1970; Pirie, 1969), especially with regard to the paucity of vitreal lipid (Krause, 1934, 1935).
X. CONCLUSION

One of the most fascinating and universal of biological processes is the biosynthesis of cellular membranes. Although a considerable amount of research effort had been directed toward the study of this process, our understanding of this aspect of biology is rather limited. Relatively little research has been focused on the problem of membrane biogenesis and its regulation in mammalian cells. The vertebrate rod cell offers a unique opportunity in this regard, since it devotes a major portion of its overall cellular metabolism to the biosynthesis of membrane constituents, the assembly of these constituents into cellular membranes (predominantly ROS membranes) and the turnover of these membranes. The general approach employed in the study of this system has been the identification of the individual membrane components, the definition of the stoichiometric relationships between these components and the study of the biosynthesis and incorporation of this components into the ROS membranes. One class of molecules which has been notably neglected in such studies is the sterols, primarily because the existing literature has suggested that sterols are present only in very small amounts in the retina and in ROS membranes. Although the results of previous studies have suggested that cholesterol is a constituent of the retina and of ROS membranes, the methods used for the identification and quantitation of the sterols were not definitive. There have been no results published concerning the biosynthesis or metabolism of sterols in the retina and the origin of the sterols found both in the whole retina and in the ROS membranes has not been determined. The research described in this dissertation was directed, therefore, toward determining the
sterol composition of the retina and of ROS membranes (qualitatively and quantitatively), followed by an examination of the sterol biosynthetic capabilities of the retina (primarily from de novo substrates).

It was found that cholesterol was essentially the only sterol in the whole bovine retina and in ROS membranes, accounting for at least 98% of the total sterol content. Traces of material having the chromatographic properties of 5α-cholest-7-en-3β-ol (lathosterol) were detected in whole retinas. In both whole retinas and in ROS membranes, traces of material having the chromatographic properties consistent with 5α-cholestan-3β-ol (cholestanol) were detected, also. Cholesterol represented only about 2% of the dry weight of both whole retinas and ROS membranes. Also, the stoichiometric molar ratio of cholesterol:rhodopsin in the ROS membranes was ≈5. Thus, cholesterol is a bona fide and invariant constituent of bovine ROS membranes and is essentially the only molecular species of its class in the membranes. Therefore, there may be certain advantages in using cholesterol as a "lipid marker" in studying the assembly and turnover of the ROS membranes, as opposed to studying the ubiquitous but highly heterogeneous phospholipids (which differ both in polar "head groups" as well as in fatty acid composition). Furthermore, it is apparent that the sterol content of the bovine retina is markedly different from that of the brain, where cholesterol is the single most ubiquitous component (besides water) and accounts for a major portion of the dry weight of that tissue (primarily as a constituent of myelin). Therefore, the results of studies concerning the biosynthesis and metabolism of brain sterols may not be applicable, necessarily, to the retina. Likewise, studies performed with the retina may not yield results which may be generalized to other neural tissues, in con-
trast to the oversimplified but prevalent view that the retina is simply "an extension of the brain".

Having defined the sterol composition of the retina and of the ROS membranes, the question of the source of retina sterols remained to be answered. The results of studies concerning the metabolism of mevalonic acid by the bovine retina, both in vitro (in "intact" retinas and in S10 retina homogenates) and in vivo (by intraocular injection), have suggested that the retina has a definite but extremely limited capacity for sterol biosynthesis. This conclusion must be accepted with the caveat that the retina sterol biosynthetic system may have been labile to the conditions employed for the preparation and/or incubation of the tissue, and that the route of administration of the substrate and the conditions under which the in vivo experiment was performed were not strictly physiological. The retinas used for most (if not all) of the incubations were derived from adult animals. Age may have been an important factor in determining the metabolism of the administered substrates; it is known that brain cholesterol biosynthesis is much more vigorous in newborn and young animals (i.e. during the period corresponding to active myelination) than in the adult (for reviews, see Kabara, 1967, 1973; Paoletti et al., 1969; Davison, 1970, 1974; Ramsey and Nicholas, 1972; Bowen et al., 1974). In all cases, the metabolism of mevalonic acid by the bovine retina led to the accumulation of various nonsaponifiable lipids as well as saponifiable components. Several of these products were identified chromatographically as known intermediates (or their derivatives) of cholesterol biosynthesis. Only minor conversion of this substrate to digitonin-precipitable material (i.e. 3β-monohydroxy sterols) was observed, and only an extremely small
portion of this material was identified (chromatographically and chemically) as cholesterol. The relative incorporation of mevalonic acid into nonsaponifiable constituents showed marked variation from incubation to incubation. Squalene was the major product of several incubations; lesser amounts of material having the chromatographic properties of C_{30} monohydroxy sterols (and possibly desmethyl sterols as well) were also prominent products of these incubations. These results are quite typical of extrahepatic tissues. Although virtually every mammalian tissue has the capacity to synthesize sterols (Srene et al., 1950; cf. Gould, 1958), the liver and gastrointestinal tract organs are the major sites of cholesterogenesis, due to both higher rates of de novo sterol biosynthesis as well as the larger overall magnitude of total sterol production by these tissues (cf. Dietschy and Siperstein, 1967). It has been reported that the levels of squalene epoxidase in various extrahepatic tissues are relatively low, whereas the levels of oxido-squalene-lanosterol cyclase (i.e. the enzyme which catalyzes the conversion of squalene-2,3-epoxide to lanosterol) in these tissues are relatively high (Astruc et al., 1977). This may account, in part, for the relatively large accumulation of squalene and the lesser amounts of "lanosterol-like" compounds in several of the incubations. In addition, there was some indication that a factor (or factors) is (are) present in the retina which can inhibit the conversion of mevalonic acid to digitonin- precipitable material; however, this finding requires further examination to confirm the nature of the "inhibitor".

Incubations of the retina S_{10} homogenates with \textsuperscript{3}H-labelled lanost-8-en-3\beta\,-ol (24,25-dihydrolanosterol) failed to exhibit conversion of
this substrate to other products, for reasons not fully understood. Although this sterol is known to be convertible to cholesterol by rat liver $S_{10}$ homogenates (Kandutsch and Russell, 1960; Avigan et al., 1967), only minimal conversion of the substrate to $C_{27}$ monohydroxy sterols was detected when "parallel" incubations of rat liver $S_{10}$ homogenates were performed under conditions similar to those used for the retina homogenate incubations. Considering the fact that conversion of mevalonic acid to $C_{27}$ sterols was noted in similar preparations of both rat liver and bovine retina homogenates (with great efficiency, in the case of the rat liver homogenates), it is most likely that the failure or relative lack of ability of these preparations to convert the $C_{30}$ sterol substrate to cholesterol or other sterols was due to some aspect of the in vitro system itself and did not accurately reflect the biosynthetic capabilities of the tissues from which the homogenates were prepared. Likewise, the limited conversion of $^3$H-labelled cholesta-8,14-dien-3β-ol to cholesterol by the retina homogenates may have been due to "extrinsic factors". A substantial portion of the diene sterol substrate was converted to monoene sterols (in one of the incubations), but little formation of cholesterol was evident. Further experimentation, involving the optimization of the in vitro system, utilizing a wider variety of labelled sterols, would be required to evaluate the relative facility of sterol interconversion in this tissue more accurately.

A novel aspect of this research was the observation that a substantial proportion of the overall conversion of mevalonic acid by the retina preparations was due to the formation of isoprenoid acids. In some incubations, these acids represented the major metabolites of the given substrate. Curiously, only $C_{15}$ and $C_{20}$ isoprenoid acids were recovered
from the incubation mixtures, and the $C_{20}$ acids accounted for 80-90% of the total labelled components of this class. The reasons for the accumulation of these products and for the relative predominance of the $C_{20}$ acids are not understood at this time. Since such isoprenoids were not detected as endogenous components of the retina, it is apparent that such "alternate" metabolism of mevalonic acid (with regard to the formation of these particular acids) is not normally a significant process in the retina; the accumulation of these products represents an interesting (but nonphysiological) aspect of the in vitro system rather than a salient feature of in vivo metabolism. In addition, the marked accumulation of more than one component having the chromatographic properties of cis isoprenoid acids in these incubations represents a very novel finding, since (with the exception of dolichol and its derivatives) the formation of cis isoprenoids by mammalian tissues had not been described heretofore. In particular, the formation of cis,cis-farnesoic acid has not been described in a tissue from any source, including plants (which are notorious for their ability to synthesize a host of unusual terpenoids and other "exotic" compounds); appreciable amounts of a labelled component having the chromatographic properties of this isoprenoid were isolated from incubations of retina $S_{10}$ homogenates. Furthermore, a major product of these incubations exhibited behavior on various chromatographic systems which was consistent with a $C_{20}$ isoprenoid acid related to geranylgeranoic acid but having at least one cis double bond; such a compound has not been described, heretofore, in mammalian tissues. The biological significance of this unusual biosynthetic capacity remains to be explored.

It was demonstrated conclusively that the metabolism of mevalonic
acid by intact bovine retinas in vitro did not result in the formation of labelled n-fatty acids. This result suggested that the retina lacks the so-called "trans-methylglutaconate shunt" which had been postulated to be effective in all tissues of neural ectoderm origin (Edmond and Popjak, 1974). The generalization of this biosynthetic pathway to all such tissues, therefore, may not be warranted.

Ocular tissues, including the retina, lens, cornea, vitreous and the retina-free eyecup, failed to convert intraocularly-administered mevalonic acid to cholesterol in vivo, under the conditions employed. Instead, the major nonsaponifiable component derived from this substrate had the chromatographic properties of squalene in all tissues examined. Although the conditions were not strictly physiological, these results tend to support the conclusion derived from the results of the in vitro studies (i.e. that the bovine retina has a rather limited capacity for sterol biosynthesis).

The results of the experiments described herein suggest that the de novo biosynthesis of cholesterol in the bovine retina is not the major source of retina sterols and is insufficient to account for the amount of cholesterol required for ROS membrane biogenesis. This is not to say that de novo biosynthesis does not play some role in the overall maintenance of the sterol composition of the retina. Such synthesis may be localized to only certain cell types or anatomical layers of the retina, or may be involved in certain restricted cellular functions. It is evident, therefore, that the retina must rely on some alternate source of cholesterol (i.e. transport), but the origin of the sterol remains to be determined. Although the circulating blood lipoproteins are a likely source of retina sterols, other possibilities
exist (see Chapters I and V). An evaluation of the relative contribution of the various potential modes by which cholesterol may arise in the retina would require considerably more extensive investigation, particularly in vivo, and would necessitate the use of an animal more conducive to laboratory experimentation than was used in these studies. In particular, it would be of interest to examine the capacity of the photoreceptor cells to synthesize cholesterol. Also, there is the distinct possibility that multiple "pools" of cholesterol exist in the retina, as had been previously suggested (Rostas, 1975). This would present a further obstacle in understanding the dynamics of sterols in the retina.

In addition to the obvious questions which remain to be answered concerning the source of retina sterols, extensions of these studies could lead to a more complete understanding of the processes of membrane biogenesis and turnover in the retina as well as providing further insights into the importance of the molecular architecture of the photoreceptor membrane to photoreceptor function. Are cholesterol and the other lipid constituents of the ROS membranes co-assembled (for instance, in the form of a bilayer into which opsin is inserted), or is the sterol component added after preliminary assembly of an "apomembrane"? Are the kinetics of cholesterol turnover parallel with those of the ROS phospholipids and rhodopsin? Is cholesterol distributed symmetrically on both halves of the disk membrane bilayer, or is there an asymmetry which may be correlated with a particular class of phospholipids? Does the low level of cholesterol in the retina and ROS membranes have a physiologically significant role and can such small amounts of cholesterol exert any effect (directly or indirectly) on rhodopsin or other
membrane constituents? Clearly, the current state of knowledge concerning the role of cholesterol in retina metabolism and physiology is quite meager at the present time, and further research in this area is indicated. In addition, a more extensive investigation of isoprenoid metabolism in this tissue may shed some light on this novel aspect of retina metabolism as well as broaden the current understanding of terpene biosynthesis in mammalian tissues, in general.
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