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STUDIES ON THE REGULATION OF THE BIOSYNTHESIS OF CHOLESTEROL

by

DWIGHT LLOYD RAULSTON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

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HOUSTON, TEXAS

MAY, 1979
ABSTRACT

STUDIES ON THE REGULATION OF THE
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Cholest-8(14)-en-3β-ol-15-one, prepared by chemical synthesis, has been shown to have significant hypcholesterolemic activity in male Sprague-Dawley rats upon subcutaneous injection in olive oil. The incorporation of labeled acetate and mevalonate into digitonin-precipitable sterols in 10,000 x g supernatant fractions of liver homogenate preparations derived from control rats and animals receiving the 15-ketosterol has been investigated.

14α-Ethylcholesterol-7-en-3β,15α-diol, prepared by chemical synthesis, has been found to cause a significant inhibition of the formation of digitonin-precipitable sterols from acetate and mevalonate upon direct addition to 10,000 x g supernatant fractions of rat liver homogenates. Under the same conditions purified cholesterol and 25-hydroxycholesterol had no effect. The inhibition of the synthesis of digitonin-precipitable sterols derived from labeled mevalonate by the ethyl diol in this in vitro system has been shown to be accompanied by the accumulation of labeled lanosterol and 24,25-dihydrolanosterol. The added diol had no effect on the individual enzymes catalyz-
ing the conversion of acetate to HMG-CoA.

[2,4-\textsuperscript{3}H]-14α-Ethylcholest-7-en-3β,15α-diol has been prepared. No conversion to cholesterol (or other less polar sterols) was observed upon incubation of the labeled diol with 10,000 x g supernatant fraction of liver homogenates from male or female rats.

Phenylmethylsulfonyl fluoride and cycloheximide at 10^{-3} M were found to inhibit the synthesis of digitonin-precipitable sterols from acetate, but not from mevalonate, in 10,000 x g supernatant fractions of rat liver homogenates. Preincubation of liver preparations with 10^{-3} M phenylmethylsulfonyl fluoride caused an inhibition of acetate thiokinase, HMG-CoA synthase, and HMG-CoA reductase but had no effect on acetoacetyl-CoA thiolase. Preincubation of liver preparations with 10^{-3} M cycloheximide caused inhibition of acetate thiokinase and HMG-CoA synthase but had no effect upon acetoacetyl-CoA thiolase and HMG-CoA reductase.
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INTRODUCTION
Cholesterol is the most ubiquitous and abundant sterol in mammalian tissues (Dempsey [1974]). Although its structure and the biological sources of its component atoms have been elucidated with considerable elegance (Popjak and Cornforth [1960]; Bloch [1965]; Clayton [1965]) and in spite of an extensive chemical knowledge of the material, relatively little is known of cholesterol's biological function on a molecular basis. And although the general outline of its biosynthesis is known (Dempsey [1974]; Frantz and Schroepfer [1967]), many of the specific steps are unclear, particularly in the part of the path following squalene cyclization (Dempsey [1965]; Galli et al. [1970]; Schroepfer et al. [1972]). Many of the enzymes catalyzing the individual biosynthetic reactions have been purified and characterized (particularly the soluble enzymes catalyzing many of the reactions involved in the conversion of acetyl-CoA to squalene), but many more remain unpurified, and their reactions are investigated only with crude preparations (Gaylor [1972]).

Much of the work on cholesterol has centered on the area of the regulation of cholesterol biosynthesis (Dempsey [1974]) and the development of hypocholesterolemic agents. The rationale for this type of work is basically threefold. Cholesterol was implicated in the development of atherosclerosis in the early 1900's when arterial lesions were found
in rabbits fed a high cholesterol diet (Anitschkow and Chalatow [1913]). The presence of cholesterol in signifi-
cant amounts in developing arterial plaques of man was
demonstrated by Biggs and Kritchevsky (1951) and high serum
cholesterol levels have been reported to be statistically
correlated with the incidence of coronary arterial disease
in man by Hill and Dvornick (1966). Further, the reduction
of serum cholesterol levels (induced by dietary restrictions)
has been reported to decrease the incidence of coronary dis-
ease relapse (Leren [1966]). Because of this and other evi-
dence suggesting that cholesterol is implicated in arterio-
sclerosis and coronary artery disease (Frantz [1955];
Schroepfer [1957]; Frantz and Moore [1969]), a method of
controlling serum cholesterol levels might be expected to
hinder or even prevent the development of these diseases.
Since heart attacks are the nation's leading cause of death,
the medical and social implications of such a process which
was free of undesirable side effects could be enormous.

Secondly, the hypercholesterolemias which are so
frequently crippling (if not lethal) are characterized by
extremely high serum sterol levels (two to ten times normal)
and by deposition of cholesterol and other sterols in joints,
tendon, and skin. Lowering the endogenous rate of synthesis
would be expected to help tremendously in mitigating or al-
leviating the severity of these conditions.

Thirdly, the high rate of sterol synthesis in cer-
tain spontaneous leukemias (Chen et al. [1973]; Chen and
Heiniger [1973]; Heiniger et al. [1976]) and spontaneous hepatomas (Kandutsch and Hancock [1971]) has raised hopes that careful control of cholesterol biosynthesis could prove to selectively inhibit the growth and spread of such tumors. However, there are other cells in the body which normally turn over rapidly (such as the intestinal epithelial cells and bone marrow cells) and these cells also produce cholesterol at an elevated rate. Thus, the control would have to be very delicate to avoid deranging the normal body machinery. This sort of problem is encountered in many of the therapeutic regimens normally followed at the present time in order to control the rate of growth and/or spread of tumors.

These basically clinical reasons, of course, are not the only reasons that so much work has been devoted to the control of sterol biosynthesis. Because of specific inhibitors of cholesterol biosynthesis, Kandutsch and Chen were able to report in 1977 that blockage of sterol synthesis in cultured cells has certain specific consequences directly related to cell division. They found that in an 80 hour incubation time, sterol synthesis was suppressed, as was DNA synthesis and cell growth. However, specific rates of uridine incorporation into RNA and of leucine into protein were not significantly altered.

Further, it appears that the activity of one of the major enzymes in cholesterol biosynthesis, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (E.C. 1.1.1.34) is
subject to a wide range of physiological controls in vivo (Rodwell et al. [1976]). The elucidation of the mechanisms of this control may increase substantially the knowledge of the process of regulation in eukaryotic cells.

A large number of potential hypocholesterolemic agents have been synthesized and tested, both for possible clinical use and for laboratory investigation. Non-steroidal agents tested for hypocholesterolemic activity in animals include neomycin, linolexamide, pectin, heparin, cholestyramine and thyroxine (see Braun and Rabinowitz [1970] for an excellent review). One important use of inhibitors of steroid biosynthesis not previously mentioned is for the blockage of specific steps in the biosynthetic pathway to enable investigators to identify the intermediate(s) which accumulate as a result. This type of approach has led to the discovery of several potential intermediates in the biosynthesis of cholesterol (Schroepfer et al. [1972]).

Initial Work

In 1951 Gould reported that cholesterol fed to rats caused inhibition of hepatic sterol synthesis from acetate. These results were soon confirmed (Tomkins et al. [1953]; Frantz et al. [1954]). Phylogenetic studies showed that this negative feedback regulation was present in members of all classes of the animal kingdom which were capable of synthesizing cholesterol (Siperstein [1965]).

Although many of the tissues in the mammalian body
appear to synthesize cholesterol (Srere et al. [1948]), the liver and ileum were found to account for nearly 90% of the synthetic activity of the entire animal (Dietschy and Siperstein [1967]). The feedback regulation process occurring in the liver has been shown to be quite sensitive to dietary cholesterol levels in both rats (Siperstein and Guest [1960]) and man (Pawliger and Shipp [1968]). In attempts to discover whether the active regulator in the negative feedback control was actually cholesterol or a derivative thereof, many investigators attempted to suppress sterol synthesis in the liver and other tissues by feeding a wide variety of steroids and bile acids to animals (Takeuchi et al. [1966]; Siperstein [1970]; Rothblat and Buchko [1971]). Sterols found to inhibit hepatic sterol synthesis when administered to animals include cholesterol, cholest-4-en-3-one, cholest-4,6-dien-3-one, cholestanol, testosterone and cholic acid (Kandutsch and Packie [1970]; Back et al. [1969]). B-Sitosterol and cholest-4-en-3-one as well as cholesterol and a number of its steroid precursors were reported to be inhibitors of sterol synthesis in fibroblast cultures (Rothblat and Buchko [1971]; Rothblat et al. [1971]; Williams and Blass [1970]).

In 1973 Kandutsch and Chen reported that cholest-5-en-3β, 7α-diol, cholest-5-en-3β, 7β-diol and cholest-5-en-3β-ol-7-one were much more effective inhibitors of both sterol synthesis from acetate and of HMG-CoA reductase activity in cultured mouse cells than were cholesterol, bile
acids, or a variety of other steroids. These 7-oxygenated sterols were reported to leave unaltered the rates of conversion of acetate to carbon dioxide and fatty acids, and the rates of protein and RNA synthesis. With the discovery that highly purified cholesterol did not inhibit HMG-CoA reductase or sterol synthesis from acetate under their conditions, Kandutsch and Chen proposed that it was the oxygenated steroid impurities in commercial cholesterol which accounted for the previous reports of its inhibition of sterol synthesis.

Another report by the same authors (Kandutsch and Chen [1974]) demonstrated similar inhibitory properties of cholesterol derivatives bearing oxygen substituents in the side chain. The most potent inhibitors in the series of steroids with oxygenated side chains tested were cholest-5-en-3β,25-diol and cholest-5-en-3β,20α-diol. The authors noted that cholest-5-en-3β,7α-diol and cholest-5-en-3β,20α-diol are known to be intermediates in animal steroid metabolism. These sterols represent, respectively, the first intermediate in the hepatic production of bile acids from cholesterol and the first intermediate in the conversion of cholesterol to the steroid hormones in the endocrine organs. The possibility of in vivo regulation of sterol synthesis by these two diols has not been rigorously investigated. But, as the authors note, it is interesting that a system for the regulation of cholesterol synthesis employing feedback regulation by cholest-5-en-3β,7α-diol and cholest-5-en-3β,20α-
dial would permit independent regulation of cholesterol synthesis by liver and endocrine organs according to the requirement for bile acids or steroid hormones.

Later papers by Kandutsch in collaboration with others continued the work on the inhibitory properties of polyoxygenated sterols (Kandutsch and Chen [1975]; Chen and Kandutsch [1976]; Breslow et al. [1975]). Brown and Goldstein (1974) reported similar results in a study of the suppression of HMG-CoA reductase activity in cultured human fibroblasts. In a later paper, these authors demonstrated the effects of these oxygenated sterols on sterol synthesis in cultured cells from individuals with homozygous familial hypercholesterolemia (Brown and Goldstein [1976]). (Unfortunately, a serious limitation of these studies was the purity of the sterols used. Most of these agents were used without rigorous purification. This renders the conclusions derived from these studies somewhat difficult to interpret.) Philippot et al. (1975) showed that oxygenated sterols were also effective in controlling sterol synthesis in neoplastic L2C guinea pig lymphocytes, which produce cholesterol at about fifty times the rate found for normal lymphocytes.

15-Oxygenated Sterols

The biological formation of 15-oxygenated steroids has been known for over ten years (Meystre et al. [1958]; Laskin et al. [1964]; Schwers et al. [1965]; Schneider [1965]; Gurpide et al. [1966]; Giannopoulos and Solomon
[1967]; Gustafsson and Sjovall [1968]; Eriksson et al. [1971]; Gustafsson and Ingelman-Sundberg [1973]). Moreover, several 15-hydroxysterols have been suggested as potential intermediates in cholesterol biosynthesis (Alexander et al. [1972]; Akhtar et al. [1969]; Phillips et al. [1976]; Martin et al. [1970]; Huntoon and Schroeper [1970]; Schroeper et al. [1970]; Schroeper et al. [1972]; Spike et al. [1974], [1978]).

In collaborative efforts between the laboratory of Dr. Andrew A. Kandutsch and that of Dr. G. J. Schroeper, Jr. the effects of 15-oxygenated sterols on sterol biosynthesis in mouse cells grown in culture in serum-free media have been investigated. A large number of these 15-oxygenated sterols and their derivatives have been found to be very potent inhibitors of sterol synthesis in L-cells and in primary cultures of fetal mouse liver cells (Schroeper et al. 1976, 1977a, 1977b, 1977c, 1977d). The same compounds were also shown to be very active in the reduction of the levels of HMG-CoA reductase activity in the same cells. For example, cholest-8(14)-en-3β-ol-15-one caused a 50% inhibition of sterol synthesis from acetate in L-cells at $10^{-7}$ M and a similar reduction in HMG-CoA reductase activity at $3 \times 10^{-7}$ M (Schroeper et al. [1976]; [1977a]). Many other 15-oxygenated sterols have been synthesized and tested for inhibition of cholesterol-genesis in the laboratories of Schroeper and Kandutsch. Two of the most potent of these are 14α-ethylcholest-7-en-3β,15α-diol (Schroeper et al.
[1977b]) and 14α-ethylcholest-7-en-15α-ol-3-one (Schroepfer et al. [1977c]).

Many of the experiments described in this thesis were undertaken to investigate the effects of cholest-8(14)-en-3β-ol-15-one and 14α-ethylcholest-7-en-3β,15α-diol on serum cholesterol levels in animals and in vitro sterol biosynthesis from 10,000 x g supernatant preparations from rat livers.

Site and Mechanism of Action of Oxygenated Sterols.

From several different systems including cultured cells and whole animals, data have been accumulated describing the effects of oxygenated sterols on various steps in the cholesterolgenic process. Results from the laboratories of Kandutsch, Philippot and Schroepfer have demonstrated that oxygenated sterols act in general by decreasing the incorporation of acetate into digitonin-precipitable sterols (Kandutsch and Chen [1973]; Philippot et al. [1975]; Kandutsch and Chen [1975]). Brown and Goldstein (1974, 1976) and Kandutsch and Chen (1973, 1975, 1976) have reported reduction of the levels of HMG-CoA reductase activity assayed directly by following the conversion of radio-labeled HMG-CoA to mevalonate. The assays were conducted on crude microsomes or detergent-solubilized cell extracts which were isolated from cells which had previously been incubated with the inhibitors. In general, the conditions required to cause 50% inhibition of sterol synthesis were comparable to those required to cause a 50% reduction in the levels of HMG-
CoA reductase activity in the same cells. These results imply that the effect of the oxygenated sterols is at the level of HMG-CoA reductase. However, no other enzyme in the cholesterol biosynthetic pathway has been directly examined. Specifically, the enzymes catalyzing the reactions in the conversion of acetate to HMG-CoA have not been investigated, although an inhibition of one or more of these enzymatic activities might also be effected by oxygenated sterols.

Brown et al. (1975) reported an additional effect of oxygenated sterols on a cholesterol-involving process. They noted that incubation of monolayers of cultured human fibroblasts with oxygenated sterols markedly enhanced the rate at which the cells esterified their endogenous cholesterol and further produced an increase in the cellular content of cholesterol esters. The enhanced esterification activity was associated with an increase in the activity of a membrane-bound fatty acyl-CoA:cholesterol acyltransferase.

Kandutsch et al. (1977) have reported that in mice given cholest-5-en-3β,25-diol or cholest-5-en-3β-ol-7-one in the diet at levels of 0.25% and 0.5%, growth of immature animals was suppressed and that mature mice lost weight. The effect on body weight was related to an apparent diminution of the appetite. This effect was not due to unpalatability of the sterols, since diets containing them were not rejected in favor of control diets.

Kandutsch et al. (1977) also reported that in mice
administered cholest-5-en-3β,25-diol (5 mg) by gavage, within two hours of administration sterol synthesis in the liver from acetate had decreased to 20% of its control level. The half-life of HMG-CoA reductase has been variously estimated at 1.5 - 4 hours (Rodwell et al. [1973]; Kirsten and Watson [1974]; Edwards and Gould [1972]).

It has already been noted that after oxygenated sterols are preincubated with various cell cultures, the activity of HMG-CoA reductase drops drastically from control levels (typically a 50% decrease at micromolar concentrations). But although the effect on the levels of reductase activity is well documented, the mechanism of action is still unresolved. There are several different modes of action by which these inhibitors might exert their effects, and some of these are discussed below.

So far there has been no published account of a direct effect on an oxygenated sterol on HMG-CoA reductase (Bell et al. [1975]; Rodwell et al. [1973], [1976]; Higgins and Rudney [1973]). Several other means of control have been suggested, however; two other possible mechanisms of regulation of HMG-CoA reductase activity are the suppression of enzyme synthesis and the enhancement of enzyme degradation. Bell et al. (1975) have reported evidence for both mechanisms, depending on the type inhibitor tested. Using hepatoma tissue culture 7288 C (HTC) cells, the authors reported that the apparent rate of enzyme degradation was increased approximately eight-fold by one-tenth micromolar
concentrations of cholest-5-en-3β-ol-7-one and cholest-5-en-3β,25-diol. Cholesteryl succinate was also reported to be an effective inhibitor of reductase activity, but to act by decreasing the apparent rate of synthesis with no effect on the apparent rate of inactivation. The authors summarize that HMG-CoA reductase activity in HTC cells appears to be regulated by two distinct mechanisms. Which of these mechanisms is operative appears to depend on the exact structure of the steroid effector molecule tested.

Higgins and Rudney (1973) have reported that in rats fed cholesterol at the beginning of the dark phase of a light-dark cycle, "...the catalytic activity of HMG-CoA reductase was quickly inactivated while the synthesis of new enzyme continued normally for several hours." The enzyme synthesis was measured using antibodies to an impure reductase preparation, however, so these results are open to some question. If the results are accurate, one explanation may be that the enzyme was modified and that cholesterol feeding increased the rate of this modification. A reversible activation and inactivation of HMG-CoA reductase was shown by Beg et al. (1973) who demonstrated that preincubation of microsomes with cAMP stimulated the inactivation of reductase activity in washed microsomes and that this activity could be partially restored by incubation with a cytosolic protein fraction. Nordstrom et al. (1977) reported that the reductase was inactivated by an enzyme-catalyzed phosphoryl-action in a process requiring Mg-ATP and that a reactivating
system present in the cytosol was inhibited by sodium fluoride, suggesting that the reactivating system might be a protein phosphatase.

The enzyme modification proposal was examined in more detail by Beg and coworkers (1978). They reported that incubation of microsomal HMG-CoA reductase with \( [\gamma-^{32}P] - \) ATP and magnesium resulted in an increase of \( ^{32}P \)-protein-bound radioactivity and a concomitant decrease in reductase activity. Phosphorylation of HMG-CoA reductase was further suggested by immuno-precipitation of partially purified \( [\gamma-^{32}P] \)-Mg-ATP-inactivated reductase with "a reductase-specific antiserum." These papers provided evidence suggestive of rapid reversible modification of HMG-CoA reductase activity by a covalent enzyme modification involving a phosphorylation-dephosphorylation mechanism. Also in 1978, Ingebritsen et al. showed that Mg-ATP-inactivated reductase could be fully reactivated by purified cytosolic phosphorylase phosphatase (E.C.3.1.3.17). Furthermore, the reductase-inactivating enzyme was solubilized and found to be inactivated by phosphorylase phosphatase. Inactivated reductase-inactivating protein could be reactivated in the presence of Mg-ATP by a protein kinase present in the microsomal cell fraction. Beirne et al. (1976) have hypothesized that an oxygenated sterol (such as cholest-5-en-3\( \beta \), 25-diol, a known autoxidation product present in commercial cholesterol) may be a "proximal effector" which initiates a cascade of events leading to enzyme modification.
MATERIALS AND METHODS
MATERIALS AND METHODS

Solvents

All solvents were of reagent grade and were obtained from Mallinckrodt, Inc. Unless stated otherwise, the composition of mixed solvents was expressed as the ratio of the volumes of the individual components.

Melting Points

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

Ultraviolet Spectroscopy

Ultraviolet spectra were recorded on a Beckman DB-GT UV-Visible Recording Spectrophotometer or on a Cary 118 Recording Spectrophotometer using matched quartz cuvettes with a one centimeter light path.

Low Resolution Mass Spectrometry

Low resolution mass spectral analyses were performed on an LKB 9000S Mass Spectrometer with the sample introduced by direct inlet. Spectra were recorded at 70 eV with an accelerating voltage of 3.5 kV, an ion source temperature of 60°C, and a filament current of 60μA. Only ions of intensity greater than one percent of the base peak were plotted in the spectra.
High Resolution Mass Spectrometry

High resolution mass spectral analyses were performed in the laboratory of Professor Charles C. Sweeley (Michigan State University).

Infrared Spectroscopy

Infrared spectra were recorded on a Beckman IR-9 Infrared Spectrophotometer. The samples were prepared in a potassium bromide disc at a concentration of 1% (w:w).

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded on a Perkin-Elmer R-12 Nuclear Magnetic Resonance Spectrometer in deuteriochloroform solution with tetramethylsilane as an internal standard.

Measurement of Radioactivity

Radioactivity was measured in a Beckman LS-250 Liquid Scintillation System using interchangeable iso-sets. Two types of scintillation counting solutions were used. For counting labeled material soluble in an organic solvent (usually benzene of hexane), a toluene counting solution was used which contained 0.4% PPO (2,5-diphenyl-oxazole, from Beckman Instruments, Inc.) and 0.005% POPOP (1,4- bis [a-(5-phenyloxazolyl)] -benzene from Packard Instrument Company, Inc.). Tritium-labeled material was counted at 50-60% efficiency and $^{14}$C - labeled material was counted at 90-95%
efficiency. To count labeled material in aqueous solution, an ethanol-toluene scintillation fluid was used. This solution was prepared by dissolving PPO (15 gm) in a solution of toluene (2.5 l) and absolute ethanol (1.25 l). Tritium-labeled material was counted at 20-40% efficiency and \(^{14}\text{C}\)-labeled compounds were counted at 80-90% efficiency. A constant volume (10 ml per vial) of scintillation counting solution was used throughout these studies.

Thin-Layer Chromatography

Glass plates coated with 0.25 mm layers of Silica Gel G were purchased from Analtech, Inc. These plates were used for analytical separations of compounds. Preparative Silica Gel G thin-layer plates of 0.50 mm thickness were sometimes used for the isolation of a desired product. For these cases, Silica Gel G (Type 60, for TLC according to Stahl, EM Laboratories Inc.) was shaken thoroughly with two volumes water and thin-layer plates were prepared by the method of Kammereck et al. (1967). The plates were allowed to dry in air for an hour and then activated by heating in an oven (100-120°C) for another hour.

Compounds on the plate were visualized by heating in an oven at 110°C after spraying with a molybdic acid reagent (modified from that described by Clark [1964]): ammonium molybdate (20 gm) was mixed with concentrated sulfuric acid (25 ml), warmed to dissolve, and diluted to 400 ml with water. For visualization of compounds isolated by preparative thin-layer chromatography, the developed plates were
sprayed with a solution of Rhodamine 6G (0.8 mg/ml in ethanol) and the bands visualized under ultraviolet light. Compounds were eluted from the silica gel into anhydrous ether. Provided the ether was free of ethanol, the Rhodamine 6G remained bound to the silica gel during extraction of the material with ether.

Gas-Liquid Chromatography

Gas-liquid chromatographic analyses were performed on either a Hewlett-Packard Model 402 or a Hewlett-Packard 5730 Gas Chromatograph unit with glass columns (4 mm i.d.) which were six feet in length. Both chromatographs used helium as the carrier gas with a flow rate of approximately 66 or 60 ml/min respectively. Both units were equipped with flame ionization detectors. The injection port and detector were maintained at 30-50°C above the column temperature. The columns employed were silanized and packed with Gas-Chrom Q (100-200 mesh) which had been previously coated with 3% OV-1 or 3% OV-17.

Gas-Liquid Radiochromatographic Analysis

When determination of the radioactive purity or identity of a labeled sample was required, the distribution of its radioactivity throughout the gas-liquid chromatogram was measured by collecting timed (generally one minute) fractions of the effluent gas (through a splitter attached to the column outlet) in glass tubes (30 x 3 mm). Recovery of the
radioactive material varied from 50-90%.

The collected material was rinsed several times with the toluene scintillation fluid into counting vials and the radioactivity was measured as described previously. A delay of approximately one minute was observed between the mass curve reading and the radioactivity observed at the splitter.

**Column Chromatography**

**Silicic Acid-Super Cel Column Chromatography.** Silicic acid-Super Cel columns were used for the separation of components of non-saponifiable lipid preparations. They were prepared by mixing of silicic acid (A.R., 100 mesh, Mallinckrodt) and Hy-Flo Super Cel (Johns-Manville Corp.) in a 2:1 ratio (w:w) and adding an appropriate solvent to form a slurry. The slurries were poured into columns (1 x 100 cm, or 1 x 50 cm) and packed under nitrogen pressure (5 p.s.i. or 10 p.s.i.). Samples were applied in a small volume (2-3 ml) of the first eluting solvent. Fractions were collected in test tubes using an automatic fraction collector (either LKB Radi-Rac, type 3403B or ISCO Model 273 Fraction Collector). Aliquots were taken for determination of radioactivity and/or sterol mass.

**Alumina-Super Cel-Silver Nitrate Column Chromatography.** Alumina-Super Cel-silver nitrate columns were used in the separation of double bond isomers or homologues of sterols in the form of their acetate derivatives. The method described by Paliokas et al. (1968) was modified in the fol-
lowing manner. Neutral alumina AG-7 without binder (30 gm; Bio-Rad Laboratories) was thoroughly mixed with Hy-Flo Super Cel (15 gm) in a lyophilization flask (one liter). Silver nitrate (9 gm) was dissolved in deionized water (75 ml) and added to the contents of the flask. The resulting slurry was mixed thoroughly. The mixture was then frozen in an acetone-dry ice bath and lyophilized at 60 - 100 microns pressure for approximately 48 hours. The flask was wrapped in aluminum foil during the lyophilization to protect the contents from light. The resulting white powder was added to a mixture of hexane and toluene (85:15) and a homogeneous slurry prepared. Columns (1 x 100 cm) were packed with the slurry under nitrogen pressure (10 p.s.i.). Steryl acetate samples were applied to the columns in small volumes (2 ml) of the eluting solvent. The eluate fractions were collected and analyzed as described previously.

Preparation of Steryl Acetates

Sterols analyzed by alumina-Super Cel-silver nitrate column chromatography were converted to steryl acetates by the method of Johnston and Bloch (1957). A mixture of dry pyridine and acetic anhydride (1:1) was added to the sterol sample (2 ml per mg sample), and the solution stoppered and kept at room temperature for 24 hours. At the end of this time, water (two volumes) was added and the solution extracted three times with hexane (two volumes each). The combined hexane extracts were dried over anhydrous magnesium sulfate.
After filtration to remove the drying agent, the hexane solution was evaporated to dryness under nitrogen and redisolved in a small volume of an appropriate solvent for alumina-Super Cel-silver nitrate column chromatography.

**Preparation of Trimethylsilyl Sterols**

For separation of certain of the sterols described in these studies, it was found desirable to convert the compounds to their trimethylsilyl ethers. The reagent described by Carter and Garver (1967) was used. Hexamethyldisilazane (3.9 ml; Applied Science Laboratories) was mixed with dry pyridine (3.0 ml) before the addition of trimethylchlorosilane (2.4 ml; Applied Science Laboratories). The solution was mixed well and the resulting precipitate was pelleted by a brief centrifugation at 1000 x g. To derivatize a sample, the silylating reagent (0.10 ml) was added to the dried sample (0.1-0.5 mg) in a small vial. The vial was tightly capped and the mixture warmed at 80-100°C for 5-10 minutes.

**Colorimetric Assay of Sterols and Steryl Acetates**

Sterols and steryl acetates were quantitated by the method of the Liebermann-Burchard colorimetric assay. The Liebermann-Burchard reagent used throughout these studies was prepared according to the modified method described by Abell et al. (1952). A mixture of acetic anhydride and concentrated sulfuric acid (20:1) was cooled in ice in a stop-
pered flask for nine minutes. At the end of this time, glacial acetic acid was added (10 volumes). The reagent was allowed to warm to room temperature. An aliquot (1.8 ml) of this reagent was added to the sterol or steryl acetate samples in colorimetric assay tubes (10 x 75 mm). Absorption was read at 620 nm (cholesterol and other sterols not containing a 14-methyl group) or at 490 nm (lanosterol and dihydrolanosterol). Color was allowed to develop thirty minutes before the absorption was read on a Coleman Junior Spectrophotometer.

**Colorimetric Assay of Protein**

Protein concentrations were determined by the method of Goa (1953), a modification of the Biuret assay. Aliquots of each sample (generally 50 ul or 100 ul) were diluted to 1 ml with the appropriate buffer. The Biuret reagent (4 ml; *vide infra*) was added to each, the solutions mixed well, and the absorbance at 550 nm read after thirty minutes. A blank of water (1 ml) and Biuret reagent (4 ml) was used in the assay. Protein concentration was determined from the absorbance readings using a standard curve prepared with bovine serum albumin solution.

The Biuret reagent was prepared by dissolving cupric sulfate pentahydrate (1.5 gm) and sodium potassium tartrate (6.0 gm) in water (500 ml) in a volumetric flask (1 l). With constant swirling, a freshly prepared solution of 10% sodium hydroxide (300 ml) was added. The reagent solution was
aqueous ethanol (5 ml), a solution of 10% aqueous acetic acid (10 drops) was added to insure complete precipitation. The samples were capped and left overnight in a dark cabinet. The precipitate was collected the following day by a centrifugation (15 minutes) in a table-top centrifuge at 1500 x g. After removal of the supernatant by aspiration, the pellet was washed once with an ether:acetone (1:1) solution (15 ml) and twice with anhydrous ether (15 ml) by resuspending the pellet in the fresh solvent and mixing well on a Vortex mixer. The pellet was collected each time by centrifugation in the table-top centrifuge. Residual ether in the final pellet was removed by evaporation under nitrogen. The pellet was then briefly dessicated in vacuo over phosphorus pentoxide.

The digitonide complex was split by the addition of dry pyridine (0.4 ml) and heating the capped samples fifteen minutes in a boiling water bath. By the end of this time the precipitate had totally dissolved. Immediately afterwards, ether (3.2 ml) was added to each sample which was well mixed on a Vortex mixer to insure complete precipitation of the free digitonin. The precipitate was collected by centrifuging at 1500 x g. Each supernatant was carefully removed and saved. The pellets were washed twice more with ether (3.2 ml portions) by resuspending the pellet and then collecting it by centrifugation. The combined ether supernatants, which contained the digitonin-precipitable sterols, were taken to dryness under nitrogen and redissolved in a
small, accurate volume of benzene so that aliquots could be taken for liquid scintillation counting and Liebermann-Burchard colorimetry.

**Incubation Buffer**

For studies involving the conversion of sodium acetate and sodium mevalonate to non-saponifiable lipids and digitonin-precipitable sterols, incubation buffer was prepared by mixing 0.20 M dipotassium hydrogen phosphate solution (400 ml) with 0.20 M potassium dihydrogen phosphate solution (100 ml) in a volumetric flask (1 l). To this solution were added nicotinamide (3.66 gm) and magnesium chloride hexahydrate (1.00 gm). Distilled water (450 ml) was added and the solids dissolved by vigorous mixing. The pH of the solution, initially 7.3, was adjusted to pH 7.4 with a 5 M potassium hydroxide solution. Finally, the buffer was made to volume (1 l) with distilled water.

**Purification of Cholesterol Through Its Dibromide**

Radioactive cholesterol recovered from an incubation experiment was purified by the method of Frantz et al. (1959). The radioactive cholesterol sample was added to unlabeled carrier cholesterol (approximately 50 mg) and dissolved in a known volume of benzene. Aliquots were removed for determination of the radioactivity and mass content. The sample was evaporated to dryness and the residue redissolved in anhydrous ether (2 ml) in a conical centrifuge tube (5 ml) cooled
in an ice bath. To this solution bromine was added dropwise until a red-brown color persisted. The solution was maintained on ice for two hours. At the end of this time, chilled glacial acetic acid (1 ml) was added with stirring. The precipitated cholesteryl dibromide was collected by centrifugation (for twenty minutes) at 1500 x g at 2 C. The pellet was washed twice with glacial acetic acid (2 ml) and collected by centrifugation in the cold.

The resulting cholesteryl dibromide was dissolved in anhydrous ether (5 ml) and the solution immersed in an ice-water bath. The solution was stirred with a magnetic stirrer while zinc dust (25 mg) was added. Stirring was continued for 15 minutes. Water was added dropwise to dissolve the zinc dibromide, and the ether layer was washed once with 0.7 N hydrochloric acid (2 ml), twice with water (2 ml), once with a 8% sodium hydroxide solution (2 ml), and with water again (2 ml portions) until the water wash was neutral. The recrystallized cholesterol was dissolved in a known volume of benzene and aliquots were removed for the determination of radioactivity and mass.

Basic Hydrolysis Conditions

In many of the experiments described in these studies, incubation samples were hydrolyzed by the addition of a 15% ethanolic potassium hydroxide solution and heating for three hours either on a steam bath or in a water bath (70-75°C). This 15% ethanolic potassium hydroxide solution was
prepared by adding potassium hydroxide (15 gm) to water (5 ml) and swirling until the water was saturated with the alkali. Then ethanol (95 ml; 100%) was added and the solution stirred vigorously until all the solid had dissolved. The solution was prepared fresh each time immediately before use.

Analysis of Serum Cholesterol by the Method of Abell et al. (1952)

Serum was prepared by allowing blood samples to clot at room temperature for two hours. The coagulated cells were pelleted by centrifugation for 15 minutes in a table-top centrifuge at 1500 x g. The serum was carefully removed using a Pasteur disposable pipet. An accurately measured aliquot (0.3 - 0.5 ml) was removed and hydrolyzed at 37°C for 55 minutes with an alcoholic potassium hydroxide solution (5 ml). This solution was prepared by dissolving potassium hydroxide (10 gm) in water (20 ml) and adding this solution (6 ml) to absolute ethanol (94 ml). After the hydrolysis, water (5 ml) was added and the serum sterols were extracted with hexane (10 ml). The hexane was divided into two or three equal aliquots and the solvent removed by evaporation under nitrogen. The residues were analyzed for cholesterol by the Liebermann-Burchard colorimetric assay.

Statistical Evaluation of Data

In many of the experiments described in this thesis,
results are expressed as a mean value ± standard error. In determining whether or not the difference between two means is significant the Student's t-Test method was employed. The formulas used in these calculations are presented on the next page.
The standard deviation for a set of data points was calculated from the following formula:

\[ S = \left( \frac{\sum_{i=1}^{n} (x - x_i)^2}{n} \right)^{1/2} \]

in which:
- \( S \) = standard deviation
- \( x \) = mean of the data points
- \( x_i \) = the \( i \)th data point
- \( n \) = number of data points in the group

The significance of the difference between the means of two groups was calculated from the following formulas:

\[ S_{x'}^2 = \frac{(S_{1x}^2)(n_1 - 1) + (S_{2x}^2)(n_2 - 1)}{(n_1 + n_2 - 2)} \]

\[ S_{x'}^- = \left( \frac{S_{x'}^2 \cdot (n_1 + n_2)}{(n_1 \times n_2)} \right)^{1/2} \]

\[ t_{calc} = \frac{\bar{x}_1 - \bar{x}_2}{S_{x'}} \]

The value \( t_{calc} \) was compared to tabulated values of \( t \) obtained from the Handbook of Mathematics (1970). The symbols used in the above formulas are explained on the following page.
\( S_1 = \text{standard deviation of group 1} \)
\( S_2 = \text{standard deviation of group 2} \)
\( \bar{x}_1 = \text{mean of the values in group 1} \)
\( \bar{x}_2 = \text{mean of the values in group 2} \)
\( n_1 = \text{number of values in group 1} \)
\( n_2 = \text{number of values in group 2} \)

**Presentation of Statistical Correlation Data**

Due to the lack of space in many of the tables in which large amounts of data are presented in this thesis, the presentation of the statistical correlations ("p" values) will generally be given as a superscripted letter immediately following the table entry. For example, \( 3.9 \pm 0.2 \) \( ^a \). The following chart gives the superscript letters used and the statistical correlations which they represent.

<table>
<thead>
<tr>
<th>Letter</th>
<th>p Value</th>
</tr>
</thead>
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<tr>
<td>a</td>
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</tr>
<tr>
<td>b</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>c</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>d</td>
<td>0.02 &lt; p &lt; 0.05</td>
</tr>
<tr>
<td>e</td>
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</tr>
<tr>
<td>f</td>
<td>p &gt; 0.10</td>
</tr>
<tr>
<td>g</td>
<td>p &gt; 0.20</td>
</tr>
<tr>
<td>h</td>
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</tr>
<tr>
<td>i</td>
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</tr>
<tr>
<td>j</td>
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</tr>
<tr>
<td>k</td>
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</tr>
<tr>
<td>l</td>
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</tr>
<tr>
<td>m</td>
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</tr>
<tr>
<td>n</td>
<td>p &gt; 0.90</td>
</tr>
</tbody>
</table>
Radioactive Substances

The following substances were obtained from New England Nuclear Corporation:

Sodium $[2^{-14}C]$-Acetate
$[1^{-14}C$-acetyl $]$-Acetyl-CoA
$[^3H$-acetyl $]$-Acetyl-CoA
$[3^{-14}C$-3-hydroxy-3-methylglutaryl] -HMG-CoA
$[2^{-14}C]$-Mevalonic Acid, DBED salt
$[4^{-14}C]$-Cholesterol
$[^3H]$-Water

The following substance was obtained from ICN Chemical and Radiosotope Division:

Sodium $[^3H]$-Acetate

The following substance was obtained from Amersham Corporation:

$[2^{-2}H]$-Mevalonolactone

Other Chemicals

The following reagents were obtained from Sigma Chemical Company:

Acetyl-CoA, Li$^+$ salt, 90-95%
Acetoacetyl-CoA, Na$^+$ salt, Grade II, 75-90%
HMG-CoA, Na$^+$ salt, Grade II, 90-95%
Coenzyme A, Li$^+$ salt, Grade I-L, 85-90%
Sodium Iodoacetate, 99%
N-ethylmaleimide

Cycloheximide, Lot 86C-0172
Phenylmethylsulfonyl Fluoride, lot 126C-0412
Bromoacetamidonitrophenol
ATP, Na⁺ salt, 99%
NAD, Grade III, 98%
NADP, Na⁺ salt, 98%
G-6-P, Na⁺ salt, 98%
Glucose-6-phosphate Dehydrogenase
Dithiothreitol, Sigma grade
The following reagent was obtained from Miles Laboratories, Inc.
  Pentex Bovine Albumin, Fatty Acid Poor, Fraction V.

**Standard Steroid Reference Compounds**

In the section on Chemical Syntheses, when reference is made to an authentic standard compound, this compound was supplied by Dr. Edward Parish, unless otherwise stated.
CHEMICAL SYNTHESIS
Purification of Cholesterol

Cholesterol (10 gm; from Nutritional Biochemicals) was dissolved in ether (300 ml) and the solution cooled in ice. Bromine was added dropwise with swirling into the cooled solution until the faint yellow solution became light orange (approximately 1.5 ml). The flask was covered and set in a refrigerator (4°C) for two hours. Glacial acetic acid (300 ml) was added to the pasty mass of the dibromide and the material centrifuged ten minutes at 600 x g at 2°C. The liquid supernatant was decanted. Fresh glacial acetic acid (300 ml) was added and the precipitate resuspended and shaken well. The material was centrifuged as before and the supernatant decanted. This washing procedure was repeated twice more. The white precipitate of cholesteryl dibromide was dissolved in absolute ether (800 ml) on a steam bath and the solution cooled in ice. Zinc dust (1.5 gm) was added in small portions with stirring. The solution was maintained on ice and stirred intermittently for fifteen minutes. It was then poured into a separatory funnel and washed four times with water (1 l each). The ether was dried over anhydrous magnesium sulfate and reduced in volume (to about 50 ml) on a rotary evaporator under reduced pressure. The concentrated solution was rinsed into a beaker with ethanol and the purified material recrystallized from ethanol. The first crop was collected by suc-
tion filtration and dessicated in vacuo over phosphorus pentoxide. The yield was 6.23 gm (62%) of material melting at 147.5-148.5°C (literature value 148.5°C, Cook [1958]).

A portion of the cholesterol was further purified by preparative thin layer chromatography on Silica Gel G plates. The plates were run in chloroform and the cholesterol visualized by spraying with Rhodamine 6-G. The major bank was carefully scraped from the plate and the sterol eluted with anhydrous ether. The recovered cholesterol was then recrystallized from ether-methanol. The yield of recrystallized material was 85% which melted at 147.5-148.5°C. This material was analyzed by gas-liquid chromatography on a column of 3% OV-17 at 250°C. Only a single peak was observed, with a retention time corresponding to that of an authentic cholesterol standard. The material was judged to be greater than 99.5% pure based on this gas-liquid chromatographic analysis (the limit of detection was approximately 0.5% under these conditions and no detectable impurity was observed). Thin-layer chromatographic analysis showed a single spot with an estimated limit of detection of 0.1%.

Purification of 25-Hydroxycholesterol

Cholest-5-en-3β,25-diol was purchased from Steraloids, Inc., and determined to be approximately 85% pure as judged by gas-liquid chromatographic analysis on a column of 3% OV-17 at 250°C. The impurity was also visible in thin-layer chromatography of the material using chloroform as the de-
veloping solvent. The sterol was purified by preparative thin layer chromatography on Silica Gel G plates. The developing solvent was chloroform; the bands were visualized with Rhodamine 6-G. The minor, less polar band had an R<sub>f</sub> equal to that of cholesterol. The major, more polar band was scraped from the plate and the sterol eluted from the silica gel with anhydrous ether. The recovered sterol was recrystallized from acetone-water and melted at 180-181°C (literature value, 182°C, Cook [1958]). Gas-liquid chromatographic analysis of the purified sterol on a column of 3% OV-17 at 250°C showed only one peak corresponding in retention time to that of the major component of the original sample. Thin-layer chromatographic analysis showed only a single spot using chloroform as the developing solvent (the limit of detection was estimated at approximately 0.1%).

Synthesis of Cholest-8(14)-en-3β-ol-15-one

Cholest-8(14)-en-3β-ol-15-one was one of the compounds employed in these studies and was also a key intermediate in the synthesis of several other compounds used in these studies, particularly 14α-ethylcholest-7-en-3β,15α-diol. The synthetic scheme described below is essentially that of Parish et al. (1977) and is illustrated in Figure 5.

7-Dehydrocholesteryl Benzoate

7-Dehydrocholesterol (45 gm, Sigma) was added to a round-bottomed flask (500 ml). Pyridine (110 ml) and ben-
Figure 5. Synthesis of Cholest-8(14)-en-3β-ol-15-one after Parish et al. (1977); Abbreviations: BzOCl - Benzoyl Chloride; MCPBA - m-Chloroperbenzoic Acid.
zoyl chloride (110 ml) were added and a reflux condenser attached. The flask was heated at the reflux temperature for two hours. At the end of this time, the solution was poured over crushed ice (500 gm) and allowed to stand overnight. After filtration through a sintered glass funnel, the wet precipitate was dissolved in chloroform (250 ml) on a steam bath. The solution was poured into a separatory funnel and washed twice with equal volumes of water, twice with 5% aqueous sodium carbonate solution, and twice more with water. The chloroform solution was returned to the steam bath and the volume reduced to about 325 ml. Enough methanol (100 ml) was added to cause a permanent turbidity at the elevated temperature, and the solution was set aside to cool to room temperature. After this initial cooling, the solution was placed in the freezer (-20°C) overnight to crystallize. The crystals were collected the following day on a sintered glass funnel and washed with three portions (100 ml) of ice-cold methanol. The crystals were recrystallized from chloroform-methanol to give white crystals melting at 140-141°C (literature value, 143°C, Cook [1958]). The yield was 49.25 gm (89%). The compound showed a single component in thin-layer chromatographic analysis with two different solvent systems (chloroform and 10% ether in benzene). The following spectra were recorded: infrared, $v_{\text{max}}$ = 1730, 1455-1470 doublet, 1255-1275 doublet, 1120, 710 cm$^{-1}$; nuclear magnetic resonance, 5.05 (b, lH, C-3-H), 5.60 (d, lH, C-7-H), 5.72 (d, lH, C-6-H), 7.62 and 8.19 (m, 5H, aromatic); ultravio-
let, $\lambda_{max.} = 262 (10,330), 271 (14,300), 282 (14,650), 293 (7,590)$; mass, 381 (40%); M - C6H5CO, 351 (9%), 253 (25%), 158 (100%), 143 (78%), 105 (93%).

**Cholesta-7,14-dien-3β-yl benzoate**

7-Dehydrocholesteryl benzoate (48 gm) was dissolved in chloroform (400 ml) in an Erlenmeyer flask (1 l). The flask was immersed to the level of the solution in a dry ice-acetone-ethylene glycol bath. Gaseous hydrogen chloride was bubbled through a drying tower filled with concentrated sulfuric acid and thence through the steroid solution for two hours. The solution gradually became dark purple over the course of the reaction. At the end of two hours, the mixture was poured into a separatory funnel and allowed to sit at room temperature eighty minutes in a hood. It was then washed with seven equal volume portions of water. During the washings the color lightened through rose and light green to an orange-yellow. The organic layer was dried over anhydrous magnesium sulfate and filtered to remove the drying agent. The solution was reduced in volume to 200 ml by evaporation under nitrogen while being heated on a steam bath. Methanol (250 ml) was added and the solution set aside to crystallize. After fifteen minutes, the mixture was set in a freezer (-20°C). Several hours later, the product was collected by suction filtration, washed three times (60 ml) with ice-cold methanol and dessicated in vacuo over calcium chloride. The product recovered was 33.5 gm (70% yield).
which melted at 148-149°C (literature values, 148-150°C; Knight et al. [1966] and Parish et al. [1977]). The compound showed a single component on thin layer chromatographic analysis in two solvent systems (35% ethyl acetate in chloroform and 10% ether in benzene). The following spectra were recorded: infrared, \( \lambda_{\text{max}} = 1724, 1688, 1605, 1588, 1281, 717\ \text{cm}^{-1} \); nuclear magnetic resonance, 4.95 (m, 1H, C-3-H), 5.60 (m, 1H, C-15-H), 5.85 (m, 0.8H, C-7-H), 7.75 (m, 5H, aromatic); ultraviolet, \( \lambda_{\text{max}} = 232 (26,000) \); mass, 488 (40%; M), 473 (6%; M-CH\(_3\)), 375 (83%; M-side chain), 366 (16%), 361 (37%), 253 (100%). Analysis by nuclear magnetic resonance spectroscopy of the signal due to the C-7 vinyl proton present in cholesta-7,14-dien-3β-yl benzoate, but absent in the 8,14-diene isomer, indicated that the sample contained approximately 75-80% of the 7,14-diene; the remainder was assumed to be the 8,14-diene isomer (viz. Parish et al., [1977]).

**Cholest-7-en-14α,15α-epoxy-3β-yl Benzoate**

The hydrogen chloride isomerization product from the previous step (32 gm) was dissolved in previously dried ether (1500 ml) on a steam bath. When the solution had cooled to 18°C in an ice-water bath, a solution of technical m-chloroperbenzoic acid (26.3 gm; Aldrich) in absolute ether (300 ml) was added. The combined mixture was swirled for a few minutes in the ice bath and more ice was added to totally immerse the solution in the bath. The top of the flask was covered and the mixture set aside in the bath for six hours,
with ice added to the bath at intervals. At the end of this time the flask was placed in a freezer (-20°C) for sixteen hours. The long needles which crystallized from the solution during this period were collected by suction filtration and placed in a desiccator in vacuo over calcium chloride. The yield of crystalline material was 10.0 gm (31% yield) which melted at 198-200°C (literature value, 200-201°C, Parish et al. [1977]). The compound showed a single component on thin-layer chromatographic analysis in two different solvent systems (35% ethyl acetate in chloroform and 10% ether in benzene). The material was subjected to the following spectral analyses: infrared, \( \lambda_{\text{max.}} = 1718, 1604, 1586, 1282 \text{ cm}^{-1} \); nuclear magnetic resonance, 3.69 (s, 1H, C-15-H), 4.97 (m, 1H, C-3-H), 5.64 (s, 1H, C-7-H), 7.75 (m, 5H, aromatic); mass, 504 (100%; M), 489 (8%; M-M3), 382 (10%), 367 (9%).

**Cholest-8(14)-en-15-one-3β-yl Benzoate**

Cholest-7-en-14α,15α-epoxy-3β-yl benzoate (9.5 gm) was dissolved in chloroform (200 ml). Methanol (500 ml) was added and concentrated hydrochloric acid (40 ml). The mixture was refluxed fifteen minutes. At the end of the reflux, the mixture was concentrated to a syrup on a rotary evaporator under reduced pressure. Hexane was added (150 ml) and the solution was dried over anhydrous magnesium sulfate. After the desiccant had been removed by filtration, the solution was placed in a freezer (-20°C) for 48 hours. The orange-yellow crystals which formed during this time were
collected and recrystallized from hexane. A second crop of crystals was obtained by removing most of the hexane on a rotary evaporator under reduced pressure and adding a small amount of fresh hexane. The combined yield of the twice-recrystallized crops was 8.3 gm (88% yield).

This material was subjected to chromatography on a silica gel column (2 in. x 36 in., packed with 60-200 mesh silica gel in benzene). The crude ketone was applied to the top of the column in benzene (50 ml). When the material had drained onto the column bed, a solvent bulb (500 ml) was attached and filled with benzene. Fractions (100 ml) were collected. The first eight fractions were combined and yielded 1.62 gm of a yellowish material found to be a mixture of relatively non-polar materials when analyzed on a 12% silver nitrate-Silica Gel G thin-layer chromatographic system (benzene was the developing solvent). Fractions 10-20 were combined and yielded pure white material, which when recrystallized from acetone-water gave 6.6 gm (70% yield) of cholest-8(14)-en-15-one-3β-yl benzoate melting at 157-158°C (literature values, 157-158°C, Parish et al. [1977], Huntoon and Schroepfer [1960]; and 156°C Woodward et al. [1957]). The compound showed a single component on thin-layer chromatographic analysis in two solvent systems (chloroform and 10% ether in benzene). The following spectra were recorded: infrared, \( \lambda_{\text{max.}} = 1710, 1610, 1580, 710 \) cm\(^{-1}\); nuclear magnetic resonance, 4.20 (m,1H,C-7-H), 5.00 (m,1H,C-3-H); ultraviolet, \( \lambda_{\text{max.}} = 258 \) (14,500), 232 (15,200);
mass, 504 (50%; M), 486 (35%; M-\text{H}_2\text{O}), 373 (75%), 349 (66%), 251 (50%), 105 (100%).

**Cholest-8(14)-en-3\beta-ol-15-one**

Cholest-8(14)-en-15-one-3\beta-yl benzoate (2.0 gm) was dissolved in ethanol (400 ml). Water (20 ml) and concentrated sulfuric acid (60 ml) were added to the solution and the mixture heated under reflux for ten hours. After the solution cooled, it was reduced in volume to approximately 100 ml on a rotary evaporator under reduced pressure. The sterol was precipitated by the addition of 0.5 M sodium chloride solution (300 ml). The resulting precipitate was crystallized three times from acetone-water to give 1.65 gm of cholest-8(14)-en-3\beta-ol-15-one (80% yield) which melted at 147-149°C (literature values, 147.5-149°C, Raulston et al. [1976], Schroepfer et al. [1977], and 145-146°C, Wintersteiner and Moore [1943]). The compound showed a single component on thin-layer chromatographic analyses in two solvent systems (10% ether in benzene and 35% ethyl acetate in chloroform). The following spectra were recorded: infrared, \( \lambda_{\text{max.}} = 3350, 1704, 1620 \text{ cm}^{-1} \); nuclear magnetic resonance, 3.66 (m,1H,C-3-H), 4.18 (m,1H,C-7-H); ultraviolet, \( \lambda_{\text{max.}} = 258 \) (13,600); mass, 400 (29%; M), 385 (9%; M-Me), 382 (5%; M-H_2O), 367 (18%; M-Me-H_2O), 287 (17%), 269 (61%).

**Synthesis of 14\alpha- Ethyl-Cholest-7-en-3\beta,15\alpha-diol**

The synthesis of cholest-8(14)-en-3\beta-ol-15-one has
been described above. That compound, in addition to being used in several of the experiments described in these studies, also served as the starting material for the synthesis of several 14-alkylated sterols which were also used in the experiments reported herein. Chief among these sterols was 14α-ethylcholesterol-7-en-3β,15α-diol, whose synthesis is described below and is illustrated in Figure 6.

**14α-Ethylcholesterol-7-en-3β-ol-15-one**

Potassium metal (9 gm) was added to t-butanol (500 ml). When the metal had completely reacted, cholest-8(14)-en-3β-ol-15-one (5 gm) was added to the solution which was stirred at room temperature under nitrogen for thirty minutes. Ethyl iodide (60 ml) was added in one portion to the stirred solution and the stirring continued overnight. The material was poured into a mixture of ether (500 ml) and water (300 ml) in a separatory funnel. After thorough mixing, the ether layer was separated and washed twice with water (200 ml). The original water layer was re-extracted with ether twice more (500 ml). The combined ether extracts were dried over anhydrous magnesium sulfate. After removal of the drying agent by filtration, the ether was removed on a rotary evaporator under reduced pressure. The residue was subjected to chromatography on a silica gel column (2.5 x 50 cm). Using 10% ether in benzene as the eluting solvent, fractions of 10 ml volume were collected. Fractions 70-90 were pooled and after evaporation of the solvent under re-
Figure 6. Synthesis of 14α-Ethylcholest-7-en-3β,15α-diol.
duced pressure, the material was recrystallized from acetone-water to give 2.2 gm 14α-ethylcholesterol-7-en-3β-ol-15-one (44% yield) melting at 137-138°C (literature value, 137.5-138.5°C, Schroepfer et al. [1977a]). The compound showed a single component on thin-layer chromatographic analysis in two solvent systems (10% ether in benzene and 35% ethyl acetate in chloroform). The following spectral characteristics were recorded: infrared, $\lambda_{\text{max.}}$ = 3425, 1736, 1630, 1054 cm$^{-1}$; nuclear magnetic resonance, 3.60 (m, 1H, C-3-H), 6.50 (m, 1H, C-7-H); mass, 428 (15%; M), 410 (9%; M-H$_2$O), 399 (18%; M-Et), 371 (20%), 357 (11%), 353 (38%).

14α-Ethylcholesterol-7-en-3β, 15-diol

14α-Ethylcholesterol-7-en-3β-ol-15-one (1 gm) was dissolved in anhydrous ether (100 ml) and lithium aluminum hydride (2.0 gm; Aldrich) was added to the solution. The mixture was covered with filter paper and stirred two hours at room temperature. At the end of this time, ice was added carefully to decompose unreacted hydride. The lithio-aluminate salts were dissolved by the addition of small portions of 6 N sulfuric acid until the grey solids had dissolved in the water layer. The mixture was poured into a separatory funnel and the ether layer was removed. The aqueous phase was extracted with two portions of ether (100 ml). The combined ether extracts were dried over anhydrous magnesium sulfate and the drying agent removed by filtration. The ether was removed on a rotary evaporator under reduced pres-
sure. The residue was dissolved in a mixture of acetic anhydride and pyridine (1:1; 50 ml) and the resulting mixture was allowed to sit at room temperature overnight. The reaction was washed with equal volume portions of 5% aqueous hydrochloric acid, 5% aqueous sodium bicarbonate, and water. The ether solution was dried over anhydrous magnesium sulfate and, after removal of the drying agent by filtration, the ether was removed on a rotary evaporator under reduced pressure to yield 1.16 gm of white material. This material was separated into two bands by preparative thin layer chromatography on 0.50 mm Silica Gel G plates developed in benzene. The two bands were scraped separately from the plates and the steroids eluted from the silica gel with anhydrous ether. The less polar band co-migrated with authentic 14α-ethylcholest-7-en-3β,15α-diyl diacetate. The more polar band co-migrated with authentic 14α-ethylcholest-7-en-15β-ol-3β-y1 acetate.

The material from each band was dissolved in anhydrous ether (100 ml) and lithium aluminum hydride (0.50 gm) was added to each sample in small portions. After stirring well, the samples were covered and left overnight. The following day, excess hydride was decomposed by the dropwise addition of water. Sufficient 6 N sulfuric acid was added to solubilize the lithio-aluminate salts and the ether layer separated from the water layer in a separatory funnel. The water layer was extracted twice with ether (100 ml) and the combined ether extracts were dried over anhydrous magnesium
sulfate. After removal of the drying agent by filtration, the ether was removed on a rotary evaporator under reduced pressure. Each residue was recrystallized from acetone-water. The 14α-ethylcholest-7-en-3β,15α-diol (0.50 gm; 50% yield) melted at 202-203°C (literature value, 202-203°C, Schreopfer et al. [1977a]). The isomeric 14α-ethylcholest-7-en-3β,15β-diol (0.20 gm; 20% yield) melted at 83-84°C (literature value, 83.5-84.5°C, Schreopfer et al. [1977a]).

The 3β,15α-diol showed a single spot on thin-layer chromatographic analyses in two solvent systems (10% ether in benzene and 35% ethyl acetate in chloroform). The following spectral characterizations were recorded: infrared, $\lambda_{max} = 3410, 1650, 1045$ cm$^{-1}$; nuclear magnetic resonance, 3.60 (m, 1H,C-3-H), 4.35 (m, 1H,C-15-H), 5.52 (m, 1H,C-7-H); mass, 430 (3%; M), 401 (20%; M-Et), 383 (M-Et-H$_2$O).

The 3β,15β-diol also showed a single component in two solvent systems, with slightly different $R_f$ values from the 3β,15α-isomer (10% ether in benzene and 35% ethyl acetate in chloroform). The following spectral characterizations were recorded: infrared, $\lambda_{max} = 3430, 1645, 1045$ cm$^{-1}$; nuclear magnetic resonance, 3.55 (m, 1H,C-3-H), 4.10 (d, 1H, C-15-H), 5.60 (m, 1H,C-7-H); mass, 430 (9%; M), 412 (11%; M-H$_2$O), 401 (88%; M-Et), 383 (100%; M-Et-H$_2$O), 299 (19%).

**Synthesis of [2,4-$^3$H]-14α-Ethylcholest-7-en-3β,15α-diol**

In order to carry out both *in vitro* metabolism stud-
Figure 7. Synthesis [2,4-³H]-14α-Ethylcholest-7-en-3β,15α-diol.
ies and in vivo tissue uptake, distribution and metabolism studies, the availability of appropriately labeled substrates was required. Since studies of this sort were anticipated for 14α-ethylcholesterol-7-en-3β,15α-diol, this compound was prepared labeled with tritium. Of several possible approaches, the one described below (see Figure 7) was chosen because it allowed the introduction of stably-bound isotope in an unambiguous location on the sterol nucleus without the necessity of carrying a radioactive precursor through a lengthy synthetic scheme.

14α-Ethylcholesterol-7-en-15α-ol-3-one

To component 1 (250 ml) of the Bio-Dynamics Cholesterol oxidase solution (16 ml, component 3 of the Auto Test) and the resulting mixture was diluted with distilled water (734 ml). 14α-Ethylcholesterol-7-en-3β,15α-diol (100 mg) in isopropanol was added and the resulting mixture was incubated with shaking at 37°C for 4.5 hours. The mixture was extracted twice with chloroform (100 ml) and the volume of the combined extracts was reduced to approximately 6 ml on a rotary evaporator under reduced pressure. This material was subjected to preparative thin-layer chromatography using ether as the developing solvent. The desired ketone was eluted from the plate with ether. The residue obtained upon evaporation of the solvent under reduced pressure was recrystallized twice from methanol-ether to give 14α-ethylcholesterol-7-en-15α-ol-3-one (85.3 mg, 85% yield) melting
at 114-115°C. The following spectral characterizations were performed: infrared, $\lambda_{\text{max}} = 3350, 2930, 1715, 1645, 1375, 1260, 1120, 1050 \text{ cm}^{-1}$; nuclear magnetic resonance, 4.35 (m, 1H,C-15-H), 5.56 (m,1H,C-7-H); mass, 428 (3%, M), 410 (3%; M-H$_2$O), 399 (25%; M-Et), 381 (100%; M-Et-H$_2$O), 204 (19%); high resolution mass spectral analysis 428.3667 (calculated for C$_{29}$H$_{48}$O$_2 = 428.3654$). The compound showed a single component on gas-liquid chromatographic analysis on a 3% OV-17 column at 250°C with a retention time (relative to cholestane) of 7.50. It also showed a single component on thin-layer chromatographic analysis in chloroform-ethyl acetate (65:45) with an $R_f = 0.52$. The mass, infrared, and nuclear magnetic resonance spectra are reproduced in Figures 8-10. The presence of the ketone function on a six-membered ring (ring A) as opposed to a five-membered ring (ring D) is clearly shown by the infrared carbonyl stretching frequency of 1715 cm$^{-1}$. The isomeric 3$\beta$-ol-15-one shows a corresponding carbonyl stretching frequency of 1736 cm$^{-1}$ (Schroepfer et al.[1977c]). This demonstrates that the hydroxyl group oxidized was the one at C-3, rather than the one at C-15. The isomeric 3$\beta$-ol-15-one is also well resolvable from the 15$\alpha$-ol-3-one by gas-liquid chromatographic analysis on 3% OV-17 at 250°C. Under these conditions the two compounds have retention times (relative to cholestane) of 6.15 and 7.50 respectively.
Figure 8. Mass Spectrum of 14α-Ethylcholest-7-en-15α-ol-3-one.
Figure 9. Infrared spectrum of 14α-Ethylcholest-7-en-15α-ol-3-one.
Figure 10. Nuclear Magnetic Resonance Spectrum (60 MHz) of 14\(\alpha\)-Ethylcholest-7-en-15\(\alpha\)-ol-3-one.
[2,4-\(^3\)H]-14α-Ethylcholest-7-en-15α-ol-3-one

14α-Ethylcholest-7-en-15α-ol-3-one (20 mg) was placed in a test tube with anhydrous sodium methoxide (approximately 50 mg). Redistilled dioxane (4 ml; previously stored over molecular sieves) was added and a septum used to close the tube. The sample was shaken on a vortex mixer intermittently for about five minutes to insure solution of the steroid. The sodium methoxide remained insoluble. Tritium-labeled water (20 ul of a 1 Ci/ml solution) was added through the septum and the sample reshaken. The mixture was allowed to sit three days at room temperature and mixed on a vortex mixer at irregular intervals.

The sample was poured into a separatory funnel which contained ether (50 ml) and water (50 ml). The tube was rinsed with ether and with water and the washings added to the contents of the separatory funnel. The layers were mixed together well, and after separation of the layers, the aqueous phase was reextracted three times with ether (50 ml). The combined ether layers were washed twice with water (75 ml). The washed ether was dried over anhydrous magnesium sulfate which was removed by filtration. The labeled ketone was reduced in situ by lithium aluminum hydride as described in the following section.

[2,4-\(^3\)H]-14α-Ethylcholest-7-en-3β,15α-diol

The labeled keto-steroid in ether solution obtained
from the previous reaction was reduced in situ by addition of lithium aluminum hydride (0.50 gm) added in spatula-tip portions. The hydride-ether suspension was stirred well, covered, and left to sit overnight. The following day, excess hydride was decomposed by the cautious dropwise addition of water. The lithio-aluminate salts were dissolved by the addition of 6 N sulfuric acid solution until the pH of the aqueous phase was between pH 1-2. The layers were separated in a separatory funnel, and the water layer was reextracted three times with ether (50 ml). The combined ether layers were washed with water twice (100 ml) and then dried over anhydrous magnesium sulfate.

The material obtained by evaporation of the ether was recrystallized from acetone-water. The recrystallized sterol melted at 201-203°C and was recovered in a yield of 60% (12 mg). Analysis by gas-liquid radiochromatography on a column of 3% OV-1 at a temperature of 270°C, showed that there were two peaks of radioactivity in a ratio of 14:86. The larger peak coincided in retention time with authentic 14α-ethylcholest-7-en-3β,15α-diol. The smaller peak was presumed to be the 3α-hydroxy isomer arising as the minor product of the reduction of the 3-ketone.

Separation of the two compounds was possible by preparative thin-layer chromatography of their bis-trimethylsilyl ethers. The derivatized steroids were prepared by reaction of the radioactive sample with Carter's silylating reagent (0.25 ml) as described in the Methods section. The
reagent and the sample were heated to 80-90°C for ten minutes in a sealed tube to insure complete derivatization. The soluble portion of the reaction mixture was spotted on pre-treated Silica Gel G plates (vide infra) and the plates developed in hexane. The bands were visualized by brief exposure to iodine vapor. The band co-migrating with an authentic standard of the bis-trimethylsilyl ether of 14α-ethylcholest-7-en-3β,15α-diol was scraped from the plate and the steroid was eluted from the silica gel with ether. Gas-liquid radiochromatography of this material on a column of 3% OV-1 at a temperature of 270°C showed a single peak of radioactivity whose retention time corresponded to that of the bis-trimethylsilyl ether of a 14α-ethylcholest-7-en-3β,15α-diol standard. The gas-liquid radiochromatographic profiles of the crude material and the purified material are shown in Figures 11 and 12.

The Silica Gel G plates used in separating the bis-trimethylsilyl ethers of the isomeric reduction products were pre-treated in the following manner. A plate was developed in 2% pyridine in hexane. When the solvent had reached the top of the plate, the plate was removed from the chromatography tank and the hexane allowed to evaporate. The sample was then immediately applied to the plate and the plate developed in hexane alone as described above.
Figure 11. Gas-liquid Radiochromatographic Profile of Crude [2,4-$^3$H] - 14α-Ethylcholest-7-en-3β,15α-diol.
Figure 12. Gas-liquid Radiochromatographic Profile on the bis-Trimethylsilyl Ether of Purified 14α-Ethylcholest-7-en-3β,15α-diol. The mass tracing is from the bis-trimethylsilyl ether of an authentic sample of 14α-ethylcholest-7-en-3β,15α-diol.
IN VIVO STUDIES:

I. The Administration of Cholest-8(14)-en-3β-ol-15-one in Olive Oil to Rats
The Effects of Subcutaneous Administration of Cholest-8(14)-en-3β-ol-15-one in Olive Oil to Male Sprague-Dawley Rats on Serum Cholesterol Levels and Hepatic Sterol Synthesis

The following experiments were performed in order to determine the effects of subcutaneous administration of cholest-8(14)-en-3β-ol-15-one in olive oil (USP) on the serum cholesterol levels and hepatic sterol synthesis of male Sprague-Dawley rats. The animals in these experiments were all maintained on a strict light-dark cycle (0600-1800 light) for at least a week before the experiments were begun. During this time the animals were allowed access to food and water ad libitum. The diet used was Purina Rat Chow (the name of this diet was later changed to Purina Formula # 5008 Laboratory Chow).

Experiment 1

Twenty-four male Sprague-Dawley rats (100-150 gm; from TIMCO Laboratories) were divided into four groups of six animals each. The control group was given USP olive oil (0.2 ml) in subcutaneous injection daily at 0900. One of the experimental groups received similar injections of a solution of cholest-8(14)-en-3β-ol-15-one (10 mg/ml) in olive oil (0.2 ml injections) daily. The other experimental groups received different amounts of this steroid solution daily (0.5 ml = 5 mg, and 1.0 ml = 10 mg). Blood samples were taken from the tail of each rat every third day. The
serum cholesterol concentration was determined by the method of Abell et al. (1952) (see Materials and Methods). Table II shows the serum cholesterol values of each group as a function of time. The rats were weighed daily throughout the experiment. There was no significant difference in the weights of the four groups of animals at the beginning \((p > 0.30)\) or at the end \((p > 0.10)\) of the experiment. A graph of the animals' average weights by groups is presented in Figure 13.

The initial decrease (DAY0 vs. DAY3) in serum cholesterol levels in the control group (presumably due to the effect of the olive oil vehicle) was significant at 0.02 < p < 0.05. There was no significant difference between the initial serum cholesterol levels of any two groups \((p > 0.20)\). On each day after DAY 0, the serum cholesterol levels of the groups receiving 5 mg/day and 10 mg/day of cholest-8(14)-en-3β-ol-15-one were significantly lower than those of the control group \((p < 0.02\) or better). The serum cholesterol levels of the group receiving 2 mg/day cholest-8(14)-en-3β-ol-15-one were consistently lower than those of the control group, but due to the large standard deviations, the effects were only significant at 0.02 < p < 0.05. It should be noted that the control animals received the same volume of olive oil as the groups receiving 2 mg/day of the steroid; the groups receiving 5 mg/day and 10 mg/day of the steroid received correspondingly greater amounts of olive oil.
Figure 13. The Average Daily Weights of Control Animals (o—o), and Animals Receiving Cholest-3(14)-en-38-ol-15-one by Daily Subcutaneous Injections (+——+, 2 mg/day), (x—x—x, 5 mg/day), (Δ——Δ——Δ, 10 mg/day).
### TABLE II.

The Effect of Subcutaneous Administration of Cholest-8(14)-en-3β-ol-15-one in Olive Oil on Serum Cholesterol Levels of Male Rats (N=6).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>DAY</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>88.0 ± 2.4</td>
<td>79.0 ± 3.9</td>
<td>74.7 ± 3.2</td>
<td>75.9 ± 1.4</td>
<td>75.1 ± 2.4</td>
</tr>
<tr>
<td>2 mg/day</td>
<td>0</td>
<td>85.1 ± 3.2</td>
<td>68.2 ± 3.0</td>
<td>66.1 ± 2.5</td>
<td>63.0 ± 2.2</td>
<td>64.9 ± 2.4</td>
</tr>
<tr>
<td>5 mg/day</td>
<td>0</td>
<td>86.3 ± 4.3</td>
<td>61.6 ± 3.6</td>
<td>63.0 ± 4.5</td>
<td>56.6 ± 2.3</td>
<td>56.1 ± 3.8</td>
</tr>
<tr>
<td>10 mg/day</td>
<td>0</td>
<td>83.9 ± 1.3</td>
<td>67.9 ± 2.3</td>
<td>60.1 ± 2.5</td>
<td>57.4 ± 3.7</td>
<td>55.3 ± 2.8</td>
</tr>
</tbody>
</table>

The values of the serum cholesterol levels for each group on each day are expressed in mg cholesterol/100 ml serum (mg percent). The statistical correlations for the control group are compared with DAY 0 for the control group. The statistical correlations for the other groups are compared to the control group on the same day.
Experiment 2

Sixteen male Sprague-Dawley rats (100-150 gm, from TIMCO Laboratories) were divided into two groups of eight animals each. The control group received USP olive oil (0.5 ml) in subcutaneous injections daily at 0900. The experimental animals received similar injections of a solution of cholest-8(14)-en-3β-ol-15-one (10 mg/ml) in olive oil (0.5 ml injections) daily. Blood samples were taken from the tail of each rat every third day. The serum cholesterol content was determined by the method of Abell et al. (1952) (see Materials and Methods). The rats were weighed daily throughout the experiment. There was no significant difference in weight between the two groups at the beginning (p > 0.40) or at the end (p > 0.20) of the experiment. A graph of the animals' daily weights is presented in Figure 14. Table III records the serum cholesterol levels of each group. There was no significant effect of the steroid on the serum cholesterol levels until Day 9, but from that day until the end of the experiment (with the exception of Day 18 at which p < .10), the differences between the average serum cholesterol values of each group were statistically significant (p < 0.01 or better).

On Day 25, the animals were killed by decapitation beginning at 0800. The following organs were removed, rinsed with physiological saline, damp-dried and weighed: liver, adrenals, kidneys, and the proximal 60 cm of the small intestine. After they had been weighed, the organs were placed
Figure 14. The Average Daily Weights of Control Animals (x—x—x) and Experimental Animals (o—o—o).
TABLE III.

The Effect of Subcutaneous Administration of Cholest-8(14)-en-3β-ol-15-one in Olive Oil on Serum Cholesterol Levels of Male Rats (N=8).

<table>
<thead>
<tr>
<th>DAY:</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>15</th>
<th>18</th>
<th>24</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>80.5±3.0</td>
<td>76.1±2.2</td>
<td>73.4±2.6</td>
<td>82.7±2.0</td>
<td>79.1±1.5</td>
<td>75.4±0.7</td>
<td>72.5±1.6</td>
<td>75.2±1.8</td>
<td>80.4±1.7</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>84.1±2.3h</td>
<td>76.5±2.2n</td>
<td>76.5±1.4g</td>
<td>72.6±3.3d</td>
<td>65.3±4.4b</td>
<td>68.9±2.5a</td>
<td>69.1±1.1e</td>
<td>64.1±1.1a</td>
<td>67.4±2.6a</td>
</tr>
</tbody>
</table>

The statistical correlations are discussed in the text.
in vials and frozen by immersion in liquid nitrogen. Table IV records
the average weights of the organs removed from the animals in each
group. In no case were the differences in weights of the organs
significant.

The sterols in the serum samples of the individual animals were
analyzed by gas-liquid chromatography on a column of 3% OV-1 at
250 C. Cholesterol was found to comprise greater than 99% of the sterol
fraction of the sera from the control and experimental animals. The
serum samples were those taken on the day of sacrifice.
TABLE IV.

The Effect of Subcutaneous Administration of Cholest-8(14)-en-3β-ol-15-one in Olive Oil on the Weights of Selected Organs in Male Rats (N=8).

<table>
<thead>
<tr>
<th>Group</th>
<th>Organs (weights in gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Control</td>
<td>11.41 ± 0.92</td>
</tr>
<tr>
<td>Experimental</td>
<td>11.26 ± 0.42^n</td>
</tr>
</tbody>
</table>

The statistical correlations showed no significant differences between the weights of any of the organs in the experimental compared to the control groups.
Experiment 3

In this experiment the effect of daily administration of cholest-8(14)-en-3β-ol-15-one in olive oil to male Sprague- Dawley rats on hepatic sterol synthesis was monitored as a function of time.

Eight male Sprague-Dawley rats (Sprague-Dawley, WI) in each group were maintained on a cholesterol-free diet and water ad libitum for eight days prior to the experiment. The animals were kept on a strict light-dark cycle (0600-1800 light) throughout this time. The control animals were given subcutaneous injections of U.S.P. olive oil (0.25 ml) at either 8, 24, 48 or 72 hours before sacrifice. The experimental animals received an equal volume of olive oil containing cholest-8(14)-en-3β-ol-15-one (5 mg) in similar subcutaneous injections at corresponding times. The 8 hr and 24 hr groups received one injection. The 48 hr group received two injections 24 hours apart, and the 72 hr group received three injections 24 hours apart. The animals in the 8 hr groups (control and experimental) were sacrificed at 2230; the other groups were sacrificed between 0800-0900.

After the animals had been sacrificed by decapitation, their livers were excised, washed with physiological saline, damp-dried and weighed. A 2.5 x volume of incubation buffer (see Materials and Methods) was added to each liver which was minced with scissors and homogenized ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle at 560 rpm. The livers and homogenates were
kept on ice throughout these operations until all the animals' livers had been prepared. The homogenates were centrifuged five minutes at 500 x g (2°C) and the supernatant fractions derived therefrom were centrifuged thirty minutes at 10,000 x g (2°C).

Incubation tubes (screw-capped) were prepared for each liver preparation containing the following solutions: a cofactor stock solution (0.10 ml); and a substrate stock solution (0.10 ml) of either sodium $[^3]$H-acetate (10 mCi/4 mg/ml) or sodium [2-$^{14}$C]-mevalonate (10 uCi/60 ug/ml). The final concentrations of the substrates were 2.44 mM and 17.5 µM respectively. To the incubation tubes containing these solutions were added the 10,000 x g supernatant preparations from each rat (1.8 ml). After the liver preparation had been added and the samples mixed on a Vortex mixer, the tubes were flushed five seconds with a strong stream of oxygen, capped, and incubated two hours in a water bath (37°C) with shaking (90 osc/min).

At the conclusion of the incubation, a solution of cholesterol in ethanol (1 ml; 4 mg/ml) and a 15% ethanolic potassium hydroxide solution (3 ml) were added to each sample. The tubes were tightly recapped, mixed well, and heated three hours in a water bath (70-75°C). After this hydrolysis, water (6 ml) was added to each sample and the non-saponifiable lipids were isolated by three extractions with hexane (15 ml). The combined hexane layers were washed twice with water (10 ml). The hexane was removed by evapora-
tion under nitrogen and the residue used for the isolation of digitonin-precipitable sterols (see Materials and Methods). Table V shows the incorporation of acetate and mevalonate into digitonin-precipitable sterols as a function of time.

The difference between the incorporation of mevalonate in preparations from control and experimental animals was insignificant ($p > 0.50$ or better) at each time point, whereas the difference in incorporation of acetate was significant ($p < 0.001$) at each time point.

The difference between the incorporation of acetate in preparations from control animals killed at different time points was significant only for the 24 hr point ($p < 0.001$) compared to each other group. In preparations from experimental animals, the difference between the incorporation of acetate at 8 hr and 24 hr was insignificant ($p > 0.20$) as was the difference in incorporation at 24 hr and 48 hr, the difference at 48 hr and 72 hr, and the difference at 24 hr and 72 hr ($p > 0.20$). However, the incorporation at 8 hr was significantly ($p < 0.01$) lower than that at 48 hr or 72 hr.

There was no significant difference between the incorporation of mevalonate into digitonin-precipitable sterols at 24 hr and 48 hr in preparations from either the control or experimental animals. However, in preparations from both groups of animals, the incorporations at 8 hr and at 72 hr were significantly different from each other ($p < 0.001$) and from the incorporations at 24 hr and 48 hr ($p < 0.001$).
TABLE V.

The Effect of Subcutaneous Administration of Cholest-8(14)-en-3β-ol-15-one (5 mg/day) on Hepatic Sterol Synthesis in Male Rats (N=8).

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>[³H]-Acetate</th>
<th></th>
<th>[²-¹⁴C]-Mevalonate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>8</td>
<td>360 ± 70</td>
<td>1360 ± 110&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1770 ± 320 k</td>
<td>1630 ± 80&lt;sup&gt;k&lt;/sup&gt;</td>
</tr>
<tr>
<td>24</td>
<td>4320 ± 560&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1940 ± 650&lt;sup&gt;a,g&lt;/sup&gt;</td>
<td>7720 ± 330&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7540 ± 540&lt;sup&gt;1,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>48</td>
<td>770 ± 170&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2440 ± 900&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7740 ± 570&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7160 ± 750&lt;sup&gt;1,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>72</td>
<td>900 ± 160&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2680 ± 680&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5640 ± 490&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5750 ± 650&lt;sup&gt;m,a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

In the statistical correlations, the comparisons in each control group are to the value at the 8 hour time point. In the experimental groups, the first correlation is to the value in the control group at the corresponding time point; the second correlation is to the value in the experimental group at the 8 hour time point.
Experiment 4

In this experiment the results of the previous study were reinvestigated.

Eight male Sprague-Dawley rats (Sprague-Dawley, WI) in each group were maintained on a diet of Purina formula # 5008 Laboratory Chow and water ad libitum for five days before the start of the experiment. During this time, the animals were maintained on a strict light-dark cycle (0600-1800 light). The control animals were injected with U.S.P. olive oil (0.20 ml) subcutaneously; the experimental animals received an equal volume of olive oil containing cholest-8(14)-en-3β-ol-15-one (5 mg) in similar subcutaneous injections. The 6 hr, 18 hr, and 24 hr groups were injected once at 1700, 0500, and 2300 respectively of the day and the night before they were sacrificed at 2300. The other groups received daily injections at 2300 until the day of their sacrifice.

The animals were sacrificed at 2330-2345 on the appropriate day and liver preparations made as described in the previous experiment. These preparations were used to assay sterol synthesis from sodium \(^3\text{H}\)-acetate just as described above (Experiment 3). Table VI shows the effect of the subcutaneous administration of cholest-8(14)-en-3β-ol-15-one (5 mg/day) in olive oil on the incorporation of sodium acetate into digitonin-precipitable sterols as a function of time.

The difference in incorporation of acetate into
digitonin-precipitable sterols between the control and experimental groups was significant at each time point \( p < 0.001 \). The differences in incorporation of acetate into digitonin-precipitable sterols in the experimental groups were significant between each set of time points \( p < 0.001 \) except at 18 hr and 24 hr \( p > 0.40 \). The differences in incorporation of acetate into digitonin-precipitable sterols in the control groups were significant \( p < 0.001 \) between each set of time points except at 5 days and 15 days \( 0.02 < p < 0.05 \) at which points the difference was of less significance.
TABLE VI.


<table>
<thead>
<tr>
<th>Time</th>
<th>Experimental (dpm/mg protein)</th>
<th>Control (dpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr</td>
<td>$99,800 \pm 4,000^a$</td>
<td>$77,450 \pm 5,090$</td>
</tr>
<tr>
<td>18 hr</td>
<td>$188,600 \pm 8,700^{a,a}$</td>
<td>$104,200 \pm 8,700^a$</td>
</tr>
<tr>
<td>24 hr</td>
<td>$185,200 \pm 6,600^{a,a}$</td>
<td>$162,900 \pm 6,600^a$</td>
</tr>
<tr>
<td>36 hr</td>
<td>$210,300 \pm 19,200^{a,a}$</td>
<td>$112,200 \pm 10,000^a$</td>
</tr>
<tr>
<td>48 hr</td>
<td>$244,800 \pm 16,300^{a,a}$</td>
<td>$147,200 \pm 2,500^a$</td>
</tr>
<tr>
<td>5 days</td>
<td>$102,400 \pm 11,000^{a,i}$</td>
<td>$31,500 \pm 2,000^a$</td>
</tr>
<tr>
<td>10 days</td>
<td>$77,600 \pm 2,400^{a,b}$</td>
<td>$29,000 \pm 700^a$</td>
</tr>
<tr>
<td>15 days</td>
<td>$60,700 \pm 3,600^{a,b}$</td>
<td>$33,300 \pm 1,000^a$</td>
</tr>
</tbody>
</table>

In the control group, the statistical correlations are between the values at each time point and that of the 6 hour point. In the experimental group, the first correlation is between the value of the experimental group and the value at the corresponding time in the control group; the second correlation is between the value at each time point and that of the 6 hour point.
Experiment 5

In this experiment, the work described in Experiment 4 was repeated in order to determine whether or not it was reproducible.

Eight male Sprague-Dawley rats (Sprague-Dawley, WI) in each group were maintained on a diet of Purina formula # 5008 Laboratory Chow and water ad libitum for twelve days before the start of the experiment. During this time, the animals were maintained on a strict light-dark (0600-1800 light) cycle. The control animals were injected subcutaneously with U.S.P. olive oil (0.20 ml); the experimental animals received an equal volume of olive oil containing cholest-8(14)-en-3β-ol-15-one (5 mg) in similar injections. Except for the 6 hr and the 36 hr groups, all rats were injected at 2230-2300. The 6 hr groups were injected at 1730 of the day of sacrifice; the 36 hr groups were injected at 1130 of the day prior to the sacrifice and again at 1130 of the day of sacrifice. All the animals were sacrificed at 2330-2345 on the appropriate day.

When the animals were sacrificed, the same experimental procedure was followed in preparing the liver 10,000 x g equivalent fractions as was used in Experiment 3. The same assay described in that experiment was used to monitor the incorporation of sodium $[^3H]$-acetate into digitonin-precipitable sterols. Table VII shows the effect of subcutaneous administration of cholest-8(14)-en-3β-ol-15-one (5 mg/day) in olive oil on the incorporation of sodium acetate
TABLE VII.

The Effect of Subcutaneous Administration of Cholest-8(14)-en-3β-ol-15-one (5 mg/day) on the Incorporation of Sodium [3H]-Acetate into Digitonin-Precipitable Sterols in Male Rats' Liver Preparations (N=8).

<table>
<thead>
<tr>
<th>Time</th>
<th>Experimental (dpm/mg protein)</th>
<th>Control (dpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr</td>
<td>144,000 ± 9,000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>161,000 ± 9,100</td>
</tr>
<tr>
<td>24 hr</td>
<td>243,000 ± 7,400&lt;sup&gt;a,a&lt;/sup&gt;</td>
<td>83,000 ± 7,200&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>36 hr</td>
<td>224,000 ± 7,000&lt;sup&gt;a,a&lt;/sup&gt;</td>
<td>134,000 ± 3,500&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 days</td>
<td>195,000 ± 13,000&lt;sup&gt;a,a&lt;/sup&gt;</td>
<td>128,000 ± 7,600&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 days</td>
<td>144,000 ± 3,800&lt;sup&gt;a,n&lt;/sup&gt;</td>
<td>76,000 ± 3,800&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 days</td>
<td>105,000 ± 3,800&lt;sup&gt;a,a&lt;/sup&gt;</td>
<td>54,000 ± 2,000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>15 days</td>
<td>155,000 ± 1,900&lt;sup&gt;a,a&lt;/sup&gt;</td>
<td>25,000 ± 1,900&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

In the control group, the statistical correlation is between the value at each time point and that of the 6 hour point. In the experimental group, the first correlation is between the value of the experimental group and the value of the control group at the corresponding time; the second correlation is between the value at each time point and that of the 6 hour time point.
into digitonin-precipitable sterols as a function of time.

The difference in incorporation of acetate into digitonin-precipitable sterols between the control and the experimental groups was significant at each time point (p < 0.01 for the 6 hr group; p < 0.001 for all other time points). The differences in incorporation of acetate into digitonin-precipitable sterols in the experimental groups were significant between each set of time points (p < 0.001) except at 6 hr and 5 day (p > 0.90). The differences in incorporation of acetate into digitonin-precipitable sterols in the control groups were significant between each set of time points (p < 0.001) except at 36 hr and 2 day (0.02 < p < 0.05), at which times the difference was of less significance.

The results of Experiments 4 and 5 may be combined since the animals were all on the same regimen and were all sacrificed at the same point in their light-dark cycle. To allow for possible systemic variations throughout the course of one experiment, Table VIII presents the combination of the results of these experiments expressed as the ratio of the acetate incorporation into digitonin-precipitable sterols of the experimental groups to that of the control group.
TABLE VIII.

The Effect of Subcutaneous Administration of Cholest-8(14)-en-3β-ol-15-one (5 mg/day) on the Ratio of the Incorporation of Acetate into Digitonin-Precipitable Sterols in the Experimental Groups to that of the Control Groups (N=8, or N=16).

<table>
<thead>
<tr>
<th>Time</th>
<th>Ratio of Experimental to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr</td>
<td>1.28</td>
</tr>
<tr>
<td>18 hr</td>
<td>1.81</td>
</tr>
<tr>
<td>24 hr</td>
<td>2.03 (2.68, 1.38)</td>
</tr>
<tr>
<td>2 day</td>
<td>1.59 (1.64, 1.54)</td>
</tr>
<tr>
<td>5 day</td>
<td>2.57 (3.05, 2.09)</td>
</tr>
<tr>
<td>10 day</td>
<td>2.31 (2.05, 2.57)</td>
</tr>
</tbody>
</table>

The 6 hr and 18 hr time points are from one experiment only. The other time points were included in both experiments and the value reported is the mean value of the two experiments. The value in parentheses are the individual values from the two experiments.
Figure 15. The Effect of Subcutaneous Administration of Cholest-8(14)-en-3β-ol-15-one (5 mg/day) on the Ratio of the Incorporation of Acetate into Digitonin-Precipitable Sterols in the Experimental Groups to that of the Control Groups.
The results of the experiments described in this section have demonstrated that subcutaneous administration of cholest-8(14)-en-3β-ol-15-one (5 mg/day) in olive oil (0.5 ml) produces a significant (p< 0.01) reduction in the serum cholesterol levels of rats (approximately 20-25%) when compared to control animals receiving injections of olive oil not containing the steroid.

Hepatic sterol synthesis from acetate, assayed in 10,000 x g supernatant liver preparations, was found to be increased approximately two-to-three-fold in rats injected with cholest-8(14)-en-3β-ol-15-one at times as early as 18 hours after the initial injection. This elevated sterol synthesis continued for as long as 15 days in animals given daily injections of the steroid. However, in one experiment, at 24 hours after the initial injection, the sterol synthesis in the experimental group was less than that in the control group. The reasons for this singularity are not clear. Hepatic sterol synthesis from mevalonate was not affected at times from 8 hours to 72 hours after the initial injection in animals receiving daily injections of the steroid.

There was no effect of the steroid on the weight increases of the experimental animals compared to those of the control animals over the course of the treatments.

After the observation that cholest-8(14)-en-3β-ol-15-one inhibited sterol synthesis from acetate and caused a reduction in the level of HMG-CoA reductase activity in cul-
tured mammalian cells (Kandutsch and Chen [1975]), it was thought that the agent might be effective in lowering the serum cholesterol levels of experimental animals. Initial work indicated that this assumption was valid (Raulston et al. [1976]). The results of the experiments presented in this section further demonstrated the validity of this hypothesis. This effective lowering of the serum cholesterol levels in rats has led to the hope that the agent (or a derivative) may eventually be useful in the clinical treatment of hypercholesterolemias. An enormous amount of further testing and experimentation is obviously required, however, before any clinical trials could be expected. Still, as a result of these initial encouraging studies, extensive further examination is currently underway in the laboratory of Dr. G. J. Schroepfer, Jr.

Due to the effects on sterol synthesis from acetate and HMG-CoA reductase reported by Schroepfer et al. (1976, 1977a) administration of cholest-8(14)-en-3β-ol-15-one to experimental animals was expected to result in decreased hepatic sterol synthesis from acetate in liver preparations from animals treated with the compound. In one experiment, this result was obtained, and liver preparations from animals receiving the compound (2 mg/day) did show reduced incorporation of acetate into digitonin-precipitable sterols (30% of control values); the incorporation of mevalonate into digitonin-precipitable sterols was unchanged between preparations from control and experimental animals (Raulston et
al. [1976]). The experiments in this section have demonstrated that the lack of effect on mevalonate incorporation into digitonin-precipitable sterols was unchanged between preparations from control and experimental animals (Raulston et al. [1976]). The experiments in this section have also demonstrated that the lack of effect on mevalonate incorporation into digitonin-precipitable sterols was reproducible. However, the incorporation of acetate into digitonin-precipitable sterols was increased rather than decreased in liver preparations from experimental animals compared to control animals. This result was obtained in several experiments. It is still unclear why it was not obtained in the initial work. It is possible that repeated bleeding of the rats in the initial experiment induced a metabolic stress which caused the sterol to have an unusual effect on the animals. It is also possible that the effect might be due to substrain differences, since the rats in the initial experiment were obtained from a different source than those in the later experiments. The cofactor concentrations used in the original work were ten-fold lower than those used in the later time-course experiments. Although that difference was shown to be unimportant in incubations using liver preparations from untreated animals, it has not been shown that the difference is unimportant in incubations using liver preparations from experimental animals. Finally, the dosage used in the earlier experiments (2 mg/day) was lower than that used in the later experiments (5 mg/day). It is not known whether or not this difference is significant.
The increased incorporation of acetate into sterols is all the more unusual since the serum cholesterol levels were lowered concomitantly. This apparent paradox might have one of the following explanations. Cholest-8(14)-en-3β-ol-15-one may have different effects on sterol production in different tissues. Synthesis in the ileum, which is thought to be a major source of serum cholesterol (Dietschy et al. [1971]), may be depressed even though synthesis in the liver is elevated. An alternate possibility is that the agent may inhibit the synthesis of one or more classes of lipoproteins which are required for serum transport of cholesterol (Henderson [1963]; Shiff et al. [1971]; Mjos et al. [1975]). This would result in lower serum cholesterol levels regardless of the effect on sterol synthesis in either the liver or ileum, since only a limited amount of cholesterol would be able to be packaged for export to the blood.

It has yet to be determined which explanation best accounts for the experimental observations reported in the studies in this section. It is not unlikely that a combination of effects is responsible, and the ultimate solution to the problem may be something totally different from the ideas presented above, although these seem to be the most likely based on the evidence available at this time. But whatever the explanation for the effect, the increase in sterol synthesis from acetate in liver preparations from animals to which the sterol had been administered,
is opposite to the inhibition observed in L-cells cultured in the presence of the sterol (Schroepfer et al., [1976], [1977a]). This is the first of several instances in this thesis in which the results obtained from cell culture studies have not been extrapolatable to studies in whole animals or in vitro cell-free systems.

In a large number of dietary experiments in which cholest-8(14)-en-3β-ol-15-one was administered to rats as a powder mixed with their diet (generally at 0.1% concentration), a large effect on the serum cholesterol levels of the experimental animals was demonstrated (Kisic et al., unpublished results [1977]; Miller and Raulston, unpublished results, [1978]; Schroepfer et al. [1977d]). However, these decreases in the animals' serum cholesterol levels (generally 40% - 60% decreases) were accompanied by large decreases in the food intake of the experimental animals (approximately 50%). The weights of the experimental animals also decreased over the course of these experiments (approximately 1-2 gm/day) whereas the weights of the ad libitum control animals increased over the course of these experiments (4-5 gm/day). Pair fed animals (whose food intake was restricted to match that of the experimental animals) showed similar weight losses to those of the experimental animals, but the level of their serum cholesterol was not significantly different from the ad libitum control animals'. Thus, although the steroid causes a reduction in the serum cholesterol levels of experimental animals when administered in the diet
and by subcutaneously injection, the other effects (on food consumption, weight gain, for example) of the compound may differ according to its mode of administration.

Work from the laboratory of Professor Kandutsch has demonstrated that other oxygenated sterols such as cholest-5-en-3β,25-diol and cholest-5-en-3β-ol-7-one stimulate the synthesis of digitonin-precipitable sterols from acetate without any effect on the serum cholesterol levels of experimental animals (mice). Cholest-8(14)-en-3β-ol-15-one is, however, the first poly-oxygenated sterol reported to both lower serum cholesterol levels in treated animals while concomitantly raising hepatic sterol synthesis from acetate.
IN VIVO STUDIES:

II. The Administration of 14α-Methylcholest-7-en-3β-ol-15-one in Olive Oil to Rats
The Effects of Subcutaneous Administration of 14α-Methylcholest-7-en-3β-ol-15-one in Olive Oil to Male Sprague-Dawley Rats on Serum Cholesterol Levels and Hepatic Sterol Synthesis

The following experiment was performed in order to determine if alkylation of cholest-8(14)-en-3β-ol-15-one at the 14-position would yield a derivative which resulted in greater lowering of serum cholesterol levels than the parent substance when administered subcutaneously to rats.

Sixteen male Sprague-Dawley rats (TIMCO Laboratories) were maintained on Purina Rat Chow and water ad libitum for five days before the start of the experiment. At the beginning of the experiment they were divided into two groups of eight animals each. The control group received subcutaneous injections of U.S.P. olive oil (0.5 ml) daily. The experimental animals were similarly injected with olive oil containing 14α-methylcholest-7-en-3β-ol-15-one (0.5 ml; 5 mg). Blood samples were taken from the tail of each animal every third day. The cholesterol content of the blood was analyzed by the method of Abell et al. (1952) (see Materials and Methods). The animals were weighed throughout the experiment. Figure 16 shows the average weight of the animals in each group as a function of time. There was no significant difference in the weights of the two groups of animals at the start (p > 0.50) or at the end (p > 0.40) of the experiment. Table IX records the average serum choles-
Figure 16. The Average Weights of Control Animals (o—o—o), and of Animals Receiving 14α-Methylcholest-7-en-3β-ol-15-one (5 mg/day) (x—x—x).
TABLE IX.

The Effects of Subcutaneous Administration of 14α-Methylcholest-7-en-3β-ol-15-one in Olive Oil (5 mg/day) on the Serum Cholesterol Levels of Male Rats (N=8).

<table>
<thead>
<tr>
<th>DAY</th>
<th>CONTROL</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75.1±2.2</td>
<td>66.4±2.4</td>
<td>67.7±1.5</td>
<td>70.7±2.8</td>
<td>69.0±1.1</td>
<td>69.2±2.2</td>
<td>74.9±1.5</td>
<td>72.5±1.3</td>
<td>70.6±1.2</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>78.1±4.3</td>
<td>69.2±3.0</td>
<td>68.5±2.7</td>
<td>68.6±3.7</td>
<td>71.0±3.5</td>
<td>70.5±1.9</td>
<td>68.6±2.7</td>
<td>67.9±4.2</td>
<td>69.0±4.3</td>
</tr>
</tbody>
</table>
terol levels of each group as a function of time. In no case was the difference in the mean serum cholesterol levels of the control and experimental groups significant (p > 0.05 or better). The parallel slopes of the lines in Figure 16 demonstrate that there was no measurable effect of the compound on the animals’ weight gains.

On Day 23 of the experiment, the animals were sacrificed and in vitro incubations were performed to determine the ability of 10,000 x g supernatant liver preparations from the animals to synthesize digitonin-precipitable sterols from sodium acetate and sodium mevalonate.

The procedure used to prepare the livers was as follows. The livers from the decapitated animals were excised, rinsed with physiological saline, damp-dried and weighed. A 2.5 x volume of incubation buffer (see Materials and Methods) was added to each liver which was minced with scissors. The preparation was homogenized ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle. The homogenate was centrifuged five minutes at 500 x g and the supernatant therefrom was centrifuged thirty minutes at 10,000 x g. Both centrifugations were performed at 2°C. The 10,000 x g supernatant was used as the enzyme source in the incubations described below.

To each incubation assay tube was added the following cofactor stock solutions (0.10 ml each): NAD (11.05 mg/7.5 ml); NADP (11.50 mg/7.5 ml); ATP (34.6 mg/5.6 ml); G-6-P (20.0 mg/9.3 ml). The appropriate substrate stock solution
was then added (0.10 ml): either sodium [2-\(^{14}\)C]-acetate (120 uCi/5 mg/ml) or sodium [2-\(^{14}\)C]-mevalonate (26.7 uCi/0.5 mg/ml). The final concentrations of the substrates were 3.05 mM and 0.15 mM respectively. The incubation was begun with the addition of the 10,000 x g supernatant preparations (1.5 ml). The solutions were mixed briefly and incubated two hours in a water bath (37°C) with shaking (90 osc/min) under a gentle oxygen flow.

At the end of the incubation, the samples were prepared for hydrolysis by the addition of a 4 mg/ml solution of cholesterol in ethanol (1 ml) and a 15% ethanolic potassium hydroxide solution (3 ml). After mixing, the sample tubes were tightly capped and heated in a water bath (70-75°C) for three hours. At the end of this time, and after the addition of water (6 ml) to each tube, the samples were extracted three times with hexane (15 ml). The combined hexane layers were washed with water (10 ml) twice and the hexane was removed by evaporation under nitrogen. Digitonin-precipitable sterols were isolated from the residue as described in the Materials and Methods section. The results of these incubations are summarized in Table X.

There was no significant difference between the incorporation of mevalonate into digitonin-precipitable sterols in the control and experimental groups (p > 0.10). However, the incorporation of acetate into digitonin-precipitable sterols was significantly higher (p < 0.001) in the experimental group.
TABLE X.


<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetate Incorporation</th>
<th>Mevalonate Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>470 ± 70</td>
<td>22,000 ± 470</td>
</tr>
<tr>
<td>Experimental</td>
<td>1480 ± 250&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22,500 ± 840&lt;sup&gt;k&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
The results of this experiment have indicated that modification of cholest-8(14)-en-3β-ol-15-one by the introduction of a 14α-methyl-group results in a compound, 14α-methylcholest-7-en-3β-ol-15-one, which had no significant effect on the serum cholesterol levels of experimental rats when administered daily (5 mg) by subcutaneous injection in olive oil (0.5 ml) for 23 days.

In spite of this lack of effect on serum cholesterol levels, there was a significant \( (p < 0.001) \) increase (300%) in the incorporation of acetate into digitonin-precitable sterols by 10,000 x g supernatant preparations in livers from the experimental animals when compared to similar preparations from control animals. The incorporation of mevalonate into digitonin-precitable sterols was not significantly different in preparations from control and experimental animals.

There was no effect of administration of this sterol on the average daily weight gains of the animals in the experimental group compared to those of the animals in the control group over the course of the experiment.
THE INHIBITION OF STEROL SYNTHESIS IN L-CELLS BY 14α-ETHYLCHOLEST-
7-EN-15α-OL-3-ONE
The Inhibition of Sterol Synthesis in L-Cells by 14α-Ethylcholester-7-en-15α-ol-3-one

14α-Ethylcholester-7-en-15α-ol-3-one was prepared by the action of cholesterol oxidase on 14α-ethylcholester-7-en-3β,15α-diol as described in the Chemical Syntheses section. This compound was tested for its effects on the incorporation of acetate into digitonin-precipitable sterols in cultured mammalian cells and on the levels of the HMG-CoA reductase activity in the same cells. The results of the experiments, performed at The Jackson Laboratory, Bar Harbor, Maine, by Dr. A. A. Kandutsch, are reported below (Schroepfer et al. [1977c]).

Mouse L-cell (a subline of NCTC clone 929 mouse fibroblasts) cultures were grown in serum-free media as described in Schroepfer et al. (1977a); Kandutsch and Chen (1973); and Kandutsch and Chen (1974). The preparation of steroid-containing media, procedures for assay of the rate of conversion of [1-14C]-acetate into digitonin-precipitable sterols and fatty acids and methods for the measurement of DNA, protein, and HMG-CoA reductase were also as described in the references cited above. The L-cell cultures were pre-incubated with the test compound for 4 hours; then [1-14C]-acetate was added at a concentration of 4 umoles (4 uCi) per ml. To determine the effects of the sterol on HMG-CoA reductase of the L-cells, the sterol was incubated with the
cultures for five hours prior to harvesting them for the
determination of microsomal HMG-CoA reductase activity.

The effects of 14α-ethylcholest-7-en-15α-ol-3-one
on the rates of incorporation of [1-14C]-acetate into
digitonin-precipitable sterols and fatty acids are shown in
Figure 1. Studies of the rates of acetate metabolism to
fatty acids were made so as to detect any possible effects
of the inhibitor of sterol synthesis on general metabolism.
In an effort to correct for variations of individual cul-
tures upon the estimation of inhibitory potency, the concen-
tration required to inhibit sterol synthesis by 50% was esti-
mated from a plot (Figure 2) of the ratio of [14C]-sterols
to [14C]-fatty acids as a function of the concentration of
the inhibitor. The results of this analysis indicated that
14α-ethylcholest-7-en-15α-ol-3-one caused a 50% inhibition
of sterol synthesis at a concentration of 6 x 10^{-9} \text{ M}. A
major site of the inhibition of sterol synthesis appears to
be at the level of HMG-CoA reductase (Figure 3) since the
compound caused a 50% reduction on the level of microsomal
HMG-CoA reductase activity in the L-cells at a concentration
of 4 x 10^{-8} \text{ M}.

Prior to the work described in this study, the most
potent 15-oxygenated sterol inhibitor of sterol synthesis in
cultured cells was reported to be 14α-ethylcholest-7-en-3β,
15α-diol, which caused a 50% inhibition of sterol synthesis
in L-cells at a concentration of 5 x 10^{-8} \text{ M} (Schroepfer et
al. [1977b]). In this study, 14α-ethylcholest-7-en-15α-ol-
3-one has been found to be significantly more potent, causing a 50% inhibition of sterol synthesis in L-cells at a concentration of $6 \times 10^{-9}$ M.

Figure 1. Effect of 14a-ethyl-5a-cholest-7-en-15a-ol-3-one on the rates of incorporation of $[\text{1}^4\text{C}]$-acetate into digitonin-precipitable sterols and fatty acids in L cells grown in serum-free media.

Figure 2. Effect of 14a-ethyl-5a-cholest-7-en-15a-ol-3-one on the ratio of the incorporation of $[\text{1}^4\text{C}]$-acetate into $[\text{1}^4\text{C}]$-digitonin-precipitable sterols to the incorporation of $[\text{1}^4\text{C}]$-acetate into fatty acids in L cells grown in serum-free media.

Figure 3. Effect of 14a-ethyl-5a-cholest-7-en-15a-ol-3-one on the level of microsomal HMG-CoA reductase activity in L cells grown in serum-free media.
IN VITRO STUDIES:

I. Preliminary Experiments
Cofactors in In Vitro Synthesis Studies

In all the experiments in this thesis to which cofactors were added (for the purpose of promoting the production of non-saponifiable lipids and digitonin-precipitable sterols from acetate or mevalonate), the cofactors were added in a concentrated stock solution, which, when diluted to the final incubation volume, gave the following final concentrations of the indicated cofactors:

ATP - 5 mM
NAD - 1 mM
NADP - 1 mM
G-6-P - 3.8 mM
The Effect of Homogenization Speed on the Incorporation of Acetate into Non-Saponifiable Lipids and Digitonin-Precipitable Sterols in 10,000 x G Supernatant Fractions of Rat Liver Homogenate Preparations

In this experiment a Sears #11480 Variable Speed Reversible Drill was used as a drive for the homogenization process. It was connected to a Variac power transformer which was used to operate the drill at different speeds in order to determine the optimal speed at which the homogenization should be performed.

Livers from three male Sprague-Dawley rats (150-200 gm; Sprague-Dawley, WI), which had been maintained on a cholesterol-free diet and water ad libitum for a week prior to sacrifice, were excised and rinsed with physiological saline solution. The livers were damp-dried, weighed, and combined. The combined tissues were minced with scissors and divided into five equal portions by weight. Each portion was added to a 2.5 x volume of incubation buffer (see Materials and Methods) and homogenized ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle. The pestle was driven by the drill whose speed was regulated by the Variac power transformer. One liver portion was homogenized at each of the following Variac settings: 30, 40, 50, 60, and 70. With the drill set on maximum, these settings corresponded nominally to 360, 480, 600, 720 and 840 rpm. Each homogenate was centrifuged five
minutes at 500 x g (2°C). The supernatants from this cen-
trifugation were centrifuged again at 10,000 x g for thirty
minutes (2°C). These supernatants were the enzyme prepara-
tions used in the assay.

To each incubation tube was added the following co-
factor stock solutions (0.10 ml): ATP (125 mg/2 ml); G-6-P
(46 mg/2 ml); NAD (30 mg/2 ml); NADP (32 mg/2 ml). The
substrate was sodium [3H]-acetate (1 mCi=0.7 mg). The final
concentration of the substrate in the incubation medium was
4.3 mM. The appropriate 10,000 x g supernatant (1.5 ml) was
added to each of three tubes per group, the tubes flushed
five seconds with a strong stream of oxygen and capped. The
samples were incubated two hours in a water bath (37°C) with
shaking (90 osc per min).

At the end of two hours, a solution of cholesterol
in ethanol (1 ml; 4 mg/ml) and a 15% ethanolic potassium hy-
droxide solution (3 ml) were added to each tube. The sam-
ples were tightly recapped, mixed and heated in a water
bath for three hours (70-75°C). At the end of this hy-
drolysis, water (6 ml) was added to each tube and the non-
saponifiable lipids extracted with three portions of hexane
(15 ml). The combined hexane layers were washed with two
portions of water (10 ml) and the hexane removed by evapora-
tion under nitrogen. The samples were redissolved in a
small amount of benzene and aliquots removed for liquid-
scintillation counting. Figure 1 shows the incorporation of
acetate into non-saponifiable lipids vs. Variac setting.
Figure 1. The Incorporation of Sodium $[^3]$H-Acetate into Non-Saponifiable Lipids as a Function of Variac Setting in 10,000 x g Supernatant Fractions from Rat Liver Preparations.
The digitonin-precipitable sterols were recovered from the non-saponifiable lipids by the method discussed in Materials and Methods. A plot of the incorporation of acetate into digitonin-precipitable sterols vs. Variac setting is presented in Figure 2.

The appearance of the peak at Variac - 60 is quite marked in both the non-saponifiable lipids and the digitonin-precipitable sterols.

In order to confirm these unexpected results, the experiment was repeated using the same protocol given above, except that the only Variac settings tested were those immediately around the peak: 50, 60 and 70. A graph of the incorporation of acetate into non-saponifiable lipids vs. Variac setting is presented in Figure 3 and it also shows a very sharp peak in the incorporation at Variac = 60.

In order to determine exactly what speed the Variac settings corresponded to, the speed of the drill in rpm was measured as a function of Variac setting. The relation between the actual speed and the Variac setting proved to be linear, although there was a lag between the applied voltage and the drill speed at low speeds. Table I gives the speeds determined for a large range of Variac settings.
Figure 2. The Incorporation of Sodium $[^3\text{H}]$-Acetate into Digitonin-Precipitable Sterols as a Function of Variac Setting in 10,000 x g Supernatant Fractions of Rat Liver Preparations.
Figure 3. The Incorporation of Sodium $[^3H]$-Acetate into Non-Saponifiable Lipids as a Function of Variac Setting in 10,000 x g Supernatant Fractions of Rat Liver Preparations.
TABLE I.

The correlation of Variac setting with actual speed (as measured by a hand tachometer) was linear from Variac = 30 to Variac = 70. The following list shows the values obtained in this correlation.

<table>
<thead>
<tr>
<th>VARIAC</th>
<th>TACHOMETER (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>240 ± 3</td>
</tr>
<tr>
<td>40</td>
<td>398 ± 6</td>
</tr>
<tr>
<td>50</td>
<td>550 ± 4</td>
</tr>
<tr>
<td>60</td>
<td>663 ± 7</td>
</tr>
<tr>
<td>70</td>
<td>793 ± 5</td>
</tr>
</tbody>
</table>
The Assay of HMG-CoA Lyase Activity in the 10,000 x G Supernatant Fraction of Rat Liver Homogenate Preparations

This experiment was performed to ascertain the extent (if any) of leakage of HMG-CoA lyase activity from the mitochondria into the 10,000 x g supernatant preparations used in the assay for non-saponifiable lipid and digitonin-precipitable sterol production from acetate and mevalonate. The presence of significant amounts of the lyase would cause the consumption of HMG-CoA produced from acetate and hence lead to lower incorporation of acetate into non-saponifiable lipids.

The assay for HMG-CoA lyase was taken from Clinkenbeard et al. (1975b) and monitors the following reaction:

\[
\text{HMG-CoA} \rightarrow \text{Acetyl-CoA} + \text{Acetoacetate}
\]

The actual measurement is the loss of radioactivity of an acidified aliquot of the assay sample when taken to dryness at 95°C in an oven. Clinkenbeard et al. (1975b) reported that under these conditions HMG (the product of acid hydrolysis of HMG-CoA) was not volatile, whereas acetoacetate was.

A male Sprague-Dawley rat (150 gm; Sprague-Dawley, WI), which had been maintained on a cholesterol-free diet and water ad libitum for more than a week prior to this experiment, was killed at 0800 and his liver removed and rinsed with a physiological saline solution. After the liver
was damp-dried and weighed, it was minced in a 2.5 x volume of 0.10 M Tris buffer, pH 8.2. The tissue was homogenized ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle. The homogenate was centrifuged five minutes at 500 g (2°C) and the supernatant therefrom was centrifuged thirty minutes at 10,000 x g (2°C). This 10,000 x g supernatant (1 ml) was preincubated for five minutes at 37°C to bring it to the assay temperature. A solution of [3-14C-3-hydroxy-3-methylglutaryl]-HMG-CoA (10 ul; 49.5 mCi/mmole - 0.18 mg/ml) was then added and the mixture shaken. The final concentration of HMG-CoA in the incubation medium was 2 μM. Aliquots (100 ul) were removed at 0, 2, 4, 6, 8, and 20 minutes. The aliquots were added to scintillation vials containing 6 N hydrochloric acid (200 ul). The vials were placed in a 110°C oven for forty minutes. At the end of this time, water (200 ul) was added to each vial and the vials were swirled vigorously to promote dissolution of any non-volatile radioactivity. Toluene-ethanol scintillation fluid (10 ml) was added to each vial and the samples counted by liquid-scintillation counting.

Figure 4 shows that there was essentially no lyase activity detected by this assay in the 10,000 x g supernatant preparation. The assay has been repeated several times and no detectable lyase activity was observed (Miller, unpublished observations [1977]).
Figure 4. Assay of HMG-CoA Lyase in a 10,000 x G Supernatant Fraction from a Rat Liver Homogenate Preparation.
The amount of radioactivity at each time point is less than 2% of the amount added as substrate at time 0. See text for details of the assay.
IN VITRO STUDIES:

II. The Effects of Various Vehicles and Oxygenated Sterols on Sterol Biosynthesis in Cell-Free Preparations
The Effects of Various Vehicles and Oxygenated Sterols on \textit{In Vitro} Sterol Biosynthesis in the 10,000 x g Supernatant Fraction of Rat Liver Homogenate Preparations

The following experiments were performed in order to determine a suitable vehicle in which selected sterols (such as cholest-8(14)-en-3\beta-ol-15-one) might be added to 10,000 x g supernatant preparations from rat liver in order to test their effects on \textit{in vitro} hepatic sterol synthesis. For each experiment, the assays were performed in accordance with the following protocol.

Male Sprague-Dawley rats (TIMCO Laboratories) were maintained on a cholesterol-free diet and water \textit{ad libitum} for at least a week before the animals were sacrificed. During this time, they were maintained on a strict light-dark (0600-1800 light) cycle. The animals were always sacrificed between 0730-0900. The animals were killed by decapitation and their livers carefully excised. The livers were rinsed with physiological saline solution, damp-dried and weighed. A 2.5 x volume of incubation buffer (see \textit{Materials and Methods}) was added to the tissue which was minced with scissors. The preparation was homogenized ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle. The homogenate was centrifuged five minutes at 500 x g (2°C) and the supernatant therefrom was centrifuged thirty minutes at 10,000 x g (2°C).
Incubation tubes were prepared with a cofactor stock solution (0.10 ml) containing ATP (315 mg/ml), NAD (74.5 mg/ml), NADP (77.5 mg/ml) and G-6-P (115 mg/ml) which was adjusted approximately to neutrality by the addition of 5 N potassium hydroxide solution (4 drops/5 ml). A substrate stock solution (0.10 ml) of sodium [2-¹⁴C]-acetate (120 uCi/5.0 mg/ml) was also added to each incubation tube. The final concentration of sodium acetate in the incubation medium was 3.0 mM. Then, to each set of tubes was added either a control solution (containing only the vehicle to be tested) or a solution containing cholest-8(14)-en-3β-ol-15-one in the vehicle to be tested (0.10 ml). Finally, to each tube was added the 10,000 x g supernatant preparation (1.8 ml). The tubes were mixed briefly, flushed five seconds with a strong stream of oxygen, capped and incubated two hours in a water bath (37°C) with shaking (90 osc/min).

At the end of the incubation, a solution of cholesterol in ethanol (1 ml; 4 mg/ml) and a 15% ethanolic potassium hydroxide solution (3 ml) were added to the tubes which were recapped, mixed thoroughly and heated three hours in a water bath (70-75°C). After the samples had been removed from the bath, water (6 ml) was added to each one and the non-saponifiable lipids were extracted with three portions of hexane (15 ml). The pooled hexane layers were washed with water (10 ml) and the hexane removed by evaporation under nitrogen. The residue was dissolved in benzene and aliquots taken for liquid-scintillation counting.
Experiment 1

In this experiment the vehicle used by Kandutsch and Chen (1973) for administering oxygenated sterols to cells in culture was used to administer cholest-8(14)-en-3β-ol-15-one solutions to the in vitro sterol synthesizing system present in 10,000 x g supernatant preparations from rats' liver.

The control solution was a 5% solution of bovine serum albumin (BSA) in water (9 ml) containing 10% ethanol (1 ml). To make the steroid solutions, the steroid was first dissolved in ethanol (1 ml) and this solution added to the 5% BSA solution (9 ml). Greater dilutions of the steroid solutions were obtained by diluting the original steroid solution (in ethanol) 1:10 and 1:100 with ethanol and using these solutions (1 ml) with the albumin solution (9 ml). Table XI shows that the cholest-8(14)-en-3β-ol-15-one was effective in lowering the incorporation of acetate into nonsaponifiable lipids at $10^{-4}$ M by 23% ($p < 0.01$) but was ineffective at lower concentrations ($p > 0.40$ at $10^{-5}$ M; $p > 0.30$ at $10^{-6}$ M). The effect on ethanol on this system was tested in a second part of this experiment. Using water as a control solution, test solutions were made containing 10% aqueous ethanol and 1% aqueous ethanol. The final concentrations of ethanol in the incubation assay mixture were 8.6 mM and 8.6 mM respectively. Table XII shows that there was a large effect of ethanol on the incorporation of acetate into non-saponifiable lipids ($p < 0.001$ at each concentration).
### TABLE XI


<table>
<thead>
<tr>
<th>[Cholest-8(14)-en-3β-ol-15-one]</th>
<th>[Ethanol]</th>
<th>Incorporation into Non-Saponifiable Lipids (dpm x 10(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 M</td>
<td>86 mM</td>
<td>1.72 ± 0.08</td>
</tr>
<tr>
<td>10(^{-4}) M</td>
<td>86 mM</td>
<td>1.32 ± 0.07(^b)</td>
</tr>
<tr>
<td>10(^{-5}) M</td>
<td>86 mM</td>
<td>1.65 ± 0.03(^i)</td>
</tr>
<tr>
<td>10(^{-6}) M</td>
<td>86 mM</td>
<td>1.48 ± 0.05(^h)</td>
</tr>
</tbody>
</table>

### TABLE XII

The Effect of Ethanol on the Incorporation of Sodium [2-\(^{14}\)C]-Acetate into Non-Saponifiable Lipids (N=4).

<table>
<thead>
<tr>
<th>[Ethanol]</th>
<th>Incorporation into Non-Saponifiable Lipids (dpm x 10(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>6.26 ± 0.14</td>
</tr>
<tr>
<td>8.6 mM</td>
<td>3.44 ± 0.10(^a)</td>
</tr>
<tr>
<td>86 mM</td>
<td>2.72 ± 0.19(^a)</td>
</tr>
</tbody>
</table>
Experiment 2

In this experiment, liposomes were used as a solubilizing agent for the steroid inhibitor.

Liposomes were prepared after the method of Batzri and Korn (1973). The lecithin used in the liposome procedure was prepared as follows. Egg yolks (24; 400 ml) were separated from the whites and blended in a Waring Blender with acetone (450 ml) for one minute. The emulsion was poured into an Erlenmeyer flask (3 l) and acetone (2.5 l) was added. The mixture was swirled vigorously and stirred with a magnetic stirrer for fifteen minutes. The mixture was collected on a Buchner funnel. The acetone extract was deep yellow in color and, except for a sample reserved for analysis by thin-layer chromatography, it was discarded. One-half the filter cake was extracted in the blender for one minute with Folch solution (300 ml; chloroform:methanol, 2:1). After similar treatment of the remainder of the filter cake, the two slurries were combined and re-filtered. The total residual solid was then re-extracted with Folch solution (300 ml). Thin-layer chromatographic analysis of the acetone and Folch extractions was performed on Silica Gel G plates. The solvent used to develop the plates was chloroform:methanol:water (75:25:4). A copy of those profiles is reproduced on the following page. Due to the presence of a large amount of lecithin in the second Folch extract, the last filter cake was extracted twice more with Folch solution (300 ml). The total Folch extracts were com-
Figure 17. Thin-layer Chromatography of Lipid Fractions Isolated in Lecithin Preparation from Egg yolk. The solvent was chloroform:methanol:water (75:25:4). The channels contain aliquots from the acetone extractions, the first Folch extraction, and the second Folch extraction (in order from left to right). The spots marked A and B correspond in mobility to neutral lipids; C corresponds in mobility to phosphatidyl ethanolamine; D corresponds in mobility to lecithin.
bined and the solvent removed on a rotary evaporator under reduced pressure. The remaining lipid was a semi-solid mass and was dried overnight in vacuo over phosphorus pentoxide.

The Folch-extractable material was further purified by column chromatography. A column (2 x 50 cm) was packed with neutral alumina (100 gm; Woelm, Activity I, from ICN) in a chloroform slurry. The final packed height of the bed was 30 cm. The dried lipid obtained from the Folch extracts was weighed and transferred to a volumetric flask (250 ml) with chloroform. The solution was made to volume with chloroform. This gave a solution of 110 gm of lipid in a 250 ml solution. This solution (10 ml; 4.4 gm lipid) was applied to the top of the alumina column. Elution with chloroform was continued until no further neutral lipid was detected by thin-layer chromatographic analysis (using the solvent system described above) of the column effluent. This required 550 ml chloroform. The solvent was then changed to 5% methanol in chloroform and fourteen fractions (30 ml) were collected. The solvent was then changed to 15% methanol in chloroform and twelve more fractions (30 ml) were collected. At the end of this procedure, pure lecithin was eluted from the column with 40% methanol in chloroform. A total of 2.75 gm neutral lipid was eluted from the column. Phosphatidyl ethanolamine remained tightly bound to the column using these eluting conditions. The fractions eluted using 40% methanol in chloroform were pooled and the solvent
removed on a rotary evaporator under reduced pressure. After overnight dessication \textit{in vacuo} over phosphorus pentoxide, the recovered lecithin was a waxy white mass of weight 585 mg. Analysis by thin-layer chromatography in the solvent system described above indicated no contamination by neutral lipids or by phosphatidyl ethanolamine.

The liposomes were prepared from the following stock solutions. Solution A contained cholesterol in ethanol (0.5 mg/ml) and solution B contained lecithin in chloroform (16.7 mg/ml). Solution A (1.15 ml) was mixed with solution B (1.4 ml) and the solvents removed by evaporation under nitrogen. The residue was redissolved in absolute ethanol (1.0 ml) giving a solution containing 30 umoles lecithin and 6 umoles cholesterol.

The lecithin-cholesterol solution (0.8 ml) was taken up in a Tuberculin syringe (1 ml) with a Luer-Lok tip and injected as rapidly as possible through a 27 gauge needle under the surface of rapidly stirred deionized water (30 ml). Stirring was continued 30 seconds after the addition of the lecithin-cholesterol solution. The solution was concentrated to approximately 3 ml volume on an Amicon-52 ultrafilter using a 43 mm diameter XM-100 A membrane at a nitrogen pressure of 10 p.s.i. After the initial concentration, the retained solution was diluted with deionized water (20 ml) and the mixture again concentrated. This washing procedure was used in order to remove most of the ethanol originally present. Aliquots of the filtrate and of the retained
liquid (diluted 1:20) were analyzed by ultra-violet spectroscopy. The following figure shows that the retained liquid showed a large absorbance at approximately 204 nm (at which the original lecithin stock solution also showed an absorbance maximum), whereas the filtrate showed only a very small absorbance at 204 nm. This indicated that the bulk of the lecithin-containing material was retained by the Amicon filter.

In order to remove the last traces of ethanol, the concentrated liposome preparation was subjected to Sephadex G-25 chromatography. A column (1.6 x 20 cm) was poured with a slurry of G-25-medium Sephadex in water and allowed to pack under gravity to a bed height of 12 cm. The void volume of this column, determined as the blue dextran-2000 exclusion volume, was 8-9 ml. The concentrated liposome solution (1.0 ml) was applied to the top of the drained column bed. The column was eluted with water, from a reservoir maintained at a height of 6 cm above the top of the column bed. Fractions (1 ml) were collected and monitored at 204 nm to determine the position of the lecithin-containing material. This peak occurred in the void volume of the column, as expected.

The preparation of cholesterol-lecithin liposomes was repeated using the procedure described above with the addition of a solution of [4-\textsuperscript{14}C]-cholesterol (2 ul=0.034 mg, specific activity 56 mCi/mmole) to solution A. Before concentration of the liposome preparation on the Amicon
ultrafilter, there were $6.54 \times 10^5$ dpm total radioactivity in the preparation. From the concentrated solution was recovered $6.40 \times 10^5$ dpm with no radioactivity above background detected in the filtrate. This amounted to 98% recovery. The remainder of the activity was presumed to be on the XM-100 A filter, which was stated by the manufacturer to absorb sterols occasionally. After Sephadex G-25 chromatography, the radioactivity eluted in a smooth peak corresponding to the void volume. Of the radioactivity applied to the column, 100% was recovered in the single peak. This material was then rechromatographed on a Sepharose 4-B column prepared and calibrated as follows.

A column (2.0 x 60 cm) was poured with a thick slurry of Sepharose 4-B (Pharmacia, lot 1761) and allowed to settle under gravity. The final bed height was 52 cm. A solution of Blue Dextran 2000 (2 ml=10 mg; Pharmacia, lot 3408) was carefully applied to the top of the drained column bed. After it had run onto the column, water was added above the bed and the column inlet closed by a stopped connected by tubing to a Mariotte flask. The total distance from the air inlet tube of the Mariotte flask to the column exit tip was 70 cm. The flow rate was about 0.3 ml/min. Six minute fractions (2.0 ml) were collected. The Blue Dextran elution profile indicated that the void volume was approximately 58 ml.

The [4-^{14}C]-cholesterol liposomes were subjected to this Sepharose 4-B chromatography. The liposome solution
Figure 18. Sepharose 4-B Column Chromatography of Lecithin-[4-\(^{14}\)C]-Cholesterol Liposomes. (---○---), radioactivity; (+--+-+), absorbance at 204 nm.
Figure 19. Ultraviolet Spectroscopy of the Retained Liposome Solution from the Amicon Ultrafiltration (A); of the Filtrate from the Amicon Ultrafiltration (B); of water (C).
from the Sephadex G-25 column pooled fractions (3.0 ml; 142,000 cpm/ml) was applied to the Sepharose column and the Mariotte flask adjusted so that the air inlet to column tip distance was 70 cm. Six minute fractions (2.0 ml) were collected and an aliquot from each fraction counted by liquid-scintillation counting. The radioactivity emerged as a smooth peak which essentially coincided with the void volume. Aliquots from each fraction were also analyzed for lecithin content by recording the absorbance at 204 nm. The lecithin-containing material eluted as a smooth peak also coinciding with the void volume.

The unexpectedly large size of the cholesterol-lecithin liposome preparations (as evidenced by the void volume elution on Sepharose 4-B) led to the suspicion that the sterol component of the liposome might be responsible for the unusually large apparent size. Therefore, a liposome preparation was made without any sterol. The procedure was the one described above, except that solution A was not used. The concentrated sample from the Amicon ultrafiltration step was chromatographed on the Sepharose 4-B column prepared previously. Six minute fractions were analyzed by ultra-violet spectroscopy at 204 nm to detect lecithin. The material absorbing at 204 nm again gave a smooth peak eluting in the void volume.

It was postulated that a 27 gauge needle was perhaps so large that the lecithin in ethanol solution was not subjected to enough shear force on leaving the tip, and that
this might be responsible for the large particle size obtained. (Batzri and Korn reported an average diameter of 25 nm for the liposomes which they prepared. These liposomes eluted at a volume approximately equal to twice the void volume when chromatographed on Sepharose 4-B.) To obviate this possibility, the liposome preparation with pure lecithin was repeated using a Hamilton syringe (100 ul) as the dispensing agent. The subsequent concentration, wash, and reconcentration were performed as described above. However, Sepharose 4-B chromatography again showed that the material absorbing at 204 nm eluted with the void volume.

Even though the liposomes prepared in this manner were larger than those obtained by Batzri and Korn (1973), they were used to administer 14α-ethylcholest-7-3n-3β,15-diol to the 10,000 x g supernatant preparations used for assaying sterol synthesis. The experiment is described below.

Lecithin (33.4 mg) and 14α-ethylcholest-7-en-3β,15-diol (3.4 mg) were dissolved in absolute ethanol (1.0 ml). The solution was injected through a 27 G needle into rapidly stirred water (30 ml). The stirring was continued thirty seconds more and the solution was concentrated on an Amicon ultrafilter using an XM-100 A membrane (to 3 ml volume). Water (20 ml) was added to the concentrate, the solution mixed by swirling, and the mixture again concentrated (to 3 ml volume). A control solution was prepared in the same manner starting with lecithin (35 mg) in absolute ethanol
(1.0 ml). An aliquot of each solution was analyzed at 204 nm. Sufficient water was added to the more strongly absorbing solution (the control) until the absorbance at 204 nm was equal for each preparation. The liposome control stock solution was this solution. The $2 \times 10^{-3}$ M inhibitor solution was the solution containing the 14α-ethylcholest-7-en-3β, 15α-diol. This latter solution was diluted with the control liposome solution 1:10 and 1:100 to obtain $2 \times 10^{-4}$ and $2 \times 10^{-5}$ solutions containing 14α-ethylcholest-7-en-3β, 15α-diol.

The control solution was either water or the control liposome solution. The inhibitor experimental solutions were the 14α-ethylcholest-7-en-3β, 15α-diol containing liposome solutions. Table XIII shows that there was a 25% decrease in the incorporation of acetate into non-saponifiable lipids in the presence of $10^{-4}$ M 14α-ethylcholest-7-en-3β, 15α-diol ($p < 0.01$). Lesser concentrations of the inhibitor were significant at $0.02 < p < 0.05$ on the incorporation of acetate into non-saponifiable lipids. The slight decrease due to the liposome preparation (when compared to the water control) was insignificant ($p > 0.20$). However, in the incorporation of acetate into digitonin-precipitable sterols, there was a very large inhibition of the liposome preparation when compared to the water control (77%; $p < 0.001$). In spite of this large effect, the inhibition caused by the 14α-ethylcholest-7-en-3β, 15α-diol was statistically significant when compared to the liposome con-
trol at all three concentrations ($p < 0.001$).
TABLE XIII

The Effect of 14α-Ethylcholest-7-en-3β,15α-diol on the Incorporation of Sodium [2-\(^{14}\)C]-Acetate into Non-Saponifiable Lipids and Digitonin-Precipitable Sterols using a Liposome Preparation as Vehicle (N=4).

<table>
<thead>
<tr>
<th>[14α-Ethylcholest-7-en-3β,15α-diol]</th>
<th>Incorporation into Non-Saponifiable Lipids (dpm)</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 M *</td>
<td>5770 ± 780</td>
<td>1660 ± 500</td>
</tr>
<tr>
<td>0 M</td>
<td>5160 ± 350(^g)</td>
<td>380 ± 10(^a)</td>
</tr>
<tr>
<td>10^-6 M</td>
<td>4170 ± 170(^b)</td>
<td>320 ± 10(^a)</td>
</tr>
<tr>
<td>10^-5 M</td>
<td>4070 ± 360(^d)</td>
<td>300 ± 10(^a)</td>
</tr>
<tr>
<td>10^-4 M</td>
<td>3850 ± 410(^d)</td>
<td>270 ± 10(^a)</td>
</tr>
</tbody>
</table>

* This sample used deionized water as the control solution; the following one used the control liposome solution. The statistical correlation of the liposome preparation is to that of the water control solution in each column. The statistical correlations of the sterol-containing solutions are to that of the liposome control solution.
Experiment 3

Due to the large effects of the vehicles used in the previous experiments, the search for a more suitable vehicle was continued. Propylene glycol was used as the control solution in this experiment. The inhibitor solutions were prepared by dissolution of 14α-ethylcholesterol-7-en-3β,15α-diol in propylene glycol to give solutions of $2 \times 10^{-3}$ M, $2 \times 10^{-4}$ M, and $2 \times 10^{-5}$ M concentration. Sodium [2-14C]-acetate was used as substrate and its incorporation into digitonin-precipitable sterols was monitored. Table XIV presents the results obtained in this experiment.

The effect of propylene glycol was to stimulate rather than to inhibit the synthesis of digitonin-precipitable sterols from acetate by various amounts in different experiments (Miller, unpublished observations, [1977]). In this experiment, the stimulation was by 80% ($p < 0.001$) compared to a control incubation containing water in place of propylene glycol. The 14α-ethylcholesterol-7-en-3β,15α-diol solutions were all extremely potent in inhibiting the synthesis of digitonin-precipitable sterols from acetate. The effects were large (65%–75%) and statistically significant ($p < 0.001$) at all concentrations tested. The differences between the effects of the three concentrations were all significant: the difference between the $10^{-5}$ M and $10^{-6}$ M solutions was significant at $0.02 < p < 0.05$ and the other differences were significant at $p < 0.001$.

The results of the experiments in this section have
TABLE XIV.

The Effect of 14α-Ethylcholesterol-7-en-3α,15α-diol on the Synthesis of Digitonin-Precipitable Sterols from Sodium [2-14C]-Acetate using Propylene Glycol as Vehicle (N=4).

<table>
<thead>
<tr>
<th>[14α-Ethylcholesterol-7-en-3α,15α-diol]</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 M*</td>
<td>12,900 ± 900</td>
</tr>
<tr>
<td>0 M</td>
<td>23,100 ± 400a</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>8,000 ± 600a</td>
</tr>
<tr>
<td>10⁻⁵ M</td>
<td>7,000 ± 300a</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>5,900 ± 300a</td>
</tr>
</tbody>
</table>

* This sample was incubated with deionized water as the control solution; the following one used propylene glycol as the control solution. The statistical correlation of the propylene glycol solution is to that of the water control solution. The statistical correlations of the sterol solutions are to that of the propylene glycol control solution.
demonstrated that propylene glycol is a more suitable vehicle for the administration of sterols to 10,000 x g supernatant liver preparations than either ethanol-BSA-water systems or liposomal preparations when the synthesis of digitonin-precipitable sterols from acetate is investigated.

Further, cholest-8(14)-en-3β-ol-15-one has been shown to significantly (p < 0.01) inhibit the incorporation of acetate but not mevalonate into non-saponifiable lipids (approximately 20%) at $10^{-4}$ M.

14α-Ethylcholest-7-en-3β,15α-diol was demonstrated to significantly inhibit the incorporation of acetate into digitonin-precipitable sterols at concentrations of $10^{-4}$ M, $10^{-5}$ M, and $10^{-6}$ M (approximately 75%, 70%, and 65% respectively).
The observation that an ethanol-containing vehicle is inappropriate for the assay of sterol synthesis from radio-labeled sodium acetate has been reported previously. The effect of the ethanol was reported to be due to the oxidation of the substance to acetate. This unlabeled acetate then diluted the specific activity of the labeled material added to the incubation. This dilution of specific activity resulted in lower incorporation of radioactive acetate. (This work is from the Ph.D thesis of Clearmond Eskelson, 1967.)

The effect of the liposome preparation on the synthesis of digitonin-precipitable sterols but not on non-saponifiable lipids is unusual and will require further experimentation to quantify and to explain. It is possible that the lipid liposomal vesicles cause preferential extraction of sterol intermediates from the microsomal enzymes which are active in the later stages of the sterol biosynthetic pathway. This would result in the depletion of the lipid soluble intermediates in the late stages of cholesterol biosynthesis, many of which are not totally digitonin-precipitable (viz. squalene, lanosterol, 4,4-dimethyl-sterols). Thus, extraction of squalene or lanosterol (which are extracted into the non-saponifiable lipids but which are not precipitated by digitonin to the same extent as cholesterol) would result in the build-up of these products which would be carried away from the microsomal enzymes and diluted into the liposomes and away from further metabolism.
It is also possible that the liposomes may interact with the microsomal enzymes and cause changes in the fluidity of the microsomes which might result in inhibition of the enzymes by forcing them into less active conformations. With the dearth of experimental evidence, however, any explanations must be purely speculative as of yet.

The inhibition of the incorporation of acetate into non-saponifiable lipids due to $10^{-4}$ M cholest-8(14)-en-3β-ol-15-one has been demonstrated in this work and has been extensively documented by further work of Miller and of Pajewski (unpublished observations [1977], [1978]). This inhibition is somewhat unexpected in view of the results of the whole animal experiments described in the previous section in which administration of the agent to animals caused an increase in the ability of the 10,000 x g supernatant fraction of hepatic preparations to incorporate acetate into digitonin-precipitable sterols.

The concentration of cholest-8(14)-en-3β-ol-15-one (relative to 14α-ethylcholest-7-en-3β,15α-diol) required for significant inhibition of sterol synthesis from acetate is relatively high. The reason for this discrepancy is not known, but the fact that 10,000 x g supernatant fractions of rat liver homogenates metabolize the ketone to cholesterol in significant amounts (Monger, unpublished results, [1978]) and do not metabolize the diol to cholesterol (vide infra) may be important. Cholesterol at a concentration of $10^{-6}$ M has been shown to be without effect on the conversion
of acetate to digitonin-precipitable sterols in the 10,000 x g supernatant fraction of rat liver homogenates (vide infra).

The inhibition of sterol synthesis produced by 14α-ethylcholest-7-en-3β,15α-diol was investigated in detail in experiments reported in the following section. The results of these experiments are presented in the following pages.
IN VITRO STUDIES:

III. The Effects of 14α-Ethylcholest-7-en-3β,15α-diol on Sterol Biosynthesis in Cell-Free Preparations
The In Vitro Effects of 14α-Ethylcholesterol-7-en-3β,15α-Diol on Sterol Synthesis in the 10,000 x G Supernatant Fraction of Rat Liver Homogenate Preparations

The following experiments concern investigations of the effects of 14α-ethylcholesterol-7-en-3β,15α-diol on the in vitro biosynthesis of non-saponifiable lipids and digitonin-precipitable sterols from acetate and mevalonate in 10,000 x g supernatant fractions of rat liver homogenate preparations. The procedures used in these experiments are described below. Any modifications of this protocol are described with the individual experiments.

Male Sprague-Dawley rats (from Sprague-Dawley Farms, WI, weighing 100-200 gm) were maintained on a diet of Purina formula # 5008 laboratory chow and water ad libitum for at least a week prior to sacrifice. During this time, the animals were maintained on a strict light-dark cycle (0600-1800 light). Animals were sacrificed in the morning (0700-0900) during the period of basal activity of HMG-CoA reductase (Rodwell et al.[1976]). The animals were sacrificed by decapitation and their livers excised. The livers were rinsed with a solution of physiological saline and excess liquid removed by damp-drying with Kim-wipes. The livers were weighed and added to a volume of incubation buffer (pre-chilled to 4°C in a refrigerator) corresponding to 2.5 times their wet weight. The tissue was minced with scissors
and homogenized for ninety seconds on ice at 660 rpm in a
glass homogenizer with a loose-fitting teflon pestle. The
homogenate was centrifuged for five minutes at 500 x g (2°C),
and the supernatant derived therefrom was centrifuged for
thirty minutes at 10,000 x g (2°C). This latter supernatant
was the enzyme source for all the experiments described
in this section.

In each experiment the incubation assay medium was
prepared in the following manner. A stock solution was pre-
pared with the following substances: adenosine triphos-
phate (ATP) 64 mg/ml; nicotinamide adenine dinucleotide (NAD)
15.8 mg/ml; nicotinamide adenine nucleotide phosphate (NADP)
16 mg/ml; glucose-6-phosphate (G-6-P) 21 mg/ml. When 0.10
ml of this cofactor stock solution was diluted to the final
incubation assay volume of 2.0 ml, the final concentration
of each cofactor was ATP 5 mM; NAD 1mM; NADP 1 mM; G-6-P
3.8 mM. Inhibitor stock solutions were prepared by dissolv-
ing the appropriate sterol (usually 14α-ethylcholest-7-en-
3β,15α-diol) in propylene glycol to a concentration twenty
times that desired in the final assay medium and adding 0.10
ml of this stock solution to the medium. The control solu-
tion in these experiments was propylene glycol. Substrate
stock solutions were also prepared; sodium [3H]-acetate (10
mCi/4.0 mg/ml) and sodium [2-14C]-mevalonate (10 uCi/60 ug/
ml). The final concentrations of the two substrates in the
incubation medium were 2.4 mM and 17.5 μM respectively.

The assays were performed by mixing the cofactor
stock solution (0.10 ml), the inhibitor or control stock solution (0.10 ml), and the appropriate substrate stock solution (0.10 ml). The reaction was started by addition of the 10,000 x g supernatant enzyme preparation (1.7 ml). The samples were mixed well, flushed with a strong stream of oxygen for five seconds, and then capped. They were then allowed to incubate in a water bath (37°C) with shaking (90 osc/min). The length of incubation was two hours when sodium acetate was the substrate and one and one-half hours when sodium mevalonate was the substrate. In the case of the time-course experiments, the samples were allowed to incubate for the various times described in the experiments.

At the end of the incubation, enzymatic activity was stopped by the addition of a 15% ethanolic potassium hydroxide solution (3 ml; see Materials and Methods). A solution of cholesterol in ethanol (1 ml; 4 mg/ml) was added, and the samples were tightly capped. After thorough mixing, they were hydrolyzed in a water bath (70-75°C) for three hours. After the hydrolysis, water (6 ml) was added to each tube and the non-saponifiable lipids were extracted with three portions of hexane or petroleum ether (15 ml each portion). The pooled hexane or petroleum ether layers were washed twice with water (10 ml each time). The hexane was removed by evaporation under nitrogen and the lipid dried briefly by dessication in vacuo over phosphorus pentoxide. The dried material was dissolved in benzene and aliquots taken for liquid scintillation counting and Liebermann-
Burchard colorimetry.

Digitonin-precipitable sterols were prepared as described in the Materials and Methods section. Colorimetry before and after the digitonide preparation was used to correct for losses during the procedure. All values are reported as a mean value ± standard error of the mean. The value given in parentheses after the title of each experiment indicates the number of replicate assays per group; e.g., (N=4) means that the values reported for each group were the means of four replicate samples.
Experiment I

In this experiment the effect of 14α-ethylcholesterol-7-en-3β,15α-diol, 25-hydroxycholesterol, and purified cholesterol on the incorporation of acetate into digitonin-precipitable sterols was investigated. Stock solutions of the three sterols were prepared in propylene glycol at the following concentrations: Cholesterol - 7.72 mg/ml; 25-hydroxycholesterol - 8.02 mg/ml; 14α-ethylcholesterol-7-en-3β,15α-diol - 8.62 mg/ml. The solution of 14α-ethylcholesterol-7-en-3β,15α-diol was used to prepare the more dilute solutions of this sterol by serial dilutions with propylene glycol. Table XV lists the results obtained from this experiment.

TABLE XV.
The Effect of Several Sterols on the Incorporation of Sodium $^3$H]-Acetate into Digitonin-Precipitable Sterols (N-4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21,900 ± 1900</td>
</tr>
<tr>
<td>+ Cholesterol (10^{-6} M)</td>
<td>22,100 ± 900n</td>
</tr>
<tr>
<td>+ 25-Hydroxycholesterol (10^{-6} M)</td>
<td>22,400 ± 4000n</td>
</tr>
<tr>
<td>+ 14α-Ethylcholesterol-7-en-3β,15α-diol (10^{-6} M)</td>
<td>9,100 ± 900a</td>
</tr>
<tr>
<td>(10^{-7} M)</td>
<td>10,400 ± 100b</td>
</tr>
<tr>
<td>(10^{-8} M)</td>
<td>22,900 ± 600n</td>
</tr>
<tr>
<td>(10^{-9} M)</td>
<td>21,900 ± 800n</td>
</tr>
</tbody>
</table>
14α-Ethylcholest-7-en-3β,15α-diol caused a 59% decrease in the incorporation of acetate into digitonin-precipitable sterols (p < 0.001) in this experiment at a concentration of $10^{-6}$ M. A similar (53%) decrease was observed at $10^{-7}$ M (p < 0.01). At greater dilution ($10^{-8}$ M, $10^{-9}$ M) there was no significant effect (p > 0.90). In the same liver preparation in which this alkyl diol was clearly effective in inhibiting the synthesis of digitonin-precipitable sterols, purified cholesterol ($10^{-6}$ M) and 25-hydroxycholesterol ($10^{-6}$ M) were without effect.

**Experiment 2**

In this experiment the effect of 14α-ethylcholest-7-en-3β,15α-diol on the incorporation of sodium mevalonate into non-saponifiable lipids and digitonin-precipitable sterols was investigated. The experiment with sodium acetate as substrate was also repeated with the concentration of the sterol inhibitor at $10^{-6}$ M. The results are expressed in the Tables XVI and XVII.
TABLE XVI.

The Effect of 14α-Ethylcholesterol-7-en-3β,15α-diol on the Incorporation of Sodium [3H]-Acetate into Non-Saponifiable Lipids and Digitonin-Precipitable Sterols (N=4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incorporation into Non-Saponifiable Lipids (dpm)</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>117,000 ± 11,000</td>
<td>16,600 ± 900</td>
</tr>
<tr>
<td>+ 14α-Ethylcholesterol-7-en-3β,15α-diol (10^-6 M)</td>
<td>152,000 ± 6,000&lt;sup&gt;C&lt;/sup&gt;</td>
<td>8,200 ± 300&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Micromolar concentration of 14α-ethylcholesterol-7-en-3β,15α-diol caused a stimulation of the incorporation of sodium acetate into non-saponifiable lipids (30%, p < 0.02) and a large reduction in the incorporation of acetate into digitonin-precipitable sterols (51%, p < 0.001).

TABLE XVII.

The Effect of 14α-Ethylcholesterol-7-en-3β,15α-diol on the Incorporation of Sodium [2-14C]-Mevalonate into Non-Saponifiable Lipids and Digitonin-Precipitable Sterols (N=4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incorporation into Non-Saponifiable Lipids (dpm)</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>534,000 ± 25,000</td>
<td>596,000 ± 31,000</td>
</tr>
<tr>
<td>+ 14α-Ethylcholesterol-7-en-3β,15α-diol (10^-6 M)</td>
<td>592,000 ± 17,000&lt;sup&gt;C&lt;/sup&gt;</td>
<td>270,000 ± 24,000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Micromolar concentration of 14α-ethylcholesterol-7-en-3β,15α-diol caused a slight stimulation of the incorporation of
sodium mevalonate into non-saponifiable lipids (11%, p < 0.02) but a large decrease in the incorporation of sodium mevalonate into digitonin-precipitable sterols (56%, p < 0.001).

**Experiment 3**

In this experiment the effect of the length of incubation time on the incorporation of sodium acetate into non-saponifiable lipids and digitonin-precipitable sterols was investigated in the presence and absence of 10^{-6} M 14\alpha-ethylcholest-7-en-3\beta,15\alpha-diol. Tables XVIII and XIX and Figures 20 and 21 present the results obtained.

**TABLE XVIII.**

The Effect of Duration of Incubation on the Incorporation of Sodium [\textsuperscript{3}H]-Acetate into Non-Saponifiable Lipids in the Presence and Absence of 10^{-6} M 14\alpha-Ethylcholest-7-en-3\beta,15\alpha-diol (N=4).

<table>
<thead>
<tr>
<th>Incubation time (minutes)</th>
<th>Incorporation into Control Samples (dpm x 10^{-4})</th>
<th>Incorporation into Diol-treated Samples (dpm x 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>10.96 ± 2.16</td>
<td>9.94 ± 0.54</td>
</tr>
<tr>
<td>60</td>
<td>15.88 ± 0.20</td>
<td>15.99 ± 4.17</td>
</tr>
<tr>
<td>90</td>
<td>25.01 ± 1.93</td>
<td>25.18 ± 1.41&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>52.67 ± 1.64</td>
<td>53.67 ± 2.12</td>
</tr>
</tbody>
</table>

There was no significant difference (p > 0.20) between the amounts of incorporation (expressed as dpm) in the control and diol-treated samples at each time point. The time course was approximately linear from 0 - 90 minutes and
curved upward at 120 minutes (Figure 20.)
Figure 20. The Time-Course of Incorporation of Acetate into Non-Saponifiable Lipids in the Presence (o——o) and Absence (x——x) of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol.
**TABLE XIX.**

The Effect of Duration of Incubation on the Incorporation of Sodium \(^{3}H\)-Acetate into Digitonin-Precipitable Sterols in the Presence and Absence of \(10^{-6}\) M 14α-Ethylcholest-7-en-3β,15α-diol (N=4).

<table>
<thead>
<tr>
<th>Incubation time (minutes)</th>
<th>Incorporation into Control Samples (dpm x 10(^{-4}))</th>
<th>Incorporation into Diol-treated Samples (dpm x 10(^{-4}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.23 ± 0.06</td>
<td>*</td>
</tr>
<tr>
<td>60</td>
<td>1.72 ± 0.33</td>
<td>0.87 ± 0.18(^b)</td>
</tr>
<tr>
<td>90</td>
<td>1.67 ± 0.28</td>
<td>0.90 ± 0.08(^b)</td>
</tr>
<tr>
<td>120</td>
<td>3.27 ± 0.37</td>
<td>1.63 ± 0.21(^b)</td>
</tr>
</tbody>
</table>

14α-Ethylcholest-7-en-3β,15α-diol at a concentration of \(10^{-6}\) M caused an average 51% ± 2% decrease in the incorporation of sodium acetate into digitonin-precipitable sterols at each time point tested. The differences were statistically significant (\(p < 0.01\)).

* No value obtained for this point due to losses in handling.
Figure 21. The Time-Course of Incorporation of Acetate into Digitonin-Precipitable Sterols in the Presence (o—o) and Absence (x—x) of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol.
Experiment 4

In this experiment the effect of the length of incubation time on the incorporation of sodium [2-14C]-mevalonate into non-saponifiable lipids and digitonin-precipitable sterols was investigated in the presence and absence of 14α-ethylcholest-7-en-3β,15α-diol (10^{-6} M). Tables XX and XXI and Figures 22 and 23 present the results obtained.

**TABLE XX.**

The Effect of Duration of Incubation on the Incorporation of Sodium [2-14C]-Mevalonate into Non-Saponifiable Lipids in the Presence and Absence of 10^{-6} M 14α-Ethylcholest-7-en-3β,15α-diol (N-4).

<table>
<thead>
<tr>
<th>Incubation time (minutes)</th>
<th>Incorporation into Control Samples (dpm x 10^{-4})</th>
<th>Incorporation into Diol-treated Samples (dpm x 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>7.40 ± 0.40</td>
<td>7.35 ± 0.32\textsuperscript{1}</td>
</tr>
<tr>
<td>60</td>
<td>16.89 ± 0.45</td>
<td>16.80 ± 0.41</td>
</tr>
<tr>
<td>90</td>
<td>17.96 ± 0.24</td>
<td>17.78 ± 0.30\textsuperscript{k}</td>
</tr>
<tr>
<td>120</td>
<td>13.50 ± 0.38</td>
<td>13.47 ± 0.28\textsuperscript{n}</td>
</tr>
</tbody>
</table>

14α-Ethylcholest-7-en-3β,15α-diol at a concentration of 10^{-6} M had no effect on the incorporation of mevalonate into non-saponifiable lipids at any time point tested. The time-course was approximately linear from 0-60 minutes, almost level from 60-90 minutes, and decreased slightly at 90-120 minutes (Figure 22).
Figure 22. The Time-Course of Incorporation of Mevalonate into Non-Saponifiable Lipids in the Presence (○—○) and Absence (×—×) of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol.
TABLE XXI.

The Effect of Incubation Length on the Incorporation of
Sodium [2-\textsuperscript{14}C]-Mevalonate into Digitonin-Precipitable Sterols in the Presence and Absence of 10\textsuperscript{-6} M 14\alpha-Ethylcholeste-

\textsuperscript{7}-en-3\beta,15\alpha-diol (N=4).

<table>
<thead>
<tr>
<th>Incubation time (minutes)</th>
<th>Incorporation into Control Samples (dpm x 10\textsuperscript{-4})</th>
<th>Incorporation into Diol-treated Samples (dpm x 10\textsuperscript{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>4.05 ± 0.01</td>
<td>1.75 ± 0.17\textsuperscript{a}</td>
</tr>
<tr>
<td>60</td>
<td>6.57 ± 0.14</td>
<td>3.01 ± 0.13\textsuperscript{a}</td>
</tr>
<tr>
<td>90</td>
<td>8.45 ± 0.80</td>
<td>3.94 ± 0.17\textsuperscript{a}</td>
</tr>
<tr>
<td>120</td>
<td>6.58 ± 0.11</td>
<td>2.00 ± 0.12\textsuperscript{a}</td>
</tr>
</tbody>
</table>

14\alpha-Ethylcholeste-

\textsuperscript{7}-en-3\beta,15\alpha-diol at a concentration of 10\textsuperscript{-6} M caused an average 58% ± 5% decrease in the incorporation of mevalonate into digitonin-precipitable sterols at each time point tested. The time-course was nearly linear from 0-90 minutes and then decreased between 90-120 minutes. The shape of the time-course was the same for the control and diol-treated samples (Figure 23). The effect on the diol-treated samples was statistically significant (p < 0.001).
Figure 23. The Time-Course of Incorporation of Mevalonate into Digitonin-precipitable Sterols in the Presence (o--o) and Absence (x--x) of $10^{-6}$ M 14α-Ethylcholester-7-en-3β, 15α-diol.
Experiment 5

In this experiment the shape of the concentration-inhibition curve between $10^{-7}$ M and $10^{-8}$ M 14α-ethylcholest-7-en-3β,15α-diol was investigated. The substrate was sodium [2-$^{14}$C]-mevalonate. The results of this experiment are presented in Table XXII and Figure 24.

TABLE XXII.

The Effect of 14α-Ethylcholest-7-en-3β,15α-diol on the Incorporation of Sodium [2-$^{14}$C]-Mevalonate into Digitonin-Precipitable Sterols at 10, 50 and 75 nM (N=4).

<table>
<thead>
<tr>
<th>14α-Ethylcholest-7-en-3β,15α-diol (nM)</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm x 10^{-6})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.29 ± 0.43</td>
</tr>
<tr>
<td>10</td>
<td>5.69 ± 0.21^{e}</td>
</tr>
<tr>
<td>50</td>
<td>4.76 ± 0.30^{b}</td>
</tr>
<tr>
<td>75</td>
<td>3.65 ± 0.41^{b}</td>
</tr>
</tbody>
</table>

The difference between the incorporation at 0 nM and 10 nM was not statistically significant ($p > 0.50$). The differences in incorporation between the other diol concentrations was significant in every case ($p < 0.01$). The graph shows that the inhibition curve is approximately linear in the region of 10 - 75 nM 14α-ethylcholest-7-en-3β,15α-diol.
Figure 24. The Effect of 14α-Ethylcholest-7-en-3β,15α-diol on the Incorporation of Mevalonate into Digitonin-Precipitable Sterols in 10,000 x g Supernatant Fractions of Rat Liver Homogenates as a Function of Concentration.
Experiment 6

In this experiment the shape of the concentration-inhibition curve for 14α-ethylcholesterol-7-en-3β,15α-diol was investigated between $10^{-8}$ and $10^{-4}$ M. The experiment was performed using solutions of 14α-ethylcholesterol-7-en-3β,15α-diol which were prepared by appropriate serial dilutions from a stock solution containing 17.2 mg/ml of the steroid in propylene glycol. Control samples were incubated using propylene glycol alone without any steroid. The results of this experiment are presented in Table XXIII and Figure 25.

TABLE XXIII.

The Effect of 14α-Ethylcholesterol-7-en-3β,15α-diol on the Incorporation of Sodium [2-14C]-Mevalonate into Digitonin-Precipitable Sterols (N=5).

<table>
<thead>
<tr>
<th>[14α-Ethylcholesterol-7-en-3β,15α-diol]</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm x $10^{-4}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM</td>
<td>64.2 ± 1.5</td>
</tr>
<tr>
<td>10 nM</td>
<td>61.0 ± 1.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>50 nM</td>
<td>57.8 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 nM</td>
<td>48.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>500 nM</td>
<td>30.8 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$10^{-6}$ M</td>
<td>24.4 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$10^{-5}$ M</td>
<td>18.7 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$10^{-4}$ M</td>
<td>17.8 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
The decrease in incorporation of mevalonate into digitonin-precipitable sterols between the control and 10 nM samples was not statistically significant \( (p > 0.10) \), nor was the difference in incorporation between the \( 10^{-5} \) M and \( 10^{-4} \) M samples \( (p > 0.70) \). The differences between the other samples were all significant \( (p < 0.01) \). The following figure shows the concentration of 14\( \alpha \)-ethylcholesterol-7-en-3\( \beta \),15\( \alpha \)-dial plotted versus the incorporation into digitonin-precipitable sterols as a percentage of the incorporation in the control samples.
Figure 25. The Effect of 14α-Ethylcholest-7-en-3β,15α-diol on the Incorporation of Mevalonate into Digitonin-Precipitable Sterols in the 10,000 x g Supernatant Fraction of Rat Liver Homogenates as a Function of Concentration.
Experiment 7

This experiment, as well as the four following ones, was designed so that the nature of the inhibition caused by 14α-ethylcholest-7-en-3β,15α-diol on in vitro cholesterol biosynthesis might be investigated. In this experiment, both non-saponifiable lipids and digitonin-precipitable sterols produced by the incubation of sodium $[^3H]$-acetate with $10^{-6}$ M 14α-ethylcholest-7-en-3β,15α-diol were analyzed by silicic acid - Super Cel column chromatography. Control samples incubated in the absence of 14α-ethylcholest-7-en-3β,15α-diol were also analyzed for the purposes of comparison.

The columns used in this experiment (1 x 50 cm) were packed under 3 p.s.i. nitrogen pressure (see Materials and Methods). The columns were packed and eluted with benzene. Twenty minute fractions (2.0 ml volume) were collected. After seventy fractions had been collected, the benzene solvent was removed and replaced with ether to strip the more polar material from the column. The column profiles for the labeled non-saponifiable lipids and digitonin-precipitable sterols formed from $[^3H]$-acetate in the presence and absence of $10^{-6}$ M 14α-ethylcholest-7-en-3β,15α-diol are presented in Figures 26-29. In each figure, the solid line represents radioactivity and the dashed line represents cholesterol assayed colorimetrically by the Liebermann-Burchard reaction. The arrow marks fraction 70, at which the solvent was changed.
Figure 26. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from Control Incubations.

(○—○—○) radioactivity; (---x---x---) cholesterol standard measured colorimetrically.
Figure 27. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from Incubations in the Presence of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol. (○○○○) radioactivity; (×--×--×--×) cholesterol standard measured colorimetrically.
Figure 28. Silicic acid Column Chromatography of the Digitonin-Precipitable Sterols Recovered from Control Incubations. (---) radioactivity; (x---x---x--) cholesterol standard measured colorimetrically.
Figure 29. Silicic acid Column Chromatography of the Digito- 
tinin-Precipitable Sterols Recovered from Incubations in the 
Presence of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol. 
(□□□□) radioactivity; (×--×) cholesterol standard 
measured colorimetrically.
Experiment 8

In this experiment silicic acid-Super Cel column chromatography was used to investigate the effect of 14α-ethylcholest-7-en-3β,15α-diol on sterol synthesis from sodium mevalonate. Ten samples were incubated in each group, both control and diol-treated. Of these, the non-saponifiable lipids from five samples were pooled and subjected to silicic acid-Super Cel column chromatography on 1 x 50 cm columns using benzene as the eluting solvent. The remaining five samples in each group were treated individually and analyzed for non-saponifiable lipids and digitonin-precipitable sterols.

The columns were packed with a benzene slurry of the silicic acid-Super Cel mixture (2:1) under 3 p.s.i. nitrogen pressure. Twenty minute fractions of 2.0 ml volume were collected. The column elution profiles are presented in the following two figures, in which the solid lines represent radioactivity and the dashed lines represent cholesterol assayed colorimetrically by the Liebermann-Burchard reaction.

The results of the incorporation into non-saponifiable lipids and digitonin-precipitable sterols are shown in Table XXIV. There was no significant difference in the incorporation into non-saponifiable lipids (p > 0.10) between the control and diol-treated samples. A 70% reduction (p < 0.001) in the incorporation into digitonin-precipitable sterols was observed in the diol-treated samples, however.
TABLE XXIV.

The Incorporation of Sodium \(^{14}\)C-Mevalonate into Non-Saponifiable Lipids and Digitonin-Precipitable Sterols in the Presence and Absence of 10\(^{-6}\) M 14α-Ethylcholest-7-en-3β,15α-diol (N=5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incorporation into Non-Saponifiable Lipids (dpm x 10(^{-5}))</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm x 10(^{-5}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.88 ± 0.25</td>
<td>6.35 ± 0.62</td>
</tr>
<tr>
<td>+ 14α-Ethylcholest-7-en-3β,15α-diol</td>
<td>12.86 ± 1.23(^\text{j})</td>
<td>1.89 ± 0.15(^\text{a})</td>
</tr>
</tbody>
</table>
Figure 30. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from Control Incubations. 
(●●●) radioactivity; (●●×) cholesterol standard measured colorimetrically.
Figure 31. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from Incubations in the Presence of $10^{-6}$ M 14α-Ethylcholest-7-en-3α,15α-diol. (○○○) radioactivity; (×---×) cholesterol standard measured colorimetrically.
Experiment 9

Because of its position in the elution profile, the unknown peak, whose accumulation was caused by the presence of $10^{-6}$ M 14α-ethylcholesterol-7-en-3β,15α-diol in the incubation mixture, was suspected to be a methyl-sterol. When in this second experiment, sodium [2-14C]-mevalonate was incubated in the presence of 14α-ethylcholesterol-7-en-3β,15α-diol and the non-saponifiable lipids examined by means of silicic acid-Super Cel column chromatography, carrier lanosterol (5 mg) was added to the sample just before application to the column bed. This column (1 x 50 cm) was prepared in the solvent hexane:benzene (1:1) and eluted with the same solvent. Twenty minute fractions (3.0 ml) were collected. The column elution profile is shown in the following figure. The unknown material produced in the incubation co-chromatographed with the carrier lanosterol. The solid line in the figure represents radioactivity and the dashed lines represent either carrier lanosterol ($A_{490}$ nm) or carrier cholesterol ($A_{620}$ nm).
Figure 32. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from an Incubation in the Presence of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol.

(○—○) radioactivity; (+----+) lanosterol standard and (x---x) cholesterol standard measured colorimetrically.
Experiment 10

In this experiment, the convertability of the unknown material to cholesterol was investigated. The radioactive material whose accumulation from sodium $[2^{-14}C]$-mevalonate was caused by $10^{-6}$ M $14\alpha$-ethylcholest-7-en-3β,15α-diol was obtained by pooling fractions 20-30 of the material isolated from the silicic acid-Super Cel column chromatography performed in Experiment 8. This material was dissolved in a propylene glycol (1.0 ml) and incubated two hours with a 10,000 x g supernatant liver preparation (25 ml) fortified with cofactors as described in the introduction to this section. The non-saponifiable lipids were isolated and dissolved in benzene (2.0 ml). Carrier lanosterol (5 mg) was added to the sample. An aliquot of this material was reserved for analysis by gas-liquid radiochromatography; the remainder was analyzed by silicic acid-Super Cel column chromatography on a 1 x 50 cm column using benzene as the eluting solvent. Twenty minute (2.0 ml) fractions were collected. The profile in Figure 33 shows that the bulk of the material was converted to material which co-chromatographed with cholesterol under these condition. There was a small amount of a metabolite less polar than cholesterol, and an even smaller amount of material which co-chromatographed with lanosterol.
Figure 33. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from the Reincubation of Fractions 20-30 of the Silicic acid Column in Experiment 8. (-----o) radioactivity; (+-----+) lanosterol standard and (x-----x) cholesterol standard measured colorimetrically.
Figure 34 shows the profile obtained by gas-liquid radiochromatographic analysis of an aliquot of the non-saponifiable lipids on a column of 3% OV-17 at 270°C. The continuous line tracing is that of a standard solution of cholesterol, lanosterol and 24,25-dihydrolanosterol. Superimposed on this tracing is a bar graph of the radioactive non-saponifiable lipids recovered from the column effluent. One minute fractions were collected. The majority of the radioactivity elutes with a retention time equal to that of cholesterol. The remainder elutes with a retention time equal to that of either dihydrolanosterol or lanosterol.

Fractions 28-45 from the silicic acid-Super Cel column were pooled and the solvent removed on a rotary evaporator under reduced pressure. The material recovered from this pooled sample (which co-chromatographed with the carrier cholesterol) was subjected to passage through the dibromide (see Materials and Methods) and recrystallization from methanol. The specific activity of the material was determined before and after this procedure. The results are reported in Table XXV.
Figure 34. Gas-liquid Radiochromatography of the Non-Saponifiable Lipids Isolated from the Reincubation of Fractions 20-30 of the Silicic acid Column in Experiment 8. The mass standards are, in order of increasing elution time, cholesterol, dihydrolanosterol, and lanosterol.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific Activity (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dibromide</td>
<td>3550 ± 100</td>
</tr>
<tr>
<td>After dibromide</td>
<td>3500 ± 150</td>
</tr>
<tr>
<td>Recrystallization from Methanol (1 x)</td>
<td>3570 ± 200</td>
</tr>
<tr>
<td>Recrystallization from Methanol (2 x)</td>
<td>3530 ± 150</td>
</tr>
</tbody>
</table>

There was no significant difference ($p > 0.40$) between the specific activity of the material before and after the dibromide purification and the recrystallizations.
Experiment 11

Sodium $[2^{14}C]$-mevalonate was incubated in the presence of $10^{-6}$ M 14α-ethylcholest-7-en-3β,15α-diol and the non-saponifiable lipids were isolated. The recovered material was subjected to silicic-acid-Super Cel column chromatography on a 1 x 100 cm column which was packed and eluted with the solvent system hexane:benzene (1:1). Carrier cholesterol and carrier lanosterol (5 mg each) were added to the sample dissolved in hexane (2.0 ml) immediately before the sample was applied to the column. Twenty minute (2.5 ml) fractions were collected. The elution profile is shown in Figure 35.

Fractions 42-64 were pooled and the solvent removed by evaporation on a rotary evaporator under reduced pressure. Additional carrier lanosterol (50 mg) was added. The sample was dissolved in benzene (50 ml) and shaken two hours under hydrogen gas (40 p.s.i.) with Adams' catalyst (10 mg) in a Parr hydrogenator. At the end of this time, the solution was filtered to remove the catalyst and the benzene removed on a rotary evaporator under reduced pressure. The reduced material was subjected to repeated crystallizations as described below. The specific activity was determined each time.

The first recrystallization was performed by dissolving the sample in a small volume of acetone (10 ml). Aliquots were taken for liquid scintillation counting and Liebermann-Burchard colorimetry. The acetone solution was
Figure 35. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from an Incubation in the Presence of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol in the 10,000 x g Supernatant Fraction of Rat Liver Homogenates. (---o) radioactivity; (+++++) lanosterol standard and (x---x) cholesterol standard measured colorimetrically.
warmed over a steam bath and water added dropwise until a faint turbidity persisted. Acetone was then added dropwise until the turbidity just cleared. The solution was placed in the freezer to crystallize (−20°C). Crystals were collected four hours later and washed with a cold solution of acetone-water (4:1). The crystals were dissolved in ether (15 ml) and aliquots taken for radioactivity and mass determination as described above.

The ether solution was warmed on a steam bath and a mixture of methanol and water (1:1) was added dropwise until a faint turbidity persisted. Ether was added back dropwise until the solution just cleared. The solution was covered with filter paper and allowed to crystallize at room temperature. After three hours the crystals were collected and sucked dry of solvent on a sintered glass funnel. After drying in vacuo, the dried crystals were redissolved in acetone (10 ml) and aliquots taken for radioactivity and mass determination.

Methanol (2 ml) was added to the acetone solution; water was then added dropwise until a faint turbidity persisted. Methanol was added dropwise until the turbidity cleared, and the solution was placed in a freezer (−20°C) overnight to crystallize. The following day, the long needles were collected by suction filtration and washed with ice-cold methanol. They were then dissolved in a small amount of toluene and aliquots taken for radioactivity and mass determination. Table XXVI shows the results of these
recrystallizations.

TABLE XXVI.

Recrystallization of Authentic Dihydrolanosterol with the Hydrogen Reduction Product of the Material Accumulated during Incubation of Sodium \([2-^{14}\text{C}]-\text{Mevalonate}\) in the Presence of \(10^{-6} \text{ M } 14\alpha\text{-Ethylcholest-7-en-3\beta,15\alpha-diols}\) (N=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (dpm/ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Recrystallization</td>
<td>170 ± 15</td>
</tr>
<tr>
<td>Acetone-water Recrystallization</td>
<td>150 ± 20(^j)</td>
</tr>
<tr>
<td>Ether-methanol-water Recrystallization</td>
<td>143 ± 24(^j)</td>
</tr>
<tr>
<td>Acetone-methanol-water Recrystallization</td>
<td>150 ± 20(^j)</td>
</tr>
</tbody>
</table>

There was no significant difference \((p > 0.50)\) between the values of the initial sample and the recrystallized samples.
Experiment 12

In this experiment, mevalonic acid lactone was incubated with a 10,000 x g supernatant preparation from male rats' liver in the presence of $10^{-6}$ M 14α-ethylcholest-7-en-3β,15α-diol. This resulted in the production of the material which had been tentatively identified as lanosterol and dihydrolanosterol from the previous experiments' results. This material was of extremely high specific activity. The characterizations described in this experiment were all performed on the same sample of material, thus obviating a possible objection to the previous experiments that the different characterizations had been performed on different samples.

[2-^3H]-Mevalonolactone (172 mCi/mMole; Amersham) was obtained as a solution in benzene (1 mCi/ml). This solution (0.20 ml) was evaporated under nitrogen in an Erlenmeyer flask (50 ml). Water (0.5 ml) was added and the radioactive material dissolved by swirling. A cofactor stock solution (0.5 ml) prepared as described in the introduction to this section was added to the flask, followed by the introduction of a solution of 14α-ethylcholest-7-en-3β,15α-diol in propylene glycol (0.5 ml of a 2 x $10^{-5}$ M solution). The incubation was begun by the addition of a 10,000 x g supernatant preparation from male rats' liver (8.5 ml). The contents of the flask were mixed by swirling and flushed 15 seconds with a strong stream of oxygen. The flask was capped and the solution incubated ninety minutes in a water bath
(37°C) with shaking (90 osc/min). At the end of this time, a 4 mg/ml solution of cholesterol in ethanol (1 ml) and a 15% ethanolic potassium hydroxide solution (11 ml) were added to the flask. A length of glass tubing was attached to the flask to serve as a reflux condenser. The contents of the flask were hydrolyzed by heating on a steam bath for three hours. At the end of the hydrolysis, an equal volume (22 ml) of water was added to the hydrolyzed sample, and the non-saponifiable lipids were extracted with three portions of hexane (90 ml). The combined hexane extracts were washed twice with water (75 ml) and then dried over anhydrous magnesium sulfate. After removal of the drying agent by filtration, the hexane was removed on a rotary evaporator under reduced pressure. The residue was dissolved in toluene (2 ml) and applied to a silicic acid-Super Cel column (1 x 100 cm, packed under 10 p.s.i. nitrogen). Forty minute fractions of approximately 2.5 ml volume were collected using toluene as the eluting solvent. After 180 fractions had been collected, the eluting solvent was changed to 20% ether in toluene and a further 50 fractions were collected. The column profile is shown in Figure 36. The major peak eluted in fractions 66-86; the cholesterol (added just prior to the hydrolysis and assayed colorimetrically) eluted in fractions 120-135. There was a final peak of radioactivity in fractions 198-210 eluted with the more polar solvent. The major peak fractions were pooled and the solvent removed on a rotary evaporator at reduced pressure. The material
was redissolved in anhydrous ether and stored in the freezer (-20°C) under nitrogen.

An aliquot of the material corresponding to the contents of fractions 60 - 90 was analyzed by gas-liquid radiochromatography on a column of 3% OV-17 at 250°C. A mass standard was also run on this column to compare to the radiochromatographic profile. Figure 37 shows both the mass tracing (continuous line) and the radiochromatographic profile (bar graph). Greater than 98% of the radioactivity co-eluted with either the lanosterol or dihydrolanosterol standard. Approximately 45% of the radioactivity co-migrated with lanosterol and approximately 55% with dihydrolanosterol.

A further portion of the material corresponding to the contents of fractions 60 - 90 was acetylated with acetic anhydride and pyridine (see Methods) and analyzed on an alumina-Super Cel-silver nitrate column.

The sample was applied in petroleum ether (2.0 ml) and a mixture of petroleum ether and toluene (85:15) was used as the eluting solvent. Forty minute fractions of approximately 4 ml volume were collected. A mixture of lanosteryl acetate and dihydrolanosteryl acetate (7 mg total) were added to the radioactive material prior to application on the column. Figure 38 shows the profile obtained from this column. The two radioactive peaks co-migrated with the unlabeled lanosteryl acetate and dihydrolanosteryl by colorimetry at 490 nm in the Liebermann-Burchard assay. No further radioactivity was eluted from the column even after 130
Figure 36. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from an Incubation of \(^{[2-^{3}H]}\)-Mevalonic Acid with the 10,000 x g Supernatant Fraction of a Liver Homogenate Preparation from Male Rats in the Presence of 10\(^{-6}\) M 14\(\alpha\)-Ethylcholeste-7-en-3\(\beta\),15\(\alpha\)-diol. (\(\bullet--\bullet\)) radioactivity; (\(\times--\times\)) cholesterol standard measured colorimetrically. The arrow indicates the solvent change from toluene to 20% ether in toluene.
Figure 37. Gas-liquid Radiochromatography of Fractions 60-90 from the Silicic acid Column Chromatography Presented in the Previous Figure. The mass standards are, in order of increasing elution time, dihydrolanosterol and lanosterol.
Figure 38. Alumina-Silver Nitrate Column Chromatography of the Acetylated Material from Fractions 60-90 of the Silicic acid Column Chromatography of Figure 36. (––) radioactivity; (+---+) dihydrolanosteryl acetate and lanosteryl acetate (in order of elution) measured colorimetrically.
fractions had been collected. Fractions 7-12 were pooled together as were fractions 13-19. The solvent was removed from both sets of pooled samples on a rotary evaporator under reduced pressure. Fractions 7-12 were combined with unlabeled authentic dihydrolanosteryl acetate (from Dr. F. F. Knapp) and extensively recrystallized as described in the following section. Fractions 13-19 were combined with authentic lanosteryl acetate (from Dr. F. F. Knapp) and similarly recrystallized.

Each sample was dissolved in a small amount of acetone and aliquots taken for mass and radioactivity assay. Then the samples were heated on a steam bath and water added dropwise until a faint turbidity just persisted. Acetone (2 drops) was then added and the samples set aside to crystallize overnight at room temperature covered with filter paper. The next day, the crystals were collected by suction filtration and washed with a small volume of acetone-water (5:1). The crystals were dessicated briefly in vacuo over phosphorus pentoxide and redissolved in ether. Aliquots were taken for the colorimetric determination of mass and the determination of radioactivity.

The samples in ether were then heated on a steam bath and methanol added dropwise until a faint turbidity persisted. The samples were then removed from the steam bath, covered with aluminum foil and set in the freezer (-20°C) to crystallize. The following day, the crystals were collected by suction filtration and washed with a small
volume of ice-cold methanol. After briefly drying in vacuo over phosphorus pentoxide, the crystals were dissolved in methanol and aliquots taken for the determination of mass and radioactivity. The samples in methanol were heated on a steam bath and the methanol removed under a stream of nitrogen until solid material began to precipitate from solution. Then a few drops of methanol were added to just bring the solid back into solution and the samples covered and placed in the freezer to crystallize. The crystals were collected the following day by suction filtration and dried several hours in vacuo over phosphorus pentoxide. They were then dissolved in benzene and aliquots taken for the determination of mass and radioactivity. Table XXVII shows the specific activities of the original samples and after each recrystallization.

TABLE XXVII.

Recrystallization of Fractions 7-12 With Dihydrolanosteryl Acetate and Fractions 13-19 With Lanosteryl Acetate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific Activity of Fractions 7-12</th>
<th>Specific Activity of Fractions 13-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial sample</td>
<td>139 dpm/ug</td>
<td>176 dpm/ug</td>
</tr>
<tr>
<td>After acetone-water recrystallization</td>
<td>136 dpm/ug</td>
<td>182 dpm/ug</td>
</tr>
<tr>
<td>After ether-methanol recrystallization</td>
<td>141 dpm/ug</td>
<td>180 dpm/ug</td>
</tr>
<tr>
<td>After methanol recrystallization</td>
<td>138 dpm/ug</td>
<td>178 dpm/ug</td>
</tr>
</tbody>
</table>

The above results demonstrate that there was essentially no
change in the specific activity of either sample upon repeated recrystallization.

A second sample of the material from the contents of fractions 60-90 was acetylated with acetic anhydride in pyridine without any added carrier and separated on a silver-nitrate-alumina-Super Cel column (1 cm x 100 cm). The sample was applied in petroleum ether (2 ml) and a mixture of petroleum ether and toluene (85:15) was used as the eluting solvent. Thirty minute fractions of approximately 3 ml volume were collected. Figure 39 shows the elution profile. The profile is essentially identical to that obtained in the presence of unlabeled carrier, except that the peaks were somewhat broader. Separation, however, was apparently better. Fractions 10-19 were combined and the solvent removed on a rotary evaporator under reduced pressure. This material was redissolved in a small amount of ether and an aliquot analyzed by gas-liquid radiochromatography on a column of 3% OV-17 at 280°C. The bulk of the radioactivity (86%) corresponded in retention time with authentic dihydrolanosterol acetate (prepared by acetylation of dihydrolanosteryl supplied by Dr. F. F. Knapp). A smaller peak which eluted slightly before dihydrolanosteryl acetate was also observed. This peak accounted for the remainder of the radioactivity eluted in the chromatogram. The radiochromatogram, superimposed on a curve showing the elution of unlabeled lanosteryl acetate and dihydrolanosteryl acetate under identical conditions, is presented in Figure 40.
Figure 39. Alumina-Silver Nitrate Column Chromatography of the Acetylated Material from Fractions 60-90 of the Silicic acid Column Chromatography of Figure 36. (○—○) radioactivity.
Figure 40. Gas-liquid Radiochromatography of Fractions 10-19 from the Alumina-Silver Nitrate Column Chromatography of Figure 39. The mass standards are, in order of increasing elution time, dihydrolanosteryl acetate and lanosteryl acetate.
The contents of fractions 21-30 were also combined and the solvent removed on a rotary evaporator under reduced pressure. This material was dissolved in a small amount of ether and analyzed by gas-liquid radiochromatography on a column of 3% OV-17 at 280°C. The majority of the radioactivity (91%) eluted at the same retention time as a sample of authentic unlabeled lanosteryl acetate. A second peak (9%) eluting at the same retention time as the minor component of the material from fractions 10-19 constituted the remainder of the radioactivity eluted from the column. The material obtained from the pooling of fractions 21-30 was hydrogenated by bubbling hydrogen gas through an ether solution of the material containing approximately 10 mg Adam’s catalyst. The hydrogenation was performed at room temperature and pressure for one hour. The catalyst was removed by filtration and the ether solution reduced in volume under a stream of nitrogen. An aliquot of this hydrogenated material was analyzed by gas-liquid radiochromatography on 3% OV-17 at 280°C. The bulk (95%) eluted with the same retention time as dihydrolanosteryl acetate. The minor component (5%) eluted at the same retention time as before hydrogenation. The elution profiles obtained by gas-liquid radiochromatography of the second peak from the silver nitrate-alumina column (pooled fractions 21-30) both before and after hydrogenation are shown in Figures 41 and 42. The radiochromatograms are plotted as bar graphs superimposed over a profile obtained from the analysis of unlabeled lanosteryl ace-
Figure 41. Gas-liquid Radiochromatography of Fractions 21-30 from the Alumina-Silver Nitrate Column Chromatography of Figure 39. The mass standards are, in order of increasing elution time, dihydrolanosteryl acetate and lanosteryl acetate.
Figure 42. Gas-liquid Radiochromatography of Fractions 21-30 from the Alumina-Silver Nitrate Column Chromatography of Figure 39 after Hydrogenation of the Material over Platinum Oxide. The mass standards are, in order of increasing elution time, dihydrolanosteryl acetate and lanosteryl acetate.
acetate and dihydrolanosteryl acetate under identical conditions.
Experiment 13

In this experiment the effects of several other 14α-alkyl-3,15-dioxygenated sterols on sterol synthesis from sodium mevalonate were investigated. The compounds were: 14α-ethylcholesterol-7-en-3β,15β-diol; 14α-ethylcholesterol-7-en-3β-ol-15-one; 14α-ethylcholesterol-7-en-15α-ol-3-one; and 14α-ethylcholesterol-7-en-3β,15α-diol. These compounds were tested for inhibition of synthesis of digitonin-precipitable sterols from sodium [2-14C]-mevalonate.

TABLE XXVIII.


<table>
<thead>
<tr>
<th>Steroid</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm x 10^-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.37 ± 0.08</td>
</tr>
<tr>
<td>+ 14α-Ethylcholesterol-7-en-3β,15α-diol (10^-6 M)</td>
<td>1.71 ± 0.08^a</td>
</tr>
<tr>
<td>+ 14α-Ethylcholesterol-7-en-3β,15β-diol (10^-5 M)</td>
<td>3.06 ± 0.08^c</td>
</tr>
<tr>
<td>+ 14α-Ethylcholesterol-7-en-3β-ol-15-one (10^-5 M)</td>
<td>1.82 ± 0.08^a</td>
</tr>
<tr>
<td>+ 14α-Ethylcholesterol-7-en-15α-ol-3-one (10^-5 M)</td>
<td>1.31 ± 0.05^a</td>
</tr>
</tbody>
</table>

The 14α-ethylcholesterol-7-en-3β,15α-diol caused a 50% reduction in the incorporation of mevalonate into digitonin-precipitable sterols (p < 0.001). The isomeric 15β-diol had
the least effect, a 10% reduction, although this was statistically significant ($p < 0.02$). The 15-one at $10^{-5}$ M caused approximately the same inhibition as the 3β,15α-diol (46%, $p < 0.001$) at $10^{-6}$ M. The isomeric 15α-ol-3-one was slightly more effective at $10^{-5}$ M (61% reduction, $p < 0.001$) than the 15α-diol at $10^{-6}$ M.

An aliquot of the material recovered from the digitonin precipitation of each sample was analyzed by gas-liquid radiochromatography. A reference tracing was obtained by injecting a solution of cholesterol, dihydrolanosterol and lanosterol on the chromatograph. This mass tracing is plotted in the following figures as a continuous line. The radioactive profile of each sample is presented as a bar graph superimposed on the mass tracing. One minute fractions were collected.
Figure 43. Gas-liquid Radiochromatography of the Digitonin-Precipitable Sterols Recovered from a Control Incubation. The mass standards are, in order of increasing elution time, cholesterol, dihydrolanosterol, and lanosterol.
Figure 44. Gas-liquid Radiochromatography of the Digitonin-Precipitable Sterols Recovered from an Incubation in the Presence of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol. The mass standards are, in order of increasing elution time, dihydrolanosterol and lanosterol.
Figure 45. Gas-liquid Radiochromatography of the Digitonin-Precipitable Sterols Recovered from an Incubation in the Presence of $10^{-5}$ M 14α-Ethylcholest-7-en-3β-ol-15-one. The mass standards are, in order of increasing elution time, cholesterol, dihydrolanosterol, and lanosterol.
Figure 46. Gas-liquid Radiochromatography of the Digitonin-Precipitable Sterols Recovered from an Incubation in the Presence of $10^{-5}$ M 14α-Ethylcholest-7-en-15α-ol-3-one. The mass standards are, in order of increasing elution time, cholesterol, dihydrolanosterol and lanosterol.
Experiment 14

In this experiment the effects of 14α-ethylcholesterol-7-en-3ß,15α-diol and 14α-ethylcholesterol-7-en-3ß,15ß-diol on the production of sterols from sodium [2-14C]-mevalonate were investigated in liver preparations from male and female rats. The concentrations of the two sterols present in the final incubation mixtures were 10^{-6} \text{ M} and 10^{-5} \text{ M} respectively. The non-saponifiable lipids from each group were analyzed by silicic acid-Super Cel column chromatography on 1 x 100 cm columns, using benzene:hexane (1:1) as the packing and eluting solvent. Carrier cholesterol (5 mg) and carrier lanosterol (5 mg) were added to the lipid samples just before application to the columns.

Figures 47 and 48 show that the majority of the radioactivity recovered from the column co-eluted with the cholesterol carrier detected colorimetrically in both the incubations from the male and female rat liver preparations in the absence of added sterol inhibitor. Figures 49 and 50 show that although 14α-ethylcholesterol-7-en-3ß,15α-diol produced nearly complete inhibition of cholesterol synthesis with a concurrent increase in the amount of material co-chromatographing with lanosterol in male rats' liver preparations, in preparations from female rats' livers, there were significant amounts of radioactive material co-chromatographing with cholesterol (about 33% of the recovered radioactivity) in addition to the material which co-chromatographed with the carrier lanosterol.
Figure 47. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from Control Incubations using a 10,000 x g Supernatant Fraction from a Liver Homogenate Preparation from Male Rats. (---) radioactivity; (---) cholesterol standards measured colorimetrically.
Figure 48. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from Control Incubations using a 10,000 x g Supernatant Fraction from a Liver Homogenate Preparation from Female Rats. (O--O) radioactivity; (X--X) cholesterol and (+--+) lanosterol standards measured colorimetrically.
Figure 49. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from an Incubation using a 10,000 x g Supernatant Fraction from a Liver Homogenate Preparation from Male Rats in the Presence of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol. (○--○) radioactivity; (+-----+) lanosterol and (X---X) cholesterol standards measured colorimetrically.
Figure 50. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from an Incubation using a 10,000 x g Supernatant Fraction from a Liver Homogenate Preparation from Female Rats in the Presence of 10^{-6} M 14α-Ethylcholest-7-en-3β,15α-diol. (o--o) radioactivity; (+---+) lanosterol and (x---x) cholesterol standards measured colorimetrically.
Figures 51 and 52 show that 14α-ethylcholest-7-en-3β,15β-diol had little effect on the conversion of mevalonate to cholesterol in either males' or females' rat liver preparations. Approximately 70-80% of the radioactivity recovered from the columns co-chromatographed with the cholesterol standard. In each case, however, there was at least one extra peak eluting between the cholesterol and lanosterol standards which was not present in either the control profiles or the profiles from the 14α-ethylcholest-7-en-3β,15α-diol treated samples.

Aliquots of the non-saponifiable lipids from the incubations of females' rat liver preparations with 14α-ethylcholest-7-en-3β,15α-diol and 14α-ethylcholest-7-en-3β,15β-diol were analyzed by gas-liquid radiochromatography on columns of 3% OV-17 at 270°C. The radioactivity elution profile is plotted as a bar graph superimposed over a standard mass tracing which is plotted as a continuous line. The lipids from the incubation containing 14α-ethylcholest-7-en-3β,15α-diol contained mostly material eluting at the same time as lanosterol and dihydrolanosterol, but there was a significant peak which eluted at the same time as cholesterol. The lipids from the incubation containing 14α-ethylcholest-7-en-3β,15β-diol contained predominantly material eluting at the same time as cholesterol, but there was a smaller peak eluting at a time between those of cholesterol and dihydrolanosterol.
Figure 51. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from an Incubation using a 10,000 x g. Supernatant Fraction from a Liver Homogenate Preparation from Male Rats in the Presence of 10^{-5} M 14α-Ethylcholest-7-en-3β,15β-diol. (-----o) radioactivity; (+-----+) lanosterol and (x-----x) cholesterol standards measured colorimetrically.
Figure 52. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from an Incubation using a 10,000 x g Supernatant Fraction from a Liver Homogenate Preparation from Female Rats in the Presence of 10^{-5} M 14α-Ethylcholest-7-en-3β,15β-diol. (---) radioactivity; (+---+) lanosterol and (x---x) cholesterol standards measured colorimetrically.
Figure 53. Gas-Liquid Radiochromatography of the Digitonin-Precipitable Sterols from an Incubation in the Presence of $10^{-6}$ M 14α-Ethylcholest-7-en-3β,15α-diol in a Liver Preparation from Female Rats. The mass standards are, in order of increasing elution time, cholesterol, dihydrolanosterol and lanosterol.
Figure 54. Gas-liquid Radiochromatography of the Digitonin-Precipitable Sterols Recovered from an Incubation in the Presence of $10^{-5}$ M 14α-Ethylcholest-7-en-3β,15β-diol in a Liver Preparation from Female Rats. The mass standards are, in order of increasing elution time, cholesterol, dihydro-lanosterol and lanosterol.
Experiment 15

In this experiment, the effects of 14α-ethylcholest-7-en-3β-ol-15-one and 14α-ethylcholest-7-en-15α-ol-3-one on the production of digitonin-precipitable sterols from sodium mevalonate in liver preparations from female rats were investigated by gas-liquid radiochromatography in order to see if there were significant differences in the profiles obtained when compared to similar experiments using liver preparations from male rats.

The profiles obtained are presented in Figures 55-57.
Figure 55. Gas-liquid Radiochromatography of the Digitonin-Precipitable Sterols Isolated from a Control Incubation of a Liver Preparation from Female Rats. The mass standards are, in order of increasing elution time, cholesterol, dihydrolanosterol and lanosterol.
Figure 56. Gas-liquid Radiochromatography of the Digitonin-Precipitable Sterols Isolated from an Incubation of a Liver Preparation of Female Rats Including $10^{-5}$ M 14α-Ethylcholest-7-en-3β-ol-15-one. The mass standards are, in order of increasing elution time, cholesterol, dihydrolanosterol and lanosterol.
Figure 57. Gas-liquid Radiochromatography of the Digitonin-Precipitable Sterols Isolated from an Incubation from a Liver Preparation from Female Rats Including 10^{-5}$ M 14α-Ethylcholest-7-en-15α-ol-3-one. The mass standards are, in order of increasing elution time, cholesterol, dihydrolanosterol and lanosterol.
The results of the experiments described in this section led to the observations described below.

14α-Ethylcholest-7-en-3β,15α-diol significantly \((p < 0.001)\) inhibited sterol synthesis from sodium acetate and sodium mevalonate (approximately 50 - 65%) at a concentration \(10^{-6} \text{ M}\) at which purified cholesterol and cholest-5-en-3β,25-diol had no effect on sterol synthesis from sodium acetate.

The production of non-saponifiable lipids from sodium acetate was linear from 0-90 minutes but curved upward at 120 minutes. There was no significant effect of 14α-ethylcholest-7-en-3β,15α-diol at \(10^{-6} \text{ M}\) on the production of non-saponifiable lipids. The production of digitonin-precipitable sterols from sodium acetate followed a similar time course, but at times after 30 minutes, the samples containing 14α-ethylcholest-7-en-3β,15α-diol \((10^{-6} \text{ M})\) showed only one-half the incorporation of acetate as did the control samples.

The production of digitonin-precipitable sterols from sodium mevalonate was nearly linear from 0-90 minutes, but decreased slightly at 120 minutes. At each time point tested, 14α-ethylcholest-7-en-3β,15α-diol caused an average 58% decrease in the incorporation of sodium mevalonate into digitonin-precipitable sterols at a concentration of \(10^{-6} \text{ M}\).

In the concentration range 10-75 nM, 14α-ethylcholest-7-en-3β,15α-diol caused a decrease in the incorporation of mevalonate into digitonin-precipitable sterols. This de-
crease varied in an approximately linear manner with the concentration of the sterol.

The nature of the inhibition in the production of digitonin-precipitable sterols from acetate and mevalonate was analyzed by subjecting the hexane-extractable lipids produced in the presence and absence of $10^{-6}$ M $14\alpha$-ethylcholest-7-en-3β,15α-diol to silicic acid column chromatography. In samples containing $14\alpha$-ethylcholest-7-en-3β, 15α-diol there was a large increase in the amount of material co-chromatographing with lanosterol and a sharp decrease (to nearly zero) in the amount of material co-chromatographing with cholesterol. In incubations in which sodium acetate was the substrate, there were many peaks in the radioactivity elution profiles of non-saponifiable lipids; cholesterol was a minor component of the control samples and was not detected in the samples which had been incubated with $14\alpha$-ethylcholest-7-en-3β,15α-diol. In incubations in which sodium mevalonate was the substrate, there were far fewer peaks in the radioactivity elution profiles than in the incubations in which sodium acetate had been used as a substrate. Material with the chromatographic properties of cholesterol was the major component of the control samples in the mevalonate incubations; material with the chromatographic properties of lanosterol was the major component of the samples which had been incubated with $14\alpha$-ethylcholest-7-en-3β,15α-diol.

The product whose accumulation from mevalonate was
caused by the presence of 14α-ethylcholest-7-en-3β,15α-diol in the incubation medium was shown to be convertible in part to material with the chromatographic properties of cholesterol upon reincubation with control liver preparations.

The identity of the accumulation product was analyzed more carefully with alumina-silver nitrate column chromatography and gas-liquid radiochromatography. These procedures resolved the material into two compounds which were identified as lanosterol and 24,25-dihydrolanosterol on the basis of their chromatographic behavior in the systems described above when compared to authentic standards. The material co-chromatographing with lanosterol was shown to be easily reduced to material co-chromatographing with dihydrolanosterol as expected (Fieser and Fieser [1959]). The acetate esters of the two compounds were prepared and crystallized with authentic lanosteryl acetate and dihydrolanosteryl acetate in several solvents. No significant change in specific activity was detected before and after these co-crystallizations.

Several additional 14α-ethyl-3,15-dioxygenated sterols were assayed for ability to inhibit the production of digitonin-precipitable sterols from mevalonate. 14α-Ethylcholest-7-en-15α-ol-3-one at 10^{-5} M was found to be as effective as 14α-ethylcholest-7-en-3β,15α-diol at 10^{-6} M; 14α-ethylcholest-7-en-3β-ol-15-one was less effective at 10^{-5} M; 14α-ethylcholest-7-en-3β,15β-diol was much less effective at 10^{-5} M. The products of the mevalonate incubations were ana-
lyzed by gas-liquid radiochromatography. Material co-chro-
momatographing with lanosterol and dihydrolanosterol was the
only material present in significant amounts (> 90%) in the
incubations containing 14α-ethylcholest-7-en-3β,15α-diol
and 14α-ethylcholest-7-en-15α-ol-3-one. Material co-chro-
momatographing with cholesterol comprised a significant frac-
tion of the sterols identified from incubations containing
14α-ethylcholest-7-en-3β-ol-15-one and especially 14α-ethyl-
cholest-7-en-3β,15β-diol.

It was discovered that the effects of 14α-ethylcho-
lesterol-7-en-3β,15α-diol were different in liver preparations
from male and female rats using sodium mevalonate as the
substrate. Although the non-saponifiable lipids in the con-
trol samples were very similar when analyzed by silicic
acid column chromatography, in the samples incubated in the
presence of 14α-ethylcholest-7-en-3β,15α-diol there were
distinct differences in the non-saponifiable lipids isolated
from liver preparations from male and female rats. In liver
preparations from female rats, material comigrating with
cholesterol was a significant (approximately 35%) component
of the radioactivity, while it was a very minor (approxi-
mately 5%) component of the radioactivity recovered in the
non-saponifiable lipids from liver preparations of male
rats. In preparations from both male and female rats con-
taining 14α-ethylcholest-7-en-3β,15β-diol, material comi-
grating with cholesterol was the major component of the
radioactivity in the non-saponifiable lipids recovered from
the incubations.

The effects of 14α-ethylcholesterol-7-en-3β-ol-15-one and 14α-ethylcholesterol-7-en-15α-ol-3-one were similar in liver preparations from male and female rats, except that there was generally a somewhat larger percentage of material co-migrating with cholesterol (as measured by gas-liquid radiochromatography) present in the lipids isolated from the liver preparations from female rats than in those isolated from liver preparations from male rats.

14α-Ethylcholesterol-7-en-3β,15α-diol has been shown to be one of the most potent inhibitors of sterol synthesis in cell culture yet described (Schroepfer et al. [1977b]). In an in vitro assay system, the compound was shown by the experiments in this section to be a very potent inhibitor of the incorporation of acetate and mevalonate into digitonin-precipitable sterols (approximately 60% inhibition) at a concentration (10^{-6} M) at which purified cholesterol and cholest-5-en-3β,25-diol had no effect on sterol synthesis from acetate. It is of particular interest to note that cholest-5-en-3β,25-diol, a very potent inhibitor of sterol synthesis from acetate in cell culture (Kandutsch and Chen [1974]), had no effect in this in vitro cell-free system at 10^{-6} M. This observation suggests that the mode of action of 14α-ethylcholesterol-7-en-3β,15α-diol may be different from that of cholest-5-en-3β,25-diol in cultured cells. The results of the experiments involving cholest-8(14)-en-3β-ol-15-one described earlier have shown that whole animal, cell cul-
ture and cell-free experiments are not always directly comparable. The lack of effect of cholesterol in this assay system is significant because it shows that 14α-ethylcholesterol-7-en-3β,15α-diol is acting as a specific agent rather than expressing a property common to all sterols.

In investigations of the mode of action of 14α-ethylcholesterol-7-en-3β,15α-diol, it was determined that the action was very rapid: the percentage inhibition of sterol synthesis from mevalonate in experimental vs. control samples was approximately 58% at times as early as 30 minutes and continued essentially unchanged at times as late as 120 minutes. During this time the overall rate of incorporation of mevalonate into sterols varied approximately two-fold. The in vitro inhibition caused by 14α-ethylcholesterol-7-en-3β,15α-diol was not affected by the presence or absence of a protein synthesis inhibitor (vide infra). This evidence all points to an effect other than control at a translational or transcriptional level in this in vitro system.

As in the experiments with cholest-8(14)-en-3β-ol-15-one, the mode of action of 14α-ethylcholesterol-7-en-3β,15α-diol in cell culture experiments and in the cell-free system used in these studies was quite different. The compound inhibited HMG-CoA reductase in L-cells preincubated with the sterol by 50% at a concentration of 0.05 μM (Schroepfer et al.[1977b]). In the experiments presented in these studies, there was very little effect on the production of non-saponifiable lipids from acetate (which is the point at which an
effect on HMG-CoA reductase would have been observed). In contrast, there was a large inhibition of the production of digitonin-precipitable sterols with a concomitant increase in the production of material with the chromatographic properties of lanosterol and dihydrolanosterol in a ratio of 0.46:0.54 respectively.

Because of the accumulation of C₃₀ sterols, it appears that 14α-ethylcholester-7-en-3β,15α-diol is a potent inhibitor of the metabolism of lanosterol and dihydrolanosterol, at least under the conditions employed in these experiments.
IN VITRO STUDIES:

IV. The Metabolism of 14α-Ethylcholest-7-en-3β,15α-diol in Cell-free Preparations
IN VITRO METABOLISM OF 14α-ETHYLCHOLEST-7-EN-3β,15α-DIOL BY 10,000 x G SUPERNATANT PREPARATIONS FROM RAT LIVER

Metabolism of 14α-Ethylcholest-7-en-3β,15α-diol by 10,000 x g Supernatant Preparations from Male Rats' Livers

Male Sprague-Dawley rats were maintained on a diet of Purina formula # 5008 laboratory chow and water ad libitum for at least a week prior to sacrifice. During this time the animals were maintained on a strict light-dark cycle (0600-1800 light). The animals were sacrificed at 0730-0830 and weighed 150-200 gm at the time of their sacrifice. The rats were killed by decapitation and their livers excised. After having been rinsed with physiological saline solution, the livers were dried with Kim-wipes and weighed. A 2.5 x volume of incubation buffer (see Materials and Methods) was added to the livers which were minced with scissors. This preparation was homogenized for ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle at 660 rpm. The homogenate was centrifuged at 500 x g for five minutes (2°C) and the supernatant derived therefrom was centrifuged for thirty minutes at 10,000 x g (2°C). To this supernatant preparation (50 ml) was added a solution of [2,4-3H]-14α-ethylcholest-7-en-3β,15α-diol (0.045 mg = 3 x 10^6 dpm) in propylene glycol (0.5 ml). A cofactor solution containing ATP (315 mg), NAD (77 mg), NADP (79 mg)
and G-6-P (115 mg) in the incubation buffer (5 ml) was adjusted to approximate neutrality by the addition of 5N potassium hydroxide solution (4 drops). This cofactor stock solution was added to the incubation mixture. The final concentrations of each cofactor were ATP (5 mM), NAD (1 mM), NADP (1 mM) and G-6-P (3.8 mM). The incubation solution was flushed 15 seconds with oxygen and incubated four hours in a water bath (37°C) with shaking (90 osc/min). At the end of the incubation, 15% ethanolic potassium hydroxide solution (50 ml) was added and the mixture was heated three hours on a steam bath. At the end of this time, the nonsaponifiable lipids were extracted with three portions of hexane (150 ml). The combined hexane layers were washed once with water (100 ml) and the hexane removed in a rotary evaporator under reduced pressure. (The material extracted by the hexane contained 85% of the initial radioactivity added to the incubation system.) To the residue was added dihydrolanosterol (5 mg) and cholesterol (5 mg). This material was dissolved in toluene (1 ml) and applied to the bed of a 1 x 100 cm column packed with silicic acid-Super Cel (2:1) in toluene under 10 p.s.i. nitrogen. After the sample had drained onto the column bed, a one liter solvent bulb was attached and filled with toluene. Twenty minute fractions (approximately 2 ml volume) were collected. After 150 fractions, the solvent was changed to 10% ether in toluene and another 70 fractions were collected. The column profile is presented in Figure 58.
Figure 58. Silicic acid Column Chromatography of the Non-Saponifiable Lipids Isolated from the Incubation of [2,4-\textsuperscript{3}H]-14\textalpha-Ethylcholesterol-7-en-3\textbeta,15\alpha-diol in Liver Preparations from Male Rats. (\textbullet---\textbullet) radioactivity; (+---+) lanosterol and (x---x) cholesterol standards measured colorimetrically. The arrow marks the solvent change from toluene to 10\% ether in toluene.
Metabolism of 14α-Ethylcholest-7-en-3β,15α-diol by 10,000 x g Supernatant Preparations from Female Rats' Livers

Female Sprague-Dawley rats were maintained on a diet of Purina formula # 5008 laboratory chow and water ad libitum for at least a week prior to sacrifice. During this time the animals were maintained on a strict light-dark cycle (0600-1800 light). The animals were sacrificed at 0730-0830 and weighed 150-200 gm at the time of their sacrifice. The rats were killed by decapitation and their livers excised. After having been rinsed with physiological saline solution, the livers were damp-dried and weighed. A 2.5 x volume of incubation buffer (see Materials and Methods) was added to the livers which were minced with scissors. This preparation was homogenized ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle at 660 rpm. The homogenate was centrifuged five minutes at 500 x g (2°C). The supernatant was removed and centrifuged thirty minutes at 10,000 x g (2°C). To this supernatant (50 ml) was added a solution [2,4-3H]-14α-ethylcholest-7-en-3β,15α-diol (0.045 mg = 3 x 10⁶ dpm) in propylene glycol (0.5 ml). A cofactor solution containing ATP (315 mg), NAD (77 mg), NADP (79 mg) and G-6-P (115 mg) in the incubation buffer (5 ml) was adjusted to approximate neutrality by the addition of 5 N potassium hydroxide solution (4 drops). This cofactor solution was added to the
incubation mixture. The final concentrations of each co-factor were ATP (5 mM), NAD (1 mM), NADP (1 mM), and G-6-P (3.8 mM). The incubation solution was flushed 15 seconds with a strong stream of oxygen and incubated four hours in a water bath (37°C) with shaking (90 osc/min). At the end of the incubation, a 15% ethanolic potassium hydroxide solution (50 ml) was added and a piece of glass tubing in a cork attached to the flask to serve as a reflux condenser. The mixture was heated three hours on a steam bath. At the end of this time, the non-saponifiable lipids were extracted with three portions of hexane (150 ml). The combined hexane layers were washed with water (100 ml) and the hexane removed in a rotary evaporator under reduced pressure. (The material extracted by hexane contained 88% of the radioactivity present at the start of the incubation.) To the residue was added dihydrolanosterol (5 mg) and cholesterol (5 mg). This material was dissolved in toluene (1 ml) and applied to the bed of a 1 x 100 cm column packed with silicic acid-Super Cel (2:1) in toluene under 10 p.s.i. nitrogen. After the sample had drained onto the column bed, a one liter solvent bulb was attached and filled with toluene. Twenty minute fractions (approximately 2 ml volume) were collected. After 150 fractions, the solvent was changed to 10% ether in toluene and another 70 fractions were collected. The column profile is presented in Figure 59.

In the experiments investigating the metabolism of [2,4-3H]-14α-ethylcholest-7-en-3β,15α-diol in rat liver
Figure 59. Silicic acid Column Chromatography of the Non-Saponifiable Lipids in Isolated from the Incubation of [2,4-\textsuperscript{3}H]-14\textalpha-Ethylcholest-7-en-3\beta,15\alpha-diol in Liver Preparations from Female Rats. (\textbullet\textbullet\textbullet\textbullet) radioactivity; (+-+) lanosterol and (x---x) cholesterol standards measured colorimetrically. The arrow marks the solvent changes from toluene to 10% ether in toluene.
10,000 x g supernatants, the following control was performed with the preparations from both male and female rats' livers. To the 10,000 x g supernatant preparation used in the experiments described above (1.8 ml) was added sodium [2-\textsuperscript{14}C]-mevalonate solution (0.10 ml; 10 uCi/60 ug/ml) and a cofactor stock solution (0.10 ml; ATP, 63 mg/ml; NAD 15.5 mg/ml; NADP, 16 mg/ml; G-6-P, 23 mg/ml). This mixture was flushed five seconds with oxygen and incubated in a water bath (37°C) with shaking (90 osc/min) for 1\frac{1}{2} hours. After the incubation, a 15% ethanolic potassium hydroxide solution (3 ml) and a solution of cholesterol in ethanol (1 ml; 4 mg/ml) solution were added and the mixture was heated for three hours in a water bath (70-75°C). After the hydrolysis, water (6 ml) was added and the non-saponifiable lipids were extracted three times with hexane (15 ml). The combined hexane layers were washed once with water (25 ml) and the hexane removed on a rotary evaporator under reduced pressure. The residues were used to prepare the digitonin-precipitable sterols as described in Materials and Methods. When the amount of incorporation of radioactivity into digitonin-precipitable sterols was calculated, it was found that for the preparations from male and female rats' liver the incorporations were 38% and 32%, respectively. The theoretical incorporation was 42%. The theoretical incorporation is calculated from the biosynthetic pathway, since there are 5 mevalonate units incorporated into lano-sterol and the C-2 of one of these units is lost in the con-
version of lanosterol to cholesterol. Further, utilization of only 50% of the labeled sodium mevalonate was anticipated inasmuch as the commercial starting material is racemic. The combination of these two pieces of information leads to the calculation that (5/6 x 50%) of the radioactivity of sodium [2-\textsuperscript{14}C]-mevalonate should appear in cholesterol. The fact that the actual incorporations were quite close to the theoretical demonstrates that the enzyme preparations were active and capable of converting authentic precursors to cholesterol (assayed as digitonin-precipitable sterols).

The results of these two experiments show that [2,4-\textsuperscript{3}H]-14α-ethylcholest-7-en-3β,15α-diol is not convertible to less polar sterols such as cholesterol by rat liver 10,000 x g preparations under conditions in which sodium mevalonate is readily converted to digitonin-precipitable sterols.

Some evidence was obtained by thin-layer radiochromatography (data not presented) that there was a slightly greater conversion of 14α-ethylcholest-7-en-3β,15α-diol to more polar products in female rats' liver preparations than in male rats' liver preparations, but the conversion was so slight (<2%) that the phenomenon was not rigorously investigated. These experiments have been repeated twice in liver preparations from both male and female rats, and in no case was there any detectable conversion of 14α-ethylcholest-7-en-3β,15α-diol to cholesterol or other less polar sterols.

The work of Eriksson (1971) and Gustafsson and
Sjovall (1968) demonstrated that female rats possess enzymes for the 15-hydroxylation of steroids which male rats do not. It was considered that females might also have enzymes capable of further reactions on 15-hydroxysterols unavailable to males. In view of this and the observation (vide supra) that 14α-ethylcholest-7-en-3β,15α-diol was more effective in inhibiting cholesterol synthesis in male than in female rats' liver preparations, it was thought that female rats might contain enzymes to metabolize 14α-ethylcholest-7-en-3β,15α-diol which were absent in males. However, in view of the lack of conversion of [2,4-³H]-14α-ethylcholest-7-en-3β,15α-diol to less polar sterols in either male or female rats' liver preparations and the similar recoveries of radioactivity in the non-saponifiable lipids in both cases, it seems that if female rats do possess such enzymatic activity, the rate of metabolism must be very slow since it was not detected in four hour incubations.
IN VITRO STUDIES:

V. The Effects of 14α-Ethylcholest-7-en-3β,15α-diol on Acetoacetyl-CoA Thiolase and on HMG-CoA Synthase in Cell-free Preparations
The Effects of 14α-Ethylcholesterol-7-en-3β,15α-diol on Two Early Enzymes in the Cholesterol Biosynthetic Pathway

The effect of 14α-ethylcholesterol-7-en-3β,15α-diol on the conversion of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA was also studied. This overall conversion proceeds in two steps. The first step is the reversible condensation of two acetyl-CoA molecules to yield acetoacetyl-CoA and free coenzyme A. This reaction is catalyzed by acetoacetyl-CoA thiolase. The second step is the essentially irreversible condensation of acetoacetyl-CoA and acetyl-CoA which yields 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and free coenzyme A. The latter reaction is catalyzed by HMG-CoA synthase. Both enzymes are present in the "soluble fraction" of the cell (Clinkenbeard et al. [1977], [1977a]).

For the studies described in the following section, male Sprague-Dawley rats were maintained on a diet of Purina formula # 5008 laboratory chow and water ad libitum for at least a week before sacrifice. During this time, the animals were kept on a strict light-dark cycle (0600-1800 light). All animals weighed between 150 and 200 gm at the time of their sacrifice and were killed between 0700-0830.

The livers of the sacrificed animals were excised, rinsed with physiological saline and damp-dried. The tissue was minced with scissors and added to a 2.5 x volume of the appropriate buffer (vide infra). The tissue was then
homogenized ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle at 660 rpm. The crude homogenate was centrifuged five minutes at 500 x g (2°C). The supernatant therefrom was centrifuged thirty minutes at 10,000 x g (2°C). This supernatant was either used as the enzyme source or, in some experiments, was further centrifuged one hour at 48,000 x g (4°C). The 48,000 x g supernatant was removed from the pelleted microsomal fraction by gentle aspiration.

\[ \text{Acetyl-CoA} \rightarrow \text{HMG-CoA} \]

The conversion of acetyl-CoA to HMG-CoA was monitored by an assay adapted from that of Clinkenbeard et al. (1973). The assay monitored the following reactions:

\[ 3 \text{ Acetyl-CoA} + H_2O \rightarrow \text{HMG-CoA} + 2 \text{ CoASH} \]

Clinkenbeard et al. (1973) reported the apparent equilibrium constant at pH 8.0 and 30°C to be 1.33 and to increase with increasing pH.

The assay employed involved the measurement of the incorporation of the label of \[^3\text{H}-\text{acetyl}]\text{-Acetyl-CoA} into HMG-CoA. The acid hydrolysis product of the substrate (acetic acid) is volatile at 100°C, whereas the product of acid hydrolysis of HMG-CoA (i.e., 3-hydroxy-3-methylglutaric acid) is not, and separation of the unreacted substrate from the enzymatic product can be effected by virtue of this difference in volatility of their acid hydrolysis products.
The buffer used for this assay contained 20 mM Tris and 30 μM EDTA at pH 8.0. The assay mixture was prepared by the addition of a 10,000 x g supernatant preparation (100 ul; vide supra), buffer (300 ul) and either propylene glycol (50 ul) or a 10^{-3} M solution of 14α-ethylcholest-7-en-3β,15α-diol in propylene glycol (50 ul). After a ten minute pre-incubation in a water bath (37°C), the assay was initiated by the addition of a [\textsuperscript{3}H-acetyl] -acetyl-CoA solution (20 ul = 0.02 uCi = 0.3 ug). The final concentration of acetyl-CoA in the incubation medium was 0.8 μM. Aliquots (50 ul) were removed after 2, 4, 6, 8, and 10 minutes and pipetted into scintillation vials containing 10 N hydrochloric acid (200 ul). The samples were dried an hour in an oven (95-105°C).

After the samples had been removed from the oven and had cooled to room temperature, water (0.5 ml) and ethanol (1.0 ml) were added and the vials shaken vigorously to ensure dissolution of the radioactive product. The samples were counted in toluene-ethanol scintillation fluid. The results of the assay, plotted as dpm of non-volatile radioactivity vs. time, are shown in Figure 60. There was no significant difference between the slopes of the lines obtained from the control samples (0.15 nmole/min) and those from the samples treated with 14α-ethylcholest-7-en-3β,15α-diol (0.14 nmole/min). This result appears to demonstrate that there is no effect of 14α-ethylcholest-7-en-3β,15α-diol at 10^{-4} M on the conversion of acetyl-CoA to HMG-CoA.
Figure 60. The Incorporation of Radioactivity from $[^3\text{H}]$-acetyl $\cdot$ Acetyl-CoA into a Non-Volatile Form after Treatment with Hydrochloric Acid and Heating at 100°C.
However, it is possible that this lack of apparent effect might be due to nearly equal but opposite effects on the two enzymes involved. To investigate this possibility, the enzymes were assayed individually.

**Acetoacetyl-CoA Thiolase**

Acetoacetyl-CoA thiolase is a soluble cytosolic enzyme which catalyzes the following reaction:

\[
\text{Acetoacetyl-CoA} + \text{CoASH} \rightarrow 2 \text{Acetyl-CoA}
\]

The reaction was assayed by a slight modification of the method of Clinkenbeard et al. (1975) and involves monitoring the coenzyme A dependent disappearance of acetoacetyl-CoA which has an absorption maximum at 300-303 nm. Although the reaction is reversible, the equilibrium lies far to the right as written. Clinkenbeard et al. (1975) reported a \( K_{app} = 5 \times 10^4 \) at pH 8.1 and 27°C. Thus thiolase activity is most easily measured in the direction of thiolytic cleavage.

The buffer used in the liver preparation contained 0.20 M Tris and 0.20 mM EDTA, pH 8.2. The enzyme source for these assays was the 48,000 x g supernatant prepared as described in the introduction to this section. Stock solutions of acetoacetyl-CoA (3.48 mg/ml; 4.0 mM) and coenzyme A (3.63 mg/ml; 4.5 mM) were prepared in water immediately before use. The assay was performed at 30°C in a Cary 118 Recording spectrophotometer. To the sample and reference
cuvettes were added Tris buffer (2.0 ml) and the 48,000 x g supernatant (10 ul, approximately 0.2 mg protein). The acetoacetyl-CoA stock solution (30 ul) was added to the sample cuvette. After mixing both cuvettes by inversion, the absorbance at 303 nm was monitored until there was no further decrease (generally ½ - 1 minute). The assay was initiated by the addition of the coenzyme A stock solution (20 ul). After rapid mixing, the cuvette was replaced in the spectrophotometer and the change in absorbance followed for approximately two minutes. From the linear portion of the reaction course, the specific activity of the enzyme was calculated using the extinction coefficient reported by Clinkenbeard et al. (1975) (3.6 x 10³ M⁻¹), the total incubation volume, and the protein concentration in the reaction mixture. The final concentrations of acetoacetyl-CoA and coenzyme A in the assay mixture were 0.06 mM and 0.04 mM respectively. 14α-Ethylcholest-7-en-3β,15α-diol was added to both the reference and sample cuvettes in propylene glycol (100 ul; 2 x 10⁻³ M solution) when appropriate. Table XXIX shows that there was no significant effect (p > 0.90) of either propylene glycol or a solution of 14α-ethylcholest-7-en-3β,15α-diol in propylene glycol (100 ul) on acetoacetyl-CoA thiolase activity as measured by this assay.
TABLE XXIX.

The Effect of $14\alpha$-Ethylcholester-7-en-3β,15α-diol ($10^{-4}$ M) on Acetoacetyl-CoA Thiolase Activity (n=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (umole/min/mg prot.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$0.24 \pm 0.01$</td>
</tr>
<tr>
<td>+ propylene glycol</td>
<td>$0.25 \pm 0.01^N$</td>
</tr>
<tr>
<td>+ $14\alpha$-Ethylcholester-7-en-3β,15α-diol</td>
<td>$0.24 \pm 0.01^N$</td>
</tr>
</tbody>
</table>

When $14\alpha$-ethylcholester-7-en-3β,15α-diol was preincubated ten minutes with the enzyme preparation at 30°C before the addition of either substrate, similar results were obtained.

TABLE XXX.

The Effect of $14\alpha$-Ethylcholester-7-en-3β,15α-diol ($10^{-4}$ M) on Acetoacetyl-CoA Thiolase Activity After Ten Minutes Preincubation (n=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (umole/min/mg prot.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + propylene glycol</td>
<td>$0.25 \pm 0.01$</td>
</tr>
<tr>
<td>$14\alpha$-Ethylcholester-7-en-3β, 15α-diol</td>
<td>$0.24 \pm 0.01^N$</td>
</tr>
</tbody>
</table>

Clinkenbeard et al. (1975) reported a specific activity of acetoacetyl-CoA thiolase from chicken liver of 0.28 umole/min/mg protein after a centrifugation of their crude homogenate preparation. Their value was very similar to the value reported above.

This assay was repeated on a new liver preparation
of a 48,000 x g supernatant using either a ten minute or a thirty minute preincubation at 30°C. In neither case was there a significant effect (p > 0.90) of 14α-ethylcholest-7-en-3β,15α-diol at 10^{-4} M final concentration on the acetoacetyl-CoA thiolase activity.

**TABLE XXXI.**

The Effect of 14α-Ethylcholest-7-en-3β,15α-diol (10^{-4} M) on Acetoacetyl-CoA Thiolase Activity After Ten Minutes and Thirty Minutes Preincubation (n=5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thiolase Activity 10 Minute Preincubation (umole/min/mg/protein)</th>
<th>Thiolase Activity 30 Minute Preincubation (umole/min/mg/protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + propylene glycol</td>
<td>0.15 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>14α-Ethylcholest-7-en-3β,15α-diol</td>
<td>0.15 ± 0.01\textsuperscript{R}</td>
<td>0.16 ± 0.01\textsuperscript{R}</td>
</tr>
</tbody>
</table>

A final experiment was designed to determine if it was necessary to perform the 48,000 x g centrifugation to obtain an enzyme preparation which gave accurate results in the thiolase assay; and whether or not freezing the protein solution once had any significant effect on thiolase activity in a 10,000 x g supernatant preparation.

A new liver preparation of a 10,000 x g supernatant was prepared and divided into two portions: half the preparation was assayed immediately and the remainder was frozen overnight (-20°C) and assayed the following day. In this
preparation, the aliquots used in the thiolase assay contained 0.45 mg protein, approximately twice the amount present in a similar aliquot of a 48,000 x g supernatant preparation. The results of this experiment are presented below.

**TABLE XXXII.**

The Effect of Freezing on Acetoacetyl-CoA Thiolase Activity in a 10,000 x g Supernatant Preparation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thiolase Activity (umole/min/mg/prot.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh 10,000 x g Supernatant (n=8)</td>
<td>0.078 ± 0.002</td>
</tr>
<tr>
<td>Frozen 10,000 x g Supernatant</td>
<td>0.081 ± 0.003</td>
</tr>
</tbody>
</table>

These data demonstrate that freezing once has no significant effect ($p > 0.40$) on acetoacetyl-CoA thiolase activity in this assay system. In addition, the thiolase activity can be accurately measured using a 10,000 x g supernatant as the enzyme source. The specific activity of both the 10,000 x g and 48,000 x g preparations was comparable when the differences in protein concentration were taken into account.

**HMG-CoA Synthase**

HMG-CoA synthase is a soluble cytosolic enzyme which catalyzes the following reaction:

$$\text{H}_2\text{O} + \text{Acetyl-CoA} + \text{Acetoacetyl-CoA} \rightarrow \text{HMG-CoA} + \text{CoASH}$$
The reaction was assayed by the method of Clinkenbeard et al. (1975a) and involves the measurement of the incorporation of the label of \( [1^-{\text{14}}\text{C-acetyl]}\)-acetyl-CoA into HMG-CoA. The reaction is essentially thermodynamically irreversible due to the hydrolysis of the coenzyme A ester.

The buffer used in the liver preparation contained 0.20 M Tris and 0.20 mM EDTA, pH 8.0. The enzyme source for these assays was the 48,000 x g supernatant prepared as described in the introduction to this section. The substrate stock solutions were acetoacetyl-CoA (0.92 mg/ml; 1.0 mM) and \( [1^-{\text{14}}\text{C-acetyl]}\)-acetyl-CoA (2.0 mM; 1.36 uCi/umole). The radioactive substrate was prepared in the following manner. Labeled acetyl-CoA from New England Nuclear (10 uCi = 0.16 mg) was diluted with unlabeled acetyl-CoA (5.93 mg) and dissolved in water (3.75 ml) to make the stock solution.

The assay mixture was prepared by adding the 48,000 x g preparation (200 ul) to the Tris buffer (100 ul) and either propylene glycol (20 ul) or a solution of 14\(\alpha\)-ethylcholest-7-en-3\(\beta\),15\(\alpha\)-diol (20 ul; 2 x 10\(^{-3}\) M). This mixture was preincubated for ten minutes at 30°C. The acetoacetyl-CoA stock solution (20 ul) was added and the assay initiated by the addition of the radioactive acetyl-CoA stock solution (40 ul). The final concentrations of acetoacetyl-CoA and acetyl-CoA in the assay mixture were 0.05 mM and 0.21 mM respectively. The incubations were performed in a 30°C water bath. At appropriate times, aliquots (50 ul) were re-
moved from the reaction mixture and transferred to scintillation vials containing 10 N hydrochloric acid (100 ul) to stop the reaction. The vials were taken to dryness in an oven (95-105°C). According to Clinkenbeard et al. (1975a), this process removed any unreacted substrate, leaving only the non-volatile product. After cooling, water (0.5 ml) and ethanol (1.0 ml) were added to each vial in order to dissolve the radioactivity in the residue. The samples were counted in toluene-ethanol scintillation fluid (10 ml). All the activity data reported have been corrected for quenching.

Figure 61 shows that, under the conditions employed, the assay was linear through twelve minutes. Another experiment (data not presented) showed that there was a definite leveling off of activity at fifteen minutes, however. From the slope of the line in the figure and the specific activity of the substrate (3.0 x 10^6 dpm/umole), the enzymatic activity under these conditions was calculated to be 2.4 x 10^-4 umoles/min.

The assay was repeated using a ten minute preincubation with control samples containing propylene glycol and samples containing 14α-ethylcholest-7-en-3β,15α-diol (10^-4 M final concentration). The results are presented in Figure 62. There was no significant effect of the steroid on the HMG-CoA synthase activity measured by this assay.

The results of the experiments described in this section demonstrated that there was no detectable effect of preincubation of 14α-ethylcholest-7-en-3β,15α-diol at 10^-4 M
Figure 61. The Incorporation of [1-{\textsuperscript{14}C-acetyl}] -Acetyl-CoA into Non-Volatile Radioactivity (HMG-CoA) as a Function of Time. The standard deviations are too small to be observed on this figure.
Figure 62. The Incorporation of \([1^{14}\text{C}-\text{acetyl}] -\text{Acetyl-CoA}\) into Non-volatile Radioactivity (HMG-CoA) as a Function of Time in the Presence (x—x) and Absence (o—o) of \(10^{-4} \text{ M} 14\alpha\text{-Ethylcholest-7-en-3\beta,15\alpha\text{-diol}}\).
concentration on the activity of either acetoacetyl-CoA thiolase or HMG-CoA synthase.

14α-Ethylcholest-7-en-3β,15α-diol, when preincubated in L-cell culture, caused a 50% decrease in HMG-CoA reductase activity at 2.3 μM, but a 50% decrease in sterol synthesis from acetate at 0.05 μM (Schroepfer et al. [1977b]). The two order of magnitude discrepancy in these concentrations suggested that there should be secondary sites of inhibition of 14α-ethylcholest-7-en-3β,15α-diol in addition to its effect on HMG-CoA reductase. Two early enzymes in the sterol biosynthetic pathway, acetoacetyl-CoA thiolase and HMG-CoA synthase, were assayed in the presence and absence of 14α-ethylcholest-7-en-3β,15α-diol to determine if the substance inhibited their in vitro activity. It did not. Even more surprising was the result that 14α-ethylcholest-7-en-3β,15α-diol did not inhibit HMG-CoA reductase activity in vitro on either a crude microsomal preparation (Miller [1978], unpublished observation) or on a purified HMG-CoA reductase preparation (R. Shapiro, personal communication). This is yet another demonstration of the difference in effect of the agent on cell culture and cell-free systems. To explain the discrepancy observed in the cell culture work, it will be necessary to conduct these assays on cell culture systems which have been preincubated with the sterol before assay.
The Effects of Phenylmethylsulfonyl Fluoride and Cyclo-heximide on the In Vitro Biosynthesis of Sterols from Acetate and Mevalonate in the Presence and Absence of 14α-Ethylcholester-7-en-3β,15α-diol.

In an attempt to investigate the mechanism of action of 14α-ethylcholester-7-en-3β,15α-diol in the inhibition of the biosynthesis of cholesterol from acetate and mevalonate, incubations were performed in the presence of phenylmethylsulfonyl fluoride (an inhibitor of proteases; Fahrney and Gold [1963]; Gold and Fahrney [1964]; Whitaker and Perez-Villasenor [1968]; Gold [1967]; Shaw [1972]) and cycloheximide (an inhibitor of protein synthesis; Ford and Leach [1948]; Whiffen [1958]; Johnson and Starkovsky [1962]; Johnson et al. [1965]).

The experiments in these studies were performed using the following protocol. Male Sprague-Dawley rats were maintained on a diet of Purina Rat Chow and water ad libitum for at least a week before sacrifice. During this time, the animals were kept on a strict light-dark (0600-1800 light) cycle. All animals weighed between 100 and 200 gm at the time of their sacrifice and were killed between 0700-0830.

The livers of the sacrificed animals were excised, rinsed with physiological saline and damp-dried. The tissue was minced with scissors and added to a 2.5 x volume of the phosphate incubation buffer described in the Materials
and Methods section. The tissue was homogenized ninety
seconds on ice in a glass homogenizer with a loose-fitting
teflon pestle at 660 rpm. The crude homogenate was centri-
fuged five minutes at 500 x g (2°C). The supernatant there-
from was centrifuged thirty minutes at 10,000 x g (2°C).
This latter supernatant was used as the enzyme source in all
the studies described in this section.

Each assay sample contained a cofactor stock solu-
tion (0.10 ml; vide supra); either sodium [3H]-acetate (0.10
ml; 10 mCi/4.0 mg/ml) or sodium [2,14C]-mevalonate (0.10 ml;
10 uCi/60 ug ml); and either propylene glycol (0.10 ml) or
a stock solution of 14α-ethylcholesterol-7-en-3β,15α-diol in
propylene glycol (0.10 ml; 2 x 10^-5 M). The final concen-
trations of acetate or mevalonate in the incubation medium
were 2.4 mM and 17.5 μM respectively. Samples containing
phenylmethylsulfonyl fluoride (PMSF) and 14α-ethylcholesterol-
7-en-3β,15α-diol were prepared by dissolving the PMSF in
the diol stock solution to a concentration of 3.48 mg/ml.
Samples containing PMSF alone were prepared by dissolving
PMSF in propylene glycol (3.48 mg/ml). These solutions
(0.10 ml) were used in the incubation samples containing
PMSF instead of either the diol stock solution or pure pro-
pylene glycol respectively. Samples containing cyclohexi-
mide were prepared by dissolving cycloheximide in the co-
factor stock solution (5.63 mg/ml) and adding this solu-
tion (0.10 ml) instead of the cofactor stock solution with-
out cycloheximide. These solutions were prepared in this
manner so that each incubation sample would contain a constant amount of propylene glycol (0.10 ml) and a constant amount of the 10,000 x g supernatant preparation (1.7 ml). After the 10,000 x g supernatant preparation had been added to the incubation samples, they were mixed well and flushed with a strong stream of oxygen for five seconds and tightly capped. The samples were then incubated in a water bath (37°C) with shaking (90 osc/min) for either two hours (when sodium acetate was used as substrate) of 1½ hours (when sodium mevalonate was used as substrate).

At the end of the incubation period, a solution of cholesterol in ethanol (1.0 ml; 4 mg/ml) and a 15% ethanolic potassium hydroxide solution (3 ml) were added to each sample. The samples were tightly recapped, mixed well and hydrolyzed by heating in a water bath (70-75°C) for three hours. At the end of this time, the non-saponifiable lipids were isolated by adding water (6 ml) to each sample and extracting the solution with hexane three times (15 ml). The combined hexane extracts were washed twice with water (10 ml) and the solvent evaporated under nitrogen. The residues were dissolved in a small amount of benzene and aliquots taken for determination of the radioactivity. Digitonin-precipitable sterols were prepared by removing the benzene under nitrogen and treating the material as described under the section on the isolation of digitonin-precipitable sterols in Materials and Methods.

This experiment investigated the effects of milli-
molar concentrations of PMSF and cycloheximide on the biosynthesis of non-saponifiable lipids and digitonin-precipitable sterols from sodium acetate in the presence and absence of micromolar 14α-ethylcholest-7-en-3β,15α-diol. The results presented in the following tables show a large effect of each substance on the incorporation of acetate into non-saponifiable lipids and digitonin-precipitable sterols. They had little effect on the inhibition produced by 14α-ethylcholest-7-en-3β,15α-diol in digitonin-precipitable sterols.

**TABLE XXXIII.**

The Effect of Phenylmethylsulfonyl Fluoride and Cycloheximide on the Incorporation of Sodium [3H]-Acetate into Non-Saponifiable Lipids in the Presence and Absence of 14α-Ethylcholest-7-en-3β,15α-diol (10^{-6} M) (N=4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation into Control samples (dpm x 10^{-4})</th>
<th>Incorporation into Diol-treated samples (dpm x 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52.7 ± 1.6</td>
<td>53.6 ± 2.1</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>25.3 ± 0.9^a</td>
<td>22.9 ± 2.4^a</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>36.7 ± 2.1^a</td>
<td>39.2 ± 1.7^a</td>
</tr>
</tbody>
</table>

PMSF (1 mM) caused approximately a 55% decrease in the incorporation of acetate into non-saponifiable lipids in the presence and absence of 14α-ethylcholest-7-en-3β,15α-diol (10^{-6} M; p < 0.001). Cycloheximide (1 mM) caused a similar but lesser decrease (approximately 30%; p < 0.001) in the incorporation of acetate into non-saponifiable lipids.
TABLE XXIV.


<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation into Control Samples (dpm x 10$^{-4}$)</th>
<th>Incorporation into Diol-treated Samples (dpm x 10$^{-4}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.27 ± 0.37</td>
<td>1.63 ± 0.21</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>0.62 ± 0.20$^a$</td>
<td>0.17 ± 0.05$^a$</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>2.23 ± 0.11$^a$</td>
<td>1.20 ± 0.24$^a$</td>
</tr>
</tbody>
</table>

PMSF (1 mM) caused an average 85% ± 5% decrease ($p < 0.001$) in the incorporation of acetate into digitonin-precipitable sterols in the presence or absence of 14α-ethylcholest-7-en-3β,15α-diol (10$^{-6}$ M) when compared to the proper control. The presence of 14α-ethylcholest-7-en-3β,15α-diol (10$^{-6}$ M) caused an average 56% ± 6% decrease ($p < 0.01$) in the incorporation of acetate into digitonin-precipitable sterols (compared to equivalently treated controls) in the presence or absence of cycloheximide (1 mM) or PMSF (1 mM). These results are presented in graphic form in the following figures.
Figure 63. The Effect of Phenylmethylsulfonyl Fluoride and Cycloheximide on the Incorporation of Sodium Acetate into Non-Saponifiable Lipids in the Presence (diagonally-lined) and Absence (clear) of 14a-Ethylcholest-7-en-3β, 15α-diol.
Figure 64. The Effect of Phenylmethylsulfonyl Fluoride and Cycloheximide on the Incorporation of Sodium Acetate into Digitonin-Precipitable Sterols in the Presence (diagonally-lined) and Absence (clear) of 14α-Ethylcholest-7-en-3β,15α-diol.
The following experiment investigated the effects of millimolar concentrations of PMSF and cycloheximide on the biosynthesis of non-saponifiable lipids and digitonin-precipitable sterols from sodium $[2^{-14}C]$-mevalonate in the presence and absence of $10^{-6}$ M 14α-ethylcholest-7-en-3β,15α-diol. Fresh stock solutions of cycloheximide (5.63 mg/ml) and PMSF (3.48 mg/ml) were prepared as described in the experiment immediately preceding. Assay samples were prepared as described before, except that in place of the sodium $[^3H]$-acetate stock solution, a sodium $[2^{-14}C]$-mevalonate stock solution (0.10 ml; 10 uCi/60 ug/ml) was used as the substrate. The following tables show that there was no significant effect of either cycloheximide or PMSF on the synthesis of either non-saponifiable lipids or digitonin-precipitable sterols from sodium mevalonate. Neither agent significantly affected the inhibition of digitonin-precipitable sterol synthesis caused by 14α-ethylcholest-7-en-3β,15α-diol (66% ± 5% decrease). These results are also presented in graphic form in the figures following the tables.
TABLE XXXV.

The Effect of Phenylmethylsulfonyl Fluoride and Cycloheximide on the Incorporation of Sodium [2-$^{14}$C]-Mevalonate into Non-Saponifiable Lipids in the Presence and Absence of 14α-Ethylcholesterol-7-en-3β,15α-diol (10^-6 M) (N=4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation into Control Samples (dpm x 10^-5)</th>
<th>Incorporation into Diol-treated samples (dpm x 10^-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.12 ± 0.17</td>
<td>3.21 ± 0.15</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>2.78 ± 0.16^e</td>
<td>2.94 ± 0.10^e</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>3.05 ± 0.14^i</td>
<td>3.15 ± 0.09^k</td>
</tr>
</tbody>
</table>

TABLE XXXVI.

The Effect of Phenylmethylsulfonyl Fluoride and Cycloheximide on the Incorporation of Sodium [2-$^{14}$C]-Mevalonate into Digitonin-Precipitable Sterols in the Presence and Absence of 14α-Ethylcholesterol-7-en-3β,15α-diol (10^-6 M) (N=4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation into Control Samples (dpm x 10^-5)</th>
<th>Incorporation into Diol-treated samples (dpm x 10^-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.56 ± 0.10</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>1.36 ± 0.19^i</td>
<td>0.41 ± 0.03^e</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>1.58 ± 0.06^e</td>
<td>0.64 ± 0.08^f</td>
</tr>
</tbody>
</table>
Figure 65. The Effect of Phenylmethylsulfonyl Fluoride and Cycloheximide on the Incorporation of Sodium Mevalonate into Digitonin-Precipitable Sterols in the Presence (diagonally-lined) and Absence (clear) of 14α-Ethylcholest-7-en-38,15α-diol.
The results of the experiments described in this section demonstrated that there was no effect of phenylmethylsulfonyl fluoride (a protease inhibitor) or cycloheximide (a protein synthesis inhibitor) on the effect of 14α-ethylcholest-7-en-3β,15α-diol on the production of digitonin-precipitable sterols from either sodium acetate or sodium mevalonate.

However, there was a large effect of phenylmethylsulfonyl fluoride (48%) and cycloheximide (30%) alone on the incorporation of acetate into non-saponifiable lipids and similar effects on the incorporation of acetate into digitonin-precipitable sterols. There was no significant effect of either phenylmethylsulfonyl fluoride or cycloheximide on the incorporation of mevalonate into digitonin-precipitable sterols.

Several laboratories (Higging et al. [1971]; Fogelman et al. [1977]; Brown et al. [1974]; Kirsten and Watson [1974]; Bell et al. [1975]) have demonstrated that de novo enzyme synthesis of HMG-CoA reductase is important in the regulation of the enzyme's activity and in cholesterologenesis in cell culture. Bell et al. (1975) have also reported that increased degradation of the enzyme (presumably by proteolysis) was responsible for the effects of cholest-5-en-3β,25-diol and cholest-5-en-3β-ol-7-one observed in cell culture. To determine if either of these mechanisms was operative to a significant extent in the inhibition produced by 14α-ethylcholest-7-en-3β,15α-diol in 10,000 x g supernatant fractions from rat liver homogenates, the ef-
fect of the agent on sterol synthesis in the presence and absence of cycloheximide (an inhibitor of protein synthesis) and phenylmethylsulfonyl fluoride (an inhibitor of serine proteases) were investigated. The inhibition of sterol synthesis caused by 14α-ethylcholest-7-en-3β,15α-diol was found to be unchanged by either cycloheximide or phenylmethylsulfonyl fluoride. These findings strongly suggest that the inhibition of sterol synthesis by 14α-ethylcholest-7-en-3β, 15α-diol in 10,000 x g supernatant fractions of rat liver homogenates involved neither inhibition of de novo protein synthesis nor stimulation of a serine-protease catalyzed degradation of HMG-CoA reductase.

However, a striking observation was made in the course of these experiments. Cycloheximide and phenylmethylsulfonyl fluoride were found to be potent inhibitors of the production of non-saponifiable lipids and digitonin-precipitable sterols from acetate, but not from mevalonate. These observations were investigated in more detail, and the results of these investigations are reported in later sections of this thesis.
IN VITRO STUDIES:

VI. The Effects of Phenylmethylsulfonyl Fluoride and Cycloheximide on Sterol Biosynthesis in Cell-free Preparations in the Presence and Absence of 14α-Ethylcholest-7-en-3β,15α-diol.
The Concentration-Response of the Inhibition of the Production of Non-Saponifiable Lipids and Digitonin-Precipitable Sterols from Acetate by Phenylmethylsulfonyl Fluoride in 10,000 x G Supernatant Fractions From Rat Liver Homogenate Preparations

In the experiments which demonstrated that 14α-ethylcholest-7-en-3β,15α-diol was equally effective in inhibiting the conversion of acetate and mevalonate to digitonin-precipitable sterols in the presence and absence of such agents as PMSF and cycloheximide, it was determined that these latter agents significantly inhibited the conversion of acetate, but not mevalonate to non-saponifiable lipids and digitonin-precipitable sterols. In this set of experiments, the concentration dependence of this inhibition by PMSF was investigated.

The enzyme preparation for both the following experiments was performed as follows. Male, Sprague-Dawley rats, previously maintained on a diet of Purina formula # 5008 laboratory chow and water ad libitum, were killed by decapitation between 0700-0830 and their livers excised. After they had been rinsed with physiological saline and damp-dried, the livers were minced with scissors in a 2.5 x volume of incubation buffer (see Materials and Methods.) The tissue preparation was homogenized ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle at a speed of 660 rpm. The homogenate was centrifuged five
minutes at 500 x g (2°C) and the supernatant therefrom centrifuged thirty minutes at 10,000 x g (2°C). This 10,000 x g supernatant was the enzyme source used in these experiments.

In each experiment, the 10,000 x g supernatant preparation (1.7 ml) was mixed with a cofactor stock solution (0.10 ml; ATP, 63 mg/ml; NAD, 15.8 mg/ml; NADP, 16 mg/ml; G-6-P, 23 mg/ml), the appropriate PMSF stock solution (0.10 ml; either 3480, 348, 34.8, 3.48, 0.348, or 0.035 µg/ml in propylene glycol - prepared by serial dilution of the most concentrated solution), and a sodium [3H]-acetate stock solution (0.10 ml; 10 mCi/4.0 mg/ml). The assay mixtures were flushed five seconds with a strong stream of oxygen, capped and incubated two hours in a shaking water bath (37°C; 90 osc/min). After two hours, the samples were removed from the water bath and a 15% ethanolic potassium hydroxide solution (3 ml) and a 4 mg/ml solution of cholesterol in ethanol (1 ml) were added. The samples were recapped and heated three hours in a water bath at 70-75°C. The non-saponifiable lipids were isolated from the hydrolyzed samples by the addition of water (6 ml) and extraction three times (15 ml) with hexane. The hexane extracts were washed with water (10 ml). The digitonin-precipitable sterols were isolated by the procedure described in the Materials and Methods section. The incorporation of acetate into non-saponifiable lipids and digitonin-precipitable sterols is shown in Table XXXVII and Figures 66 and 67.
TABLE XXXVII.

The Incorporation of Sodium \(^3\)H-Acetate into Non-Saponifiable Lipids and Digitonin-Precipitable Sterols as a Function of Phenylmethylsulfonyl Fluoride Concentration (N=4).

<table>
<thead>
<tr>
<th>(-\log [\text{PMSF}])</th>
<th>Incorporation into Non-Saponifiable Lipids (dpm x 10(^{-5}))</th>
<th>Incorporation into Digitonin-Precipitable Sterols (dpm x 10(^{-4}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(control)</td>
<td>9.00 ± 0.21</td>
<td>6.64 ± 0.10</td>
</tr>
<tr>
<td>3</td>
<td>3.54 ± 0.10(^a)</td>
<td>2.32 ± 0.05(^a)</td>
</tr>
<tr>
<td>4</td>
<td>6.19 ± 0.13(^a)</td>
<td>4.55 ± 0.09(^a)</td>
</tr>
<tr>
<td>5</td>
<td>7.39 ± 0.06(^a)</td>
<td>6.82 ± 0.10(^h)</td>
</tr>
<tr>
<td>6</td>
<td>6.94 ± 0.06(^a)</td>
<td>6.59 ± 0.12(^k)</td>
</tr>
<tr>
<td>7</td>
<td>7.47 ± 0.08(^a)</td>
<td>7.86 ± 0.15(^b)</td>
</tr>
<tr>
<td>8</td>
<td>9.45 ± 0.19(^f)</td>
<td>7.08 ± 0.09(^b)</td>
</tr>
</tbody>
</table>

The effect of PMSF on the incorporation of acetate into non-saponifiable lipids was significant (p < 0.001) for every concentration of PMSF tested except for 10\(^{-8}\) M, although the effect was particularly striking at 10\(^{-3}\) M (60% decrease) and 10\(^{-4}\) M (31% decrease). The effect of PMSF on the incorporation of acetate into digitonin-precipitable sterols was significant (p < 0.001) at 10\(^{-3}\) M (65% decrease) and 10\(^{-4}\) M (31% decrease). There was no significant decrease in incorporation at PMSF concentrations of 10\(^{-5}\) and 10\(^{-6}\) M. The slight stimulation observed at 10\(^{-7}\) M (18%) and at 10\(^{-8}\) M (7%) is statistically significant (p < 0.01).
Figure 66. The Effect of Phenylmethanesulfonyl Fluoride on the Incorporation of Acetate into Non-Saponifiable Lipids as a Function of Concentration (M).
Figure 67. The Effect of Phenylmethylsulfonyl Fluoride on the Incorporation of Acetate into Digitonin-Precipitable Sterols as a Function of Concentration (M).
The experiment was repeated using a fresh liver preparation. The cofactor and substrate solutions were prepared and mixed with the enzyme preparation as described in the previous experiment. Fresh PMSF stock solutions were prepared at the same concentrations as in the first experiment. After incubation and hydrolysis, the non-saponifiable lipids were isolated by hexane extraction. Table XXXVIII and Figure 68 present the results obtained.

**TABLE XXXVIII.**


<table>
<thead>
<tr>
<th>- log [PMSF]</th>
<th>Incorporation into Non-Saponifiable Lipids (dpm x $10^{-5}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(control)</td>
<td>8.06 ± 0.20</td>
</tr>
<tr>
<td>3</td>
<td>2.23 ± 0.05$^a$</td>
</tr>
<tr>
<td>4</td>
<td>6.05 ± 0.10$^a$</td>
</tr>
<tr>
<td>5</td>
<td>6.08 ± 0.09$^a$</td>
</tr>
<tr>
<td>6</td>
<td>6.17 ± 0.12$^a$</td>
</tr>
<tr>
<td>7</td>
<td>7.11 ± 0.14$^a$</td>
</tr>
<tr>
<td>8</td>
<td>7.73 ± 0.09$^d$</td>
</tr>
</tbody>
</table>

The effect of PMSF on the incorporation of acetate into non-saponifiable lipids was significant ($p < 0.001$) for every concentration of PMSF tested; except that at $10^{-8}$ M, its effect was significant at $0.02 < p < 0.05$. 
Figure 68. The Effect of Phenylmethysulfonyl Fluoride on the Incorporation of Acetate into Non-Saponifiable Lipids as a Function of Concentration (M).
At $10^{-3}$ M there was a 72% decrease. At $10^{-4}$ and $10^{-5}$ the decrease was approximately 25%.

The results of the experiments in this section demonstrated that phenylmethylsulfonyl fluoride is an extremely effective inhibitor of the incorporation of sodium acetate into non-saponifiable lipids and digitonin-precipitable sterols at concentrations of $10^{-3}$ and $10^{-4}$ M. The small inhibitions observed at $10^{-5}$ and $10^{-6}$ M were statistically significant in the non-saponifiable lipids but not in the digitonin-precipitable sterols.
IN VITRO STUDIES:

VII. The Effects of Phenylmethylsulfonyl Fluoride and Cycloheximide on Acetate Thio kinase, Acetoacetyl-CoA Thiolase, HMG-CoA Syn thase, and HMG-CoA Reductase in Cell-free Preparations
The Effects of Phenylmethylsulfonyl Fluoride and Cycloheximide on the Activity of Several Early Enzymes in the Sterol Biosynthetic Pathway

In the experiments which showed that 14α-ethylcholest-7-en-3β,15α-diol was equally effective in inhibiting the conversion of acetate and mevalonate to digitonin-precipitable sterols in the presence and absence of such agents as phenylmethylsulfonyl fluoride and cycloheximide, it was determined that both these agents significantly inhibited the conversion of acetate, but not mevalonate, to digitonin-precipitable sterols. The following sets of experiments were designed and executed to further investigate these unexpected effects.

This investigation was performed by assaying individually those enzymes which are required to transform acetate to mevalonate in rat liver. They are acetate thiokinase, acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase. The path for the conversion of acetate to mevalonate is presented in the following figure.

**Acetate Thiokinase (Acetyl-CoA Synthetase)**

Acetate thiokinase catalyzes the ATP-dependent condensation of acetate and coenzyme A to form acetyl-CoA. The ATP is hydrolyzed to AMP and pyrophosphate during the reaction. The assay method described herein is essentially that of Sladek et al. (1970).
Figure 69. The Pathway for the Conversion of Acetate to Mevalonate. The enzymes involved are I - Acetate Thiokinase; II - Acetoacetyl-CoA Thiolase; III - HMG-CoA Synthase; IV - HMG-CoA Reductase.
The assay involves the enzyme-catalyzed reaction of acetate, coenzyme A and ATP to form acetyl-CoA. This activated acetate reacts with hydroxylamine present in the assay mixture to form acetoxyhydroxamate. The acetoxyhydroxamate is separated from unreacted acetate on a Dowex ion-exchange resin. Materials for the assay were prepared as follows.

Dowex 2X-8 ion exchange resin (40 gm; 200-400 mesh, chloride form) was converted to the hydroxide form by washing three times with three volumes of 5 N potassium hydroxide solution. The resin was collected on a sintered glass funnel and the liquid removed from the filter cake by suction. A portion (10 gm) was saved to neutralize a hydroxylamine hydrochloride solution (vide infra), and the remainder was converted to the acetate form with three washes of three volumes of a mixture of 9 N acetic acid and 0.9 N sodium acetate solution (1:1).

Free hydroxylamine solution was prepared by dissolving hydroxylamine hydrochloride (16 gm) in water (15 ml) and adding the Dowex 2X-8 resin in its hydroxide form in spatula-tip portions (20 mg) until the resin no longer changed color after addition to the solution. (Dowex 2X-8 resin in its hydroxide form is dark orange-brown, whereas the chloride and acetate forms are light yellow.) Then an extra portion (20 mg) was added and the mixture swirled briefly. The solution was filtered and the filtrate (25 ml) was the source of the hydroxylamine used in the assay. This stock solution was approximately 9 M.
For each set of assays, the enzyme preparation was made in the following manner. A male Sprague-Dawley rat, previously maintained on Purina formula # 5008 laboratory chow and water ad libitum, was killed between 0700-0830 by decapitation and its liver excised. A 2.5 x volume of incubation buffer (for the preparation of this buffer, vide Materials and Methods) was added to the minced liver. The mixture was homogenized for ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle at 660 rpm. The homogenate was centrifuged for five minutes at 500 x g (2°C) and the supernatant therefrom was centrifuged again for thirty minutes at 10,000 x g. This 10,000 x g supernatant was the enzyme source.

A substrate stock solution was prepared by dissolving ATP (63 mg/ml) and coenzyme A (7.68 mg/ml) in the incubation buffer and adjusting the pH to approximately pH 7 with a potassium hydroxide solution. The assays were performed by mixing the enzyme preparation (1.6 ml), the substrate stock solution (0.10 ml), and either propylene glycol (0.10 ml) or a solution of PMSF in propylene glycol (0.10 ml; 3.48 mg/ml). When cycloheximide was added to the incubation medium, water (0.10 ml) was added in place of the PMSF solution. These assay samples were mixed well and preincubated for twenty minutes in a water bath (37°C) with shaking (90 osc/min). At the end of the preincubation, the samples were removed from the water bath and the hydroxylamine solution (0.10 ml) added. The assay was initiated by the addition of a sodium
[\textsuperscript{3}H]-acetate stock solution (0.10 ml; 10 mCi/4 mg/ml). The samples were mixed well and allowed to incubate twenty minutes (25°C).

At the end of twenty minutes, a 10 N hydrochloric acid solution (0.10 ml) was added to each sample to stop the reaction and precipitate the protein. A 0.8 N acetic acid solution (2.0 ml) was also added and the preparations centrifuged ten minutes at 3000 x g to pellet the precipitated protein.

The supernatant from the centrifuged assay mixture (0.5 ml) was applied to the top of a column (1 x 18 cm) poured with a slurry of the previously prepared Dowex resin, acetate form, in water. After the sample had drained onto the column bed, water (0.10 ml) was run onto the column bed and the eluate discarded. The acetohydroxamic acid product of the assay was eluted from the column with 0.8 N acetic acid solution (5.0 ml). Acetic acid is retained on the column under these conditions. When a sample of sodium [\textsuperscript{3}H]-acetate was acidified and applied to the column as described above, the water and 0.8 N acetic acid solution eluates contained less than 0.1% of the applied radioactivity. The radioactive acetic acid could be eluted from the column with 3 N sodium acetate solution (25 ml). The results of three separate experiments using separate liver preparations from different animals are reported in the following table.
TABLE XXXIX.

The Effect of Phenylmethylsulfonyl Fluoride on Acetate Thio kinase Activity (N=4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetic acid eluant (dpm x 10^{-7})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - 1</td>
<td>4.57 ± 0.35</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>0.78 ± 0.30a</td>
</tr>
<tr>
<td>Control - 2</td>
<td>1.82 ± 0.28</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>0.22 ± 0.10a</td>
</tr>
<tr>
<td>Control - 3</td>
<td>1.99 ± 0.36</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>0.41 ± 0.23a</td>
</tr>
</tbody>
</table>

These values correspond to an average decrease of 82% (p < 0.001) in the PMSF-treated samples. The control values reported in this experiment are approximately two orders of magnitude greater than the amounts of radioactivity recovered in the non-saponifiable lipid fraction after incubations using this same sodium [^{3}H]-acetate solution in the in vitro sterol synthesis assay described in earlier sections of this thesis. This implies that the activation of acetate to acetyl-CoA (which is the physiological substrate for sterol biosynthesis) is not limiting under the assay conditions employed. This is particularly apparent when it is considered that acetate thio kinase was only assayed twenty minutes; also the sterol synthesis assay is performed at
37°C instead of room temperature.

The effects of millimolar cycloheximide on acetate thiokinase were also investigated. Using the liver preparation and the assay procedure described above for the phenylmethylsulfonyl fluoride, experiments were performed using cycloheximide in the text samples. Table XL records the results obtained.

**TABLE XL.**

**The Effect of Cycloheximide on Acetate Thiokinase Activity (N=4).**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetic acid eluant (dpm x 10^{-7})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.91 ± 0.33</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>0.80 ± 0.32^{b}</td>
</tr>
</tbody>
</table>

These values correspond to an average decrease of 58% (p < 0.01) in the cycloheximide-treated samples. This experiment was repeated on another liver preparation with similar results. Table XLI records the results obtained.

**TABLE XLI.**

**The Effect of Cycloheximide on Acetate Thiokinase Activity (N=4).**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetic acid eluant (dpm x 10^{-7})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.01 ± 0.46</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>0.95 ± 0.27^{b}</td>
</tr>
</tbody>
</table>

These values correspond to an average decrease of 52% (p <
0.01) in the cycloheximide-treated samples.

The mechanism of action of phenylimethylsulfonyl fluoride and cycloheximide in inhibiting acetate thio kinase was not immediately apparent. However, since p-hydroxymer-
curibenzoate has been reported to inhibit acetate thio kinase from bacteria (Londesborough and Webster [1974]; Londesbor-
ough et al. [1972]; Sharkova [1968]), it was possible that the enzyme from rat liver was also sensitive to sulfhydryl reagents and that cycloheximide and PMSF were acting by blocking an essential thiol or thiols. The first part of this hypothesis was tested in the following experiments.

In these experiments, a 10,000 x g supernatant from rat liver was prepared as described above. The assay was carried out under the same conditions, except that the test samples contained either N-ethy1maleimide (2.50 mg/ml), bro-
moacetamidonitrophenol (5.50 mg/ml) or sodium iodoacetate (4.16 mg/ml) stock solutions (0.10 ml) in place of the PMSF or cycloheximide solutions. These stock solutions were chosen so that the final concentration of reagent in each assay was 1.0 mM. The results of these experiments are pre-
sented in Table XLIII.

Each reagent caused a significant (p < 0.01) decrease in acetate thio kinase activity as measured by this assay. However, it was determined in a separate experiment (see the section on the effects of cycloheximide and PMSF on aceto-
acetyl-CoA thiolase) that N-ethy1maleimide reacts very rapidly with free coenzyme A. Thus, the N-ethy1maleimide
TABLE XLII.

The Effect of Three Thiol-Reagents on Acetate Thiokinase Activity (N=4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetic acid eluant (dpm x 10^{-7})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - 1</td>
<td>1.06 ± 0.21</td>
</tr>
<tr>
<td>+ N-ethylmaleimide (1 mM)</td>
<td>0.35 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Bromoacetamido-nitrophenol (1 mM)</td>
<td>0.65 ± 0.12&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control - 2</td>
<td>3.65 ± 0.60</td>
</tr>
<tr>
<td>+ Sodium iodoacetate (1 mM)</td>
<td>0.28 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

result is not necessarily indicative of enzyme inhibition. Rather, it may simply be due to consumption of one of the substrates. Sodium iodoacetate was found not to react with free coenzyme A under similar conditions (see the section on the effects of cycloheximide and phenylmethylsulfonyl fluoride on acetoacetyl-CoA thiolase). Thus the iodoacetate-produced inhibition is indicative of actual enzyme inhibition.

Acetoacetyl-CoA Thiolase

Acetoacetyl-CoA thiolase catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA and free coenzyme A in a reversible reaction. The equilibrium lies far to the side of acetyl-CoA, however, so the most convenient assay measures activity in the thiolytic cleavage di-
rection using acetoacetyl-CoA and coenzyme A as substrates. The liver preparation procedure and the assay conditions were described in detail earlier in this thesis (viz. p. ff.) A 10,000 x g supernatant preparation was used in the experiments described below.

Stock solution of acetoacetyl-CoA (4.0 mM) and coenzyme A (4.5 mM) were prepared in water immediately before use. Stock solutions of PMSF (17.5 mg/ml in ethanol), cycloheximide (28.1 mg/ml in water) and sodium iodoacetate (20.8 mg/ml in water) were prepared. The assay buffer (2.0 ml), enzyme preparation (10 ul) and inhibitor stock solution (20 ul) were preincubated together twenty minutes (25°C) before assaying the acetoacetyl-CoA thiolase activity.

The assay was performed by pipetting the preincubated solution into the sample cuvette of a Cary 118 Spectrophotometer at 30°C and adding the acetoacetyl-CoA stock solution (30 ul). A blank was prepared in the reference cuvette containing buffer (2.0 ml), enzyme preparation (10 ul), and the inhibitor solution (20 ul). After the baseline had stabilized, the assay was initiated by the addition of the coenzyme A stock solution (20 ul). The decrease in absorbance at 303 nm was recorded vs. time. The final concentrations of acetoacetyl-CoA and coenzyme A in the assay medium were 0.06 mM and 0.04 mM respectively. Table XLIII shows that none of these agents were effective in inhibiting acetoacetyl-CoA thiolase under these conditions.
TABLE XLIII.

The Effect of Millimolar Concentrations of Cycloheximide, Phenylmethysulfonyl Fluoride and Sodium Iodoacetate on Acetoacetyl-CoA Thiolase Activity (N=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetoacetyl-CoA Thiolase Activity (nmole/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.8 ± 0.1</td>
</tr>
<tr>
<td>+ Ethanol (20 ul)</td>
<td>26.7 ± 0.9&lt;sup&gt;m&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>26.5 ± 1.6&lt;sup&gt;m&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>24.0 ± 0.7&lt;sup&gt;k&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Sodium iodoacetate (1 mM)</td>
<td>24.6 ± 0.7&lt;sup&gt;m&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

This experiment was repeated using a fresh rat liver preparation. The assay conditions were the same as those described above. Fresh stock solutions of the substrates and the modifying agents were prepared immediately before use. The results of the second experiment corroborated those of the first, as is shown in Table XLIV.
TABLE XLIV.

The Effect of Millimolar Concentrations of Cycloheximide, Phenylmethylsulfonyl Fluoride and Sodium Iodoacetate on Acetoacetyl-CoA Thiolase Activity (N=4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetoacetyl-CoA Thiolase Activity (nmole/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.5 ± 1.7</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>35.2 ± 1.8^g</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>34.6 ± 0.8^g</td>
</tr>
<tr>
<td>+ Sodium iodoacetate (1 mM)</td>
<td>33.1 ± 0.8^h</td>
</tr>
</tbody>
</table>

The use of N-ethylmaleimide as a modifying agent was attempted in this second assay. However, it proved to be incompatible with the assay procedure. When N-ethylmaleimide-treated protein was added to the sample cuvette, there was an increase in absorbance due to the absorbance at 303 nm of unreacted N-ethylmaleimide when compared to a reference blank without N-ethylmaleimide. Upon addition of the coenzyme A stock solution (20 ul) there was a rapid decrease in absorbance (occurring during the dead-time of sample mixing). This same effect was observed when coenzyme A was added to a solution of N-ethylmaleimide in water. This suggests that the very rapid decrease in absorbance upon addition of the coenzyme A solution was due to the reaction of the coenzyme A with N-ethylmaleimide. This reaction re-
sulted in both a removal of the maleimide chromophore (which caused the decrease in absorbance) and a covalent modification of the coenzyme A. The reaction is shown below.

\[ \text{CoASH} + \xrightarrow{\text{reaction}} \text{CoA-S} \]

This very rapid reaction of N-ethylmaleimide with coenzyme A renders it unsuitable as a modifying reagent in this assay without removal of the unreacted reagent prior to the assay.

Similarly, the lack of reaction of sodium iodoacetate with free coenzyme A under these conditions is inferred from the lack of inhibition observed upon preincubation of the enzyme preparation with sodium iodoacetate.

**HMG-CoA Synthase**

HMG-CoA synthase catalyzes the condensation of acetoacetyl-CoA with acetyl-CoA to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and free coenzyme A. The reaction is essentially thermodynamically irreversible (Clinkenbeard et al. [1975a]). The assay employed in these experiments was a spectrophotometric assay based on monitoring the disappearance of acetoacetyl-CoA caused by the addition of acetyl-CoA to the assay solution. The assay is that of Clinkenbeard et al. (1975a).

The liver preparations for these experiments were made as follows. Male Sprague-Dawley rats, previously main-
tained on Purina formula # 5008 laboratory chow and water ad libitum, were killed by decapitation at 0700-0830 and their livers excised. After rinsing with physiological saline and damp-drying, the livers were minced in a 2.5 x volume of 0.20 M Tris buffer containing 0.20 mM EDTA, pH 8.2. This mixture was homogenized for ninety seconds on ice in a glass homogenizer with a loose-fitting teflon pestle at 660 rpm. The homogenate was centrifuged five minutes at 500 x g (2°C) and the supernatant derived therefrom was centrifuged thirty minutes at 10,000 x g (2°C). The latter supernatant was the enzyme source for these assays.

Stock solutions of acetoacetyl-CoA (3.48 mg/ml; 4 mM) and acetyl-CoA (6.90 mg/ml; 8mM) and magnesium chloride (1 M) were prepared in water. Stock solutions of PMSF (17.5 mg/ml in ethanol), cycloheximide (28.1 mg/ml in water) and sodium iodoacetate (20.8 mg/ml in water) were prepared. The Tris buffer solution (2.0 ml), enzyme preparation (50 ul), magnesium chloride solution (20 ul) and inhibitor stock solution (20 ul) were preincubated together twenty minutes at room temperature. The preincubated solutions were pipetted into the sample cuvette of a Cary 118 Spectrophotometer at 30°C. A reference cuvette was prepared containing Tris buffer (2.0 ml), enzyme preparation (50 ul), magnesium chloride solution (20 ul) and inhibitor stock solution (20 ul). The acetoacetyl-CoA stock solution (25 ul) was added to the sample cuvette. After the baseline
had stabilized, the assay was initiated by the addition of the acetyl-CoA stock solution (50 ul). The decrease in absorbance at 300 nm was measured vs. time. The final concentrations of acetyl-CoA and acetoacetyl-CoA in the assay medium were 0.18 mM and 0.05 mM respectively. Enzymatic activity was calculated from the total volume of the assay solution, the protein concentration, and the extinction coefficient for acetoacetyl-CoA under these conditions (16.1 x 10^3 M^{-1} according to Clinkenbeard et al. [1975a]). Since this reaction produced free coenzyme A which is available to react with the acetoacetyl-CoA substrate in the thiolase-catalyzed reaction, and since the thiolase reaction is over an order of magnitude faster than the synthase reaction, the observed rate of disappearance of acetoacetyl-CoA (slope of the spectrophotometer tracing) was divided by two to correct for consumption of one molecule of the substrate for every molecule of product formed. This correction is described by Clinkenbeard et al. [1977a]). Table XLV shows that each of the modifying agents tested was effective in inhibiting HMG-CoA synthase activity under these conditions. The extent of inhibition was different for each agent, however.
TABLE XLV.

The Effect of Millimolar Concentration of Phenylmethyl-sulfonyl Fluoride, Cycloheximide and Sodium Iodoacetate on HMG-CoA Synthase Activity (N=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>HMG-CoA Synthase Activity (nmole/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.82 ± 0.17</td>
</tr>
<tr>
<td>+ Ethanol (20 μl)</td>
<td>1.85 ± 0.15^1</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>0.97 ± 0.05^b</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>1.24 ± 0.08^c</td>
</tr>
<tr>
<td>+ Sodium Iodoacetate (1 mM)</td>
<td>0.40 ± 0.04^b</td>
</tr>
</tbody>
</table>

Cycloheximide caused the smallest inhibition (32%, p< 0.02) of HMG-CoA synthase activity; PMSF resulted in a larger inhibition (47%, p< 0.01); sodium iodoacetate was the most effective inhibitor (78%, p< 0.01).

This experiment was repeated using a fresh rat liver preparation. The assay conditions were those described above. Fresh stock solutions of the substrates and modifying agents were prepared. The results of the second assay are presented in Table XLVI.
TABLE XLVI.

The Effect of Millimolar Concentrations of Phenylmethyl-sulfonyl Fluoride, Cycloheximide and Sodium Iodoacetate on HMG-CoA Synthase Activity (N=5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>HMG-CoA Synthase Activity (nmole/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.68 ± 0.17</td>
</tr>
<tr>
<td>+ Ethanol (20 ul)</td>
<td>3.27 ± 0.25^e</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>1.69 ± 0.06^a</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>2.03 ± 0.12^b</td>
</tr>
<tr>
<td>+ Sodium Iodoacetate (1 mM)</td>
<td>1.29 ± 0.09^a</td>
</tr>
</tbody>
</table>

Cycloheximide caused the smallest inhibition (25%, p < 0.01) of HMG-CoA synthase activity; PMSF resulted in a larger inhibition (50%, p < 0.001); sodium iodoacetate was also an effective inhibitor (52%, p < 0.001). The difference between the control and the control + ethanol was not significant (p > 0.05).

HMG-CoA Reductase

HMG-CoA reductase catalyzes the reduction of HMG-CoA to mevalonate with the concomitant production of free coenzyme A. The reaction requires two molecules of NADPH and is essentially irreversible. The assay employed in these experiments was a radioactive assay based on the thin-layer chromatographic separation of the substrate ([3-^{14}C-glutaryl]-3-hydroxy-3-methylglutaryl-CoA) from the product
([3-\textsuperscript{14}C]-mevalonic acid). The assay is that of Miller (1978).

The liver preparations for these experiments were made in the following manner. Male Sprague-Dawley rats, previously maintained on Purina formula # 5008 laboratory chow and water ad libitum, were killed by decapitation and their livers excised. After rinsing with physiological saline and damp-drying, the livers were minced in a 3.5 x volume of buffer containing 50 mM potassium phosphate, 250 mM sodium chloride, 30 mM EDTA and 1 mM dithiothreitol, pH 7.4. The samples were homogenized at 660 rpm in a glass homogenizer with a tight-fitting teflon pestle using five up-and-down strokes. The homogenate was centrifuged for fifteen minutes at 12,000 x g (2°C) and the supernatant centrifuged again for fifteen minutes at 12,000 x g (2°C). The second supernatant was centrifuged for one hour at 48,000 x g (4°C). The supernatant from this last centrifugation was discarded and the unwashed pellet resuspended in the original homogenization buffer (10 ml) by homogenization at 660 rpm for five strokes in a glass homogenizer with a tight-fitting teflon pestle.

A cofactor-substrate stock solution was prepared with the following components: glucose-6-phosphate (90 umoles/ml); glucose-6-phosphate dehydrogenase (6 I.U./ml); NADP (9.0 umoles/ml); DL-3-hydroxy-3-methyl-\textsuperscript{[3-\textsuperscript{14}C]}-glutaryl-CoA (1 umole/ml; 2000 dpm/nmole); and [5-\textsuperscript{3}H]-mevalonic acid (600,000 dpm/ml; 1.2 Ci/nmole). Stock solutions of cyclo-
heximide (13.2 mg/ml in water), PMSF (8.3 mg/ml in ethanol), N-ethylmaleimide (5.9 mg/ml in water) and sodium fluoride (7 mg/ml in water) were prepared.

The assay samples were prepared by adding to incubation tubes (400 ul) an aliquot of the resuspended 48,000 x g pellet (100 ul) and an aliquot of the appropriate inhibitor stock solution (10 ul). If the inhibitor solution was prepared in ethanol, then an aliquot of water (10 ul) was added to the incubation tube; if the inhibitor solution was prepared in water, an aliquot of ethanol (10 ul) was added to the incubation tube. In this manner, each incubation tube contained equal amounts of ethanol. The samples were mixed well on a Vortex mixer and preincubated twenty minutes in a water bath (37°C). The assay was initiated by the addition of the cofactor-substrate solution (50 ul) to each sample. After fifteen minutes, the reaction was stopped by the addition of 10 N hydrochloric acid (25 ul). The samples were incubated for another thirty minutes in the water bath (37°C) in order to insure lactonization of the product mevalonate to mevalonolactone. After centrifugation at 3000 rpm in a tabletop centrifuge to pellet the acid-precipitated protein, aliquots (approximately 75 ul) of the supernatant were spotted on Silica Gel G thin-layer chromatography plates (0.25 mm) and carefully dried. The plates were developed in acetone until the solvent had ascended 10 cm from the origin. The region corresponding to $R_f = 0.60-1.00$ was scraped into a scintillation vial and counted for
radioactivity using toluene-ethanol scintillation fluid. The tritiated mevalonate added at the beginning of the assay was used to correct for variable spotting on the thin-layer plate as well as variable recovery. Applying this correction and having the specific activity of the substrate (2000 dpm/nmole), the enzymatic activity was calculated.

Table XLVII reports the results obtained from the first experiment.

TABLE XLVII.

The Effect of Phenylmethylsulfonyl Fluoride on HMG-CoA Reductase Activity (N=5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>HMG-CoA Reductase Activity (pmole/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>145 ± 32</td>
</tr>
<tr>
<td>+ PMSF (3 mM)</td>
<td>35 ± 20</td>
</tr>
</tbody>
</table>

In this experiment, PMSF caused a 75% decrease in HMG-CoA reductase activity compared to the control samples. The difference was significant (p < 0.02), in spite of the relatively large standard errors.

The experiment was repeated with a fresh liver preparation using freshly prepared cofactor-substrate solution and inhibitor stock solutions. In addition to PMSF, cycloheximide and N-ethylmaleimide were tested for possible inhibition of enzymatic activity. Two further sets of sam-
amples were compared, one a control group containing 50 mM sodium fluoride, the other a group containing 50 mM sodium fluoride and PMSF. Sodium fluoride has been reported to inhibit an endogenous activator-protein present in HMG-CoA reductase preparations (Nordstrom et al. [1977]; Berndt et al. [1976]; Ingebritsen et al. [1978]). Thus, control and PMSF-treated incubation samples were assayed in the presence of sodium fluoride to determine whether or not the PMSF-inhibition was due to a direct effect on HMG-CoA reductase or due to an inhibition of the activator-protein. This problem is considered in more detail in the Discussion section immediately following these experiments.

Table XLVIII reports the results obtained in this third experiment. PMSF treatment again resulted in a large decrease in reductase activity (82%; p < 0.001) as measured by this assay. Cycloheximide had no significant (p > 0.80) effect on reductase activity in this experiment. Preincubation with sodium fluoride caused a significant inhibition of reductase activity in the presence (54%; p < 0.001) and the absence (25%; p < 0.02) of phenylmethylsulfonyl fluoride. Phenylmethylsulfonyl fluoride in the presence of sodium fluoride caused a significant decrease in reductase activity (40%; p < 0.01) compared to a control group also containing sodium fluoride.
TABLE XLVIII.

The Effect of Phenylmethylsulfonyl Fluoride, Cycloheximide and Sodium Fluoride on HMG-CoA Reductase Activity (N=6).

<table>
<thead>
<tr>
<th>Sample</th>
<th>HMG-CoA Reductase Activity (pmole/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>500 ± 20</td>
</tr>
<tr>
<td>+ PMSF (3 mM)</td>
<td>90 ± 30</td>
</tr>
<tr>
<td>+ Cycloheximide (3 mM)</td>
<td>490 ± 20</td>
</tr>
<tr>
<td>+ Sodium Fluoride (50 mM)</td>
<td>380 ± 40</td>
</tr>
<tr>
<td>+ Sodium Fluoride (50 mM) + PMSF (3 mM)</td>
<td>230 ± 30</td>
</tr>
</tbody>
</table>

The effects of phenylmethylsulfonyl fluoride and cycloheximide at concentrations of 1 mM on HMG-CoA reductase were also investigated. The higher (3 mM) concentrations were originally chosen in case there might be reaction of the dithiothreitol present in the assay buffer (1 mM) with one or both of the test agents. Even after reaction of both sulphydryl groups of the dithiothreitol, there would still be a significant amount of the modifying agents (1 mM) present available for reaction with the protein. It was decided to determine whether or not the reductase was inhibited by 1 mM concentrations of the agents. This would allow the comparison of the amounts of inhibition by the agents on the different enzymes between acetate and mevalonate since the experiments on the other enzymes were performed at
a concentration of the modifying agents of 1 mM. Further, the amount of protection by the dithiothreitol might be estimated.

The results of this experiment are presented in Table XLIX. At a concentration of 1 mM, phenylmethylsulfonyl fluoride caused a significant \( p < 0.02 \) reduction in the HMG-CoA reductase activity (50%) compared to that in control samples. Cycloheximide at 1 mM concentration had no significant effect \( p > 0.30 \) on reductase activity in this experiment. Since the effect of the phenylmethylsulfonyl fluoride at 2 mM was about 71% (average of the three previous experiments), it appears that dithiothreitol provided relatively little protection against the reductase inhibition by phenylmethylsulfonyl fluoride.

**TABLE XLIX.**

The Effect of Phenylmethylsulfonyl Fluoride and Cycloheximide on HMG-CoA Reductase Activity \( (N=6) \).

<table>
<thead>
<tr>
<th>Sample</th>
<th>HMG-CoA Reductase Activity (pmole/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>450 ± 100</td>
</tr>
<tr>
<td>+ PMSF (1 mM)</td>
<td>227 ± 40</td>
</tr>
<tr>
<td>+ Cycloheximide (1 mM)</td>
<td>570 ± 60</td>
</tr>
<tr>
<td>+ Sodium Fluoride (50 mM)</td>
<td>340 ± 60</td>
</tr>
<tr>
<td>+ Sodium Fluoride (50 mM) + PMSF (1 mM)</td>
<td>210 ± 60</td>
</tr>
</tbody>
</table>
RESULTS:

The results of the experiments described in this section demonstrated that preincubation with 1 mM or 3 mM phenylmethylsulfonyl fluoride produced large inhibitions in the activities of several enzymes in the early portion of the sterol biosynthetic path. Acetate thiokinase was inhibited an average of 48% (at 1 mM); HMG-CoA reductase was inhibited an average of 73% (at 3 mM); Acetoacetyl-CoA thiolase was not significantly inhibited by preincubation with the agent.

Preincubation with 1 mM or 3 mM cycloheximide also produced inhibition in the activities of several enzymes in the early portion of the sterol biosynthetic path, but the inhibitions were not as large as those produced by preincubation with phenylmethylsulfonyl fluoride. Cycloheximide inhibited acetate thiokinase an average of 60% (at 1 mM); HMG-CoA synthase was inhibited an average of 28% (at 1 mM). HMG-CoA reductase was not significantly affected by preincubation with cycloheximide at a concentration of either 1 mM or 3 mM. Acetoacetyl-CoA thiolase was not significantly inhibited by preincubation with cycloheximide at 1 mM.

Those enzymes which were inhibited by preincubation with phenylmethylsulfonyl fluoride were also strongly inhibited by preincubation with sodium iodoacetate and/or N-ethylmaleimide.
DISCUSSION

In an effort to understand the mechanism of the inhibition of sterol biosynthesis from acetate, but not from mevalonate, produced by cycloheximide and phenylmethylsulfonyl fluoride, each enzyme involved in the sequence of reactions between acetate and mevalonate was investigated. They will be discussed in order. The overall sequence of reactions is presented in Figure 70.

Acetate thio kinase (E.C.6.2.1.1) catalyzes the ATP-dependent condensation of acetate and coenzyme A to form acetyl-CoA. The other products are AMP and pyrophosphate. The enzyme is probably not important in sterol synthesis in vivo since acetate units are supplied as acetyl-CoA from glycolysis (via pyruvate); β-oxidation of fatty acids; and enzymatic degradation of several of the amino acids. However in animals on a diet high in ethanol or acetate, or in an experiment in a cell-free system in which labeled acetate is used as a substrate (in place of the more expensive and less readily accessible acetyl-CoA), the activation of acetate to acetyl-CoA by acetate thio kinase becomes important.

Berg (1956) proposed that the overall reaction proceeds via an acyl adenylate intermediate:

\[
\text{ATP} + \text{CH}_3\text{COOH} + \text{Enz} \rightleftharpoons \text{Enz-CH}_3\text{CO-AMP} + \text{PP}_i \\
\text{Enz-CH}_3\text{CO-AMP} + \text{CoASH} \rightleftharpoons \text{Enz} + \text{CH}_3\text{CO-SCoA} + \text{AMP}
\]
Figure 70. The Pathway for the Conversion of Acetate to Mevalonate. The enzymes involved are I - Acetate Thiokinase; II - Acetoacetyl-CoA Thiolase; III - HMG-CoA Synthase; IV - HMG-CoA Reductase.
An enzyme bound acetyl-adenylate was actually isolated by Webster (1963). The bacterial enzyme has been shown to be readily inhibited by sulfhydryl reagents such as p-mercuribenzoate (Londesborough et al. [1973]; Sharkova [1968]).

The studies reported in this thesis have demonstrated large inhibition of enzymatic activity by phenylmethylsulfonyl fluoride and cycloheximide, but the mechanism of action was not immediately apparent. The report of inhibition of thiokinase activity by p-mercuribenzoate suggested that phenylmethylsulfonyl fluoride and/or cycloheximide might also act by reacting with an essential thiol(s). This hypothesis was not, however, rigorously investigated (vide infra).

Acetoacetyl-CoA thiolase catalyzes the reaction between acetoacetyl-CoA and coenzyme A to yield two molecules of acetyl-CoA. The equilibrium lies far in the direction of acetyl-CoA under normal conditions, with $K_{\text{app}} = 5 \times 10^4$ at pH 8.1, 25°C (Stern et al. [1953]) for the enzyme isolated from avian liver cytosol. Species of thiolase from various sources have been shown to be inhibited by sulfhydryl reagents such as p-chloromercuribenzoate and 5,5'-dithio-bis-(2-nitrobenzoic acid). Iodoacetamide was much less effective (Nishimura et al. [1978]). In the studies in this thesis, cycloheximide and phenylmethylsulfonyl fluoride were found to be without effect on acetoacetyl-CoA thiolase activity. Sodium iodoacetate was also ineffective under the conditions employed.
HMG-CoA synthase (E.C.4.1.3.5) catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and free coenzyme A. The reaction is essentially thermodynamically irreversible (Clinkenbeard et al. [1975a]). The formation of an acetyl-bound enzyme complex was demonstrated by Middleton (1967). This complex reacts with acetoacetyl-CoA to form an HMG-CoA-S-Enz, complex which is hydrolyzed by water to form HMG-CoA and free enzyme (Miziorko and Lane [1977]). The attachment linkages of Enz-S-COCH₃ and Enz-S-HMG-CoA were demonstrated to be to enzyme sulfhydryl groups by Miziorko et al. (1975) and Miziorko and Lane (1977) respectively. The synthase (from avian liver) has been shown to be very sensitive to sulfhydryl reagents such as N-ethylmaleimide (Middleton and Tubbs [1972]) and sodium iodoacetate (Stewart and Rudney [1966]). The experiments described in this thesis have demonstrated that HMG-CoA synthase is inhibited by phenylmethylsulfonyl fluoride, cycloheximide and sodium iodoacetate.

HMG-CoA reductase (E.C.1.1.1.34) is a microsomal enzyme which catalyzes the reduction of HMG-CoA to mevalonate with the concomitant production of free coenzyme A. The reaction requires two molecules of NADPH and is essentially irreversible. The enzyme is very tightly regulated in vivo (Rodwell et al. [1976]) and is considered to be the rate-limiting enzyme in cholesterol biosynthesis in a wide range of physiological conditions (Siperstein [1970]; Rodwell et al. [1973]; White and Rudney [1970]; Dietschy and Brown
The enzyme has been shown to be sensitive to sulfhydryl reagents and to be stabilized by the presence of such agents as dithiothreitol (Rodwell et al. [1973]). The experiments described in this thesis demonstrated that phenylmethylsulfonyl fluoride is a potent inhibitor of reductase activity; the sulfhydryl reagent N-ethylmaleimide is even more potent. Cycloheximide had a marginal effect or no effect on reductase activity in different experiments.

Nordstrom et al. (1977), Berndt et al. (1976) and Ingebritsen et al. (1978) have reported investigations on a covalent reversible modification of HMG-CoA reductase involving phosphorylation-dephosphorylation reactions. HMG-CoA reductase evidently exists in two forms: an active, non-phosphorylated form, and an inactive, phosphorylated form. Phosphorylation is accomplished by a Mg-ATP-dependent protein kinase which appears to be microsomal but loosely bound. A soluble protein phosphatase was identified which was shown to be inhibited by fluoride ion and to be the protein responsible for reactivation of the phosphorylated reductase. Ingebritsen et al. (1978) reported that the phosphatase could be replaced by purified liver phosphorylase phosphatase and that total reactivation of the phosphorylated reductase could be achieved.

Due to the presence of large amounts of EDTA in the reductase homogenization and assay buffer, any Mg-ATP-dependent protein kinases are presumably inactive in the experiments described in this thesis. The addition of fluor-
ide ion to the assay system was shown in these experiments to lead to a decrease in reductase activity of approximately 50%. Thus, in the assay described herein, the reductase preparation is strongly activated during the preincubation and/or assay when fluoride is absent. The presence of EDTA and fluoride thus traps the system in whatever ratio of phosphorylated to non-phosphorylated enzyme existed at the time of fluoride addition.

Sodium fluoride caused approximately 50% lowering of reductase activity compared to the control. Phenylmethylsulfonyl fluoride + fluoride resulted in a further 50% decrease in activity compared to the samples containing fluoride alone. Since reductase activity is enhanced by phosphatase, any agent inhibiting the phosphatase activity would also be expected to inhibit the observed reductase activity. The observation that phenylmethylsulfonyl fluoride caused a decrease in reductase activity even in the presence of 50 mM sodium fluoride, in which phosphatase activity is supposed (Ingebritsen et al. [1978]) to be abolished, implies that the agent is exerting its effect in another manner than solely by inhibition of the phosphatase, even though serine-reagents (such as di-iso-propylfluorophosphate) are reported to inhibit alkaline and acid phosphatases (Hollander [1971]; Greenberg and Nachmansohn [1965]).

A puzzling observation is that in all experiments involving fluoride and phenylmethylsulfonyl fluoride, the
inhibition of reductase activity caused by phenylmethylsulfonyl fluoride in the presence of sodium fluoride is less than that caused by phenylmethylsulfonyl fluoride alone. It appears that fluoride somehow protects HMG-CoA reductase activity against inhibition by phenylmethylsulfonyl fluoride. This observation has not been further investigated.

It is of interest to note that the enzymes which were strongly inhibited by phenylmethylsulfonyl fluoride were exactly those enzymes which were also most susceptible to inhibition by sulfhydryl reagents. The results of the present studies indicate that phenylmethylsulfonyl fluoride, known to be an effective inhibitor of serine-type proteases (Fahrney and Gold [1963]; Gold and Fahrney [1964]; Whitaker and Perez-Villasenor [1968]; Gold [1967]; Shaw [1972]), is also an effective inhibitor of several sulfhydryl enzymes. The amount of inhibition was 48% - 82% and was consistently statistically significant at phenylmethylsulfonyl fluoride concentrations of 1-3 mM. The amount of inhibition was generally comparable to, albeit slightly less than, that observed with sodium iodoacetate.

The suggested mechanism of action of phenylmethylsulfonyl fluoride with active serine residues is thought to involve nucleophilic attack by the serine hydroxyl on the sulfur of phenylmethylsulfonyl fluoride with extrusion of fluoride ion (Gold and Fahrney [1964]). The resulting compound is a sulfonate ester which is quite stable to a variety of conditions (Barnard and Robertson [1961]). Since
the sulfur atom of cysteine is more nucleophilic than the hydroxyl oxygen of serine, the initial attack on the sulfonyl group of phenylmethuelsulfonyl fluoride may be even more rapid. The resulting compound is a thiol-sulfone. These compounds, containing an R-S-SO₂-R' linkage are extremely susceptible to thiol interchange and to hydrolysis, even in neutral solution (Savige and Maclaren [1966]). The rate of thiol interchange will naturally be a function of the concentration of neighboring thiol groups, which will probably be low for most proteins. The rate of hydrolysis will be a function of (among other things) the accessibility of the modified cysteine to the aqueous buffer solution. At the moderate pH's employed in these assays, hydrolysis of thiolesulfonates is reported to be rather slow (Savige and Maclaren [1966]). Thus, modification of cysteine by phenylmethylsulfonyl fluoride appears to be quite a reasonable reaction in view of the high nucleophilicity of the cysteine sulfur; whether or not the resultant moiety is stable may depend on how exposed it is to the aqueous solution. Even if the phenylmethylsulfonyl group is removed by hydrolysis, the original cysteine group may be modified to cysteine sulfinic acid and thereby rendered catalytically inactive (Savige and Maclaren [1966]; Lavine [1936]; Savige et al. [1964]). Much further work is obviously required before the nature of the phenylmethylsulfonyl fluoride inhibition can be demonstrated with certainty.

The effect of cycloheximide on these sulfhydryl en-
zymes is much less clear-cut. In the first instance, the inhibition is uniformly less than that produced by phenyl-
methylsulfonyl fluoride. In the second instance, the lack of significant effect on HMG-CoA reductase, which is strongly inhibited by phenylmethylsulfonyl fluoride, renders it more difficult to describe a general method of action.

There are few reports of simple enzyme inhibition in vitro caused by cycloheximide (Munro et al. [1968]; Zech and Domagk [1975]; Horgen and Griffin [1971]; Latuasan and Berends [1958]). Some of these initial reports have been disputed by other workers who were unable to reproduce the effects reported, especially the inhibition of alcohol de-
hydrogenase (Westcott [1964]) and the GTPase reaction involved in translocation (McKeelian and Hardey [1969]). In one experiment performed in the course of the studies described in this thesis, no effect of preincubation of cyclo-
heximide on alcohol dehydrogenase was observed (data not pre-

sented). Even the large number of studies performed on the mechanism of action of cycloheximide in the inhibition of protein synthesis have not succeeded in unequivocally estab-
lishing the site of inhibition. Cycloheximide has been de-
scribed variously as an inhibitor of chain initiation
(Baliga et al. [1969]); chain elongation (McKeelian and Har-
desty [1969]); and chain termination (Rajakshmi et al.
[1971]).

The lack of information of specific in vitro effects on enzyme activities may be due to an almost universal use
of cycloheximide to inhibit protein synthesis, especially in cell culture and in whole animals, and a general lack of concern with other possible effects. However, in view of the evidence presented in this thesis (and the reports of Horgen and Griffin [1971] and of Zech and Dormagk [1971]), it appears that the question of the effects of cycloheximide that do not involve protein synthesis should be considered when the substance is added to cell cultures and enzyme activities are subsequently assayed.

As mentioned earlier, the hypothesis that phenylmethylsulfonyl fluoride and/or cycloheximide might act to inhibit these early enzymes by reacting with one or more essential thiols was not rigorously investigated. It was proposed that this hypothesis might be tested in the following manner. Reaction of the enzyme preparations with 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) would be expected to block the free sulfhydryl groups present by forming cysteine thio-nitrobenzoate disulfide. In that case, enzymatic activity might be severely inhibited. The DTNB-treated protein would then be incubated with phenylmethylsulfonyl fluoride and the protecting groups removed afterward by reaction with dithiothreitol. Loss of activity comparable to that observed with phenylmethylsulfonyl fluoride treatment of unmodified enzyme would indicate that DTNB afforded no protection against phenylmethylsulfonyl fluoride inactivation, and that presumably the two agents acted on different amino acids: that is, that phenylmethylsulfonyl fluoride
was acting by reaction with serine (as in its reaction with serine proteases) and not with cysteine. Unfortunately, control levels of activity recovered do not exclude the possibility that phenylmethylsulfonyl fluoride is reacting with a serine group, since the protection afforded by DTNB could be due to steric hindrance of a serine close to the DTNB-protected cysteine, rather than by covalent protection of the phenylmethylsulfonyl fluoride sensitive group.

Preliminary work involving DTNB modification of acetate thiokinase preparations indicated that there might be practical difficulties with this approach as well. Although DTNB did lower enzymatic activity (data not presented), treatment of a control preparation with 5 mM dithiothreitol (used to remove the TNB blocking groups) resulted in nearly total loss of enzymatic activity. Passage of the dithiothreitol-containing protein solution through a Sephadex G-25 column to remove the dithiothreitol yielded a preparation with only low amounts of activity. Thus, in view of the uncertainty which even a positive result might include, the investigation was not pursued further. Rigorous establishment of the mode of action of phenylmethylsulfonyl fluoride and cycloheximide on acetate thiokinase and the other enzymes will require preparation of the enzymes from rat liver in a highly purified form so that amino acid analysis may be done on native and modified protein in order to determine the site(s) of modification. It is also possible that the inhibition does not involve irreversible covalent modifica-
tion. This possibility would have to be investigated as well.

The data presented in this section establish the fact of cycloheximide and phenylmethylsulfonyl fluoride produced inhibitions on several of the early enzymes in the sterol biosynthetic path. They indicate that the effect of phenylmethylsulfonyl fluoride, at least, may be due to sulfhydryl modification, but the evidence is not conclusive.
SUMMARY
The focus of the studies described in this thesis has been on the actions of certain poly-oxygenated sterols on serum cholesterol levels in experimental animals, on hepatic sterol synthesis, and on the synthesis of sterols in a cell-free preparation to which the compounds were added immediately before incubation. In the course of these studies, the enzymes catalyzing the conversion of acetate to mevalonate were assayed individually. The effects of cycloheximide and phenylmethylsulfonyl fluoride on overall sterol synthesis and on several individual enzymes were investigated and an unexpected effect of the agents in the cell-free system was documented. Finally, the metabolism of one of the poly-oxygenated sterols was investigated in cell-free preparations.

When cholest-8(14)-en-3β-ol-15-one (5 mg) was administered daily by subcutaneous injection in olive oil (0.5 ml) for up to 25 days to experimental Sprague-Dawley male rats, a significant (p < 0.01) reduction in the serum cholesterol levels of the experimental animals was observed (approximately 20%-25%). Hepatic sterol synthesis from acetate, assayed in 10,000 x g supernatant fractions from liver homogenate preparations, was increased 100%-300% in experimental animals at times as early as 18 hours after the initial injection and continued at an elevated level for as long as 15 days. In one experiment, at 24 hours after the initial injection, the hepatic sterol synthesis from acetate was decreased in the experimental animals compared to that
in control animals. This observation was of singular occurrence. Hepatic sterol synthesis from mevalonate was not affected at times from 8 hours to 72 hours after the initial injection.

A derivative of this steroid, 14α-methylcholest-7-en-3β-ol-15-one, was without effect on serum cholesterol levels of experimental animals when administered daily (5 mg) in olive oil (0.5 ml) by subcutaneous injection for 24 days. There was, however, a significant (p < 0.001) increase (300%) in the synthesis of sterols from acetate in 10,000 x g supernatant fractions of liver preparations from the experimental animals.

In the initial experiments performed on the effect of addition of certain poly-oxygenated sterols to the 10,000 x g supernatant fraction of liver homogenate preparations, several vehicles were investigated as possible agents in which the compounds could be administered. An ethanol-containing vehicle was found to result in decreased incorporation of acetate into sterols, presumably due to the facile oxidation of ethanol to acetate and the dilution of radioactive acetate substrate by this unlabeled material. Lecithin liposomes, prepared by the method of Batzri and Korn (1973), also proved to be inhibitors of sterol synthesis from acetate and thus were unacceptable. Propylene glycol proved to be the most acceptable vehicle tested. During these preliminary experiments, it was discovered that cholest-8(14)-en-3β-ol-15-one significantly inhibited (p <
0.01) the incorporation of acetate into non-saponifiable lipids by approximately 20% at $10^{-4}$ M. The substance was ineffective at $10^{-5}$ M and $10^{-6}$ M concentrations. The synthesis of non-saponifiable lipids from mevalonate was unaffected even at $10^{-4}$ M.

14α-Ethylcholest-7-en-3β,15α-diol was demonstrated to inhibit significantly the incorporation of acetate into digitonin-precipitable sterols at concentration of $10^{-4}$ M, $10^{-5}$ M, and $10^{-6}$ M by approximately 75%, 70%, and 65% respectively.

A comprehensive set of experiments was performed in order to investigate the nature of the inhibition caused by 14α-ethylcholest-7-en-3β,15α-diol on the synthesis of sterols from acetate. The inhibition was demonstrated to occur to the same extent when mevalonate was used as the substrate. At a concentration at which 14α-ethylcholest-7-en-3β,15α-diol caused a 60% inhibition in sterol synthesis from acetate and mevalonate ($10^{-6}$ M), purified cholesterol and cholest-5-en-3β,25-diol were demonstrated to be without effect on this cell-free system. The production of digitonin-precipitable sterols from mevalonate was demonstrated to be equally inhibited (58% ± 5%) at times as early as 30 minutes after the start of the incubations to times as late as 120 minutes after the start of the incubation, although the rate of overall sterol synthesis varied approximately two-fold during this time.

In the concentration range of $10^{-75}$ M, the compound
caused a decrease in the incorporation of mevalonate into
digitonin-precipitable sterols which was approximately
linear with respect to concentration.

The nature of the inhibition caused in the synthesis
of digitonin-precipitable sterols from mevalonate by 14α-
ethylcholest-7-en-3β,15α-diol was investigated by many tech-
niques, involving several different kinds of chromatography,
co-crystallization of unknowns with standard compounds and
hydrogenation of unknown compounds. The inhibition in the
synthesis of digitonin-precipitable sterols, but not non-
saponifiable lipids, from mevalonate was shown to be due to
the production of lanosterol and 24,25-dihydrolanosterol
(instead of the cholesterol which was produced in control
incubations). Under the conditions employed in these stud-
ies, lanosterol and 24,25-dihydrolanosterol were partly,
but not completely, precipitated by digitonin.

Other 14α-ethyl-3,15-dioxygenated sterols were as-
sayed for ability to inhibit the production of digitonin-
precipitable sterols from mevalonate. In these studies, it
was discovered that the oxidation state and stereochemistry
of the 15-oxygen substituent were more important than that
of the 3-oxygen substituent in determining the activity of
these sterols in the cell-free system employed.

Several of the 14α-ethyl-3,15-dioxygenated sterols
which were assayed proved to be more effective in inhibiting
the synthesis of cholesterol from mevalonate in cell-free
preparations from male rats' livers than in those from fe-
male rats' livers. The reason for this difference was not determined.

14α-Ethylcholesterol-7-en-3β,15α-diol was prepared in radio-labeled form and incubated with 10,000 x g supernatant fractions from liver preparations from male and female rats. In neither case was there any detectable conversion of the substance to less polar sterols such as cholesterol.

The effects of 14α-ethylcholesterol-7-en-3β,15α-diol on the enzymes catalyzing the conversion of acetate to HMG-CoA were also investigated. The dihydroxysterol was found to be without effect on any of these activities when preincubated with 10,000 x g supernatant fractions of liver preparations from male rats. The effect of the compound on highly purified HMG-CoA reductase activity was also assayed by Dr. Shapiro; no inhibition of the enzymatic activity was detected.

Phenylmethylsulfonyl fluoride (a serine-protease inhibitor) and cycloheximide (an inhibitor of protein synthesis) proved to be without effect on the percentage inhibition caused by $10^{-6}$ M 14α-ethylcholesterol-7-en-3β,15α-diol on the synthesis of digitonin-precipitable sterols from mevalonate.

However, both these agents proved to be extremely potent inhibitors of sterol synthesis from acetate (but not mevalonate) at concentrations of 1 mM. Phenylmethylsulfonyl fluoride was also shown to be effective, although to a lesser extent, at 0.10 mM. In order to investigate these un-
expected effects more carefully, the effects of these agents on each enzyme in the conversion of acetate to mevalonate were analyzed.

Preincubation of liver preparations with 1 mM phenylmethylsulfonyl fluoride proved to inhibit the activities of acetate thiokinase, HMG-CoA synthase and HMG-CoA reductase. Acetoacetyl-CoA thiolase, the remaining enzyme in the conversion of acetate to mevalonate, was unaffected by this treatment. Preincubation of liver preparations with 1 mM cycloheximide also produced inhibitions in the activities of several enzymes in the acetate to mevalonate path, but the inhibitions were not as large as those produced by phenylmethylsulfonyl fluoride. Acetate thiokinase and HMG-CoA synthase were inhibited by this pretreatment. Acetoacetyl-CoA thiolase and HMG-CoA reductase were not.

The detailed nature of these inhibitions was not explored, but it was observed that those enzymes which were inhibited by preincubation with phenylmethylsulfonyl fluoride were also strongly inhibited by preincubation with sodium iodoacetate and/or N-ethylmaleimide.
REFERENCES


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