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Metabolism of 5α-cholest-8(14)-en-3β-ol-15-one in 15-ketosterol-adapted cells

Kirkpatrick, Nanda Duhé, M.A.

Rice University, 1990
RICE UNIVERSITY

METABOLISM OF 5α-CHOLEST-8(14)-EN-3β-OL-15-ONE IN 15-KETOSTEROLO-ADAPTED CELLS

by

NANDA DUHE KIRKPATRICK

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

MASTER OF ARTS

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HOUSTON, TEXAS

APRIL, 1990
ABSTRACT

Metabolism of 5α-Cholest-8(14)-en-3β-ol-15-one in 15-Ketosterol-Adapted Cells

by

Nanda Duhe Kirkpatrick

5α-Cholest-8(14)-en-3β-ol-15-one is a potent inhibitor of cholesterol biosynthesis in cultured cells. Cells derived from the CHO K-1 cell line have been isolated based on their ability to grow in high concentrations of 5α-cholest-8(14)-en-3β-ol-15-one (up to 40 µM). These cells, designated as the 15-ketosterol-adapted or K(15) cells, accumulate cytoplasmic lipid-rich regions that appear as inclusions when observed by light microscopy. Results of studies presented herein show that K(15) cells incubated at 40 µM 5α-cholest-8(14)-en-3β-ol-15-one incorporated almost seven times as much of the 15-ketosterol as K(15) cells incubated at 15 µM 15-ketosterol. Upon incubation of K(15) cells in lipid-rich medium containing 40 µM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one, ~96% of the radioactivity recovered in an isolated floating lipid layer corresponded to 15-ketosteryl esters. Upon the addition of sodium [1-14C]oleate (82 µM) to the incubation medium, the radioactivity recovered in the floating lipid layer corresponded to both 15-ketosteryl esters and triacylglycerols. Following removal of the 15-ketosterol from the growth medium, the K(15) cells excreted 66-88% of the incorporated 15-ketosterol into the medium.
Acknowledgements

I would like to express my gratitude to Dr. George J. Schroepfer, Jr. for his support and guidance throughout this research. I am particularly thankful for his patience and support of my decision to leave research and enter the teaching profession.

I would like to thank Dr. Rick Pinkerton and Dr. Arthur Chu for their many hours of teaching and sharing of their expertise. I would like to express a special thanks to Rick for his support that extends beyond the years of the work presented here.

I would also like to express my gratitude to Dr. Bill Wilson not only for the enumerable times that he has patiently answered my computer questions, but also for his willingness to go beyond his own research and maintain the computer software and hardware of this laboratory.

I would also like to thank Dr. Alemka Kiscic for her kindness and assistance in endless ways during the last few years.

I would also like to thank my graduate student colleagues, Daniel Petersen, Ita Yuen, and Tina Meng for their special friendship during these last three years.

Finally, I would like to thank my husband, Rod, for his enormous love and patience throughout this project.

The unlabeled 5α-cholest-8(14)-en-3β-ol-15-one and [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one were prepared under NIH grant HL-22532, and the cell culture studies were supported by NIH grant HL-15276. Support from American Cyanamid Company is also gratefully acknowledged.
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<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>cm</td>
<td>centimeter</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>dl</td>
<td>deciliter</td>
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<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>F12FCS5</td>
<td>Ham's F12 medium containing 5% fetal calf serum</td>
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<tr>
<td>F12NCS5</td>
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<td>HDL</td>
<td>high density lipoproteins</td>
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<td>HPLC</td>
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<td>K(15)</td>
<td>15-ketosterol-adapted cell line, derived from CHO K-1 cells</td>
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<td>low density lipoproteins</td>
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<td>µmol</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>pmol</td>
<td>pmole(s)</td>
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<tr>
<td>psi</td>
<td>pounds per square inch</td>
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<td>radio-TLC</td>
<td>thin-layer radiochromatography</td>
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<td>s. d.</td>
<td>standard deviation</td>
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<td>TLC</td>
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Chapter I

Introduction
Cholesterol is an important component of the plasma membrane of eukaryotic cells (Chapman et al., 1985) and is the major sterol present in mammalian cells (Demei and de Kruyff, 1976). Cholesterol is the precursor of bile acids and steroid hormones (Lehninger, 1975; Hadley, 1984). Bile acids are formed in the liver and are secreted into the small intestine where they play an important role in the absorption of dietary lipids and fat-soluble vitamins. Steroid hormones are produced by steroidogenic tissues in both the adrenal glands and the gonads (Hadley, 1984). Gonadal steroid hormones are required for normal development and reproduction, while adrenal steroid hormones are necessary for carbohydrate metabolism and electrolyte balance. Provided with the appropriate precursors, the placenta also produces steroid hormones necessary for the maintenance of pregnancy (Hadley, 1984).

Studies of the tissue distribution of cholesterol in rats have indicated that cholesterol is found in the greatest concentration in the spinal cord, sciatic nerve, brain, adrenal glands, and ovaries (Andersen and Dietschy, 1978; D'Hollander and Chevallier, 1969). Although the concentration of cholesterol in the skin and muscles is not outstanding, the large total mass of these tissues results in these tissues representing large reservoirs of cholesterol in the body, together comprising about 30% of the total body cholesterol (Jones and Glomset, 1985).

The blood and tissues of animals contain both unesterified cholesterol and cholesteryl esters (Jones and Glomset, 1985). Most of the cholesteryl esters are formed from long-chain fatty acids such as palmitic acid, oleic acid, linoleic acid, or arachidonic acid (Jones and Glomset, 1985). In the rat, cholesteryl esters comprise about one-eighth of the total body cholesterol (D'Hollander and Chevallier, 1969). The adrenal glands, blood plasma, and ovaries have the highest ratio of cholesteryl ester to unesterified cholesterol (Jones and Glomset, 1985).
On a cellular level, cholesterol is found primarily in the plasma membranes (Lange and Ramos, 1983). Cholesteryl esters, however, are found mainly in lipid droplets in the cytoplasm (Brown and Goldstein, 1981). Cholesteryl esters are only slightly soluble within phospholipid membranes (Small and Shipley, 1974; Hamilton and Small, 1982). Small and Shipley provide evidence that at 37 °C, lecithin is saturated with only 2-3% cholesteryl esters and that cholesteryl esters above this concentration separate into oily phase lipid droplets. Similarly, in blood plasma lipoproteins, unesterified cholesterol appears to be associated with the phospholipids of the lipoprotein particle surface, whereas the non-polar cholesteryl esters are normally associated with the particle core (Kroon, 1981; Goldstein and Brown, 1977). Cholesteryl ester accumulated in lipid droplets in cells or in lipoproteins often contain a small amount of triacylglycerol and unesterified cholesterol (Calvert and Abbey, 1985; Brown and Goldstein, 1977; Rothblat, 1974; Rothblat et al., 1966).

Intracellularly, the enzymes important in the metabolism of cholesteryl esters are the cholesteryl ester-synthesizing enzyme, acyl CoA: cholesterol acyltransferase (ACAT) and the cholesteryl ester-degrading enzymes, for example, acid cholesteryl ester hydrolase (CEH) and neutral CEH (Jones and Glomset, 1985). Blood plasma and the extracellular fluid contain also a cholesteryl ester-synthesizing enzyme, lecithin: cholesterol acyltransferase (LCAT) and a cholesteryl ester transfer protein (CETP). The intracellular enzyme, ACAT, is associated with the endoplasmic reticulum (Mitropoulos et al., 1978; Spector, 1979; Lichtenstein and Brechner, 1980). Cholesteryl esters formed as a result of the reaction catalyzed by ACAT form lipid droplets within the cytoplasm (Brown and Goldstein, 1983).
Jones and Glomset (1985) described the following features of the cholesteryl ester cycle in a fibroblast-like cell. LDL containing cholesteryl esters first bind to apo B/E receptors located on the cell surface and the lipoprotein-receptor complex is subsequently internalized by endocytosis (Jones and Glomset, 1985; Brown and Goldstein, 1976, 1980, 1983). The lipoprotein cholesteryl ester is then hydrolyzed by a lysosomal CEH and then the products, free cholesterol and fatty acids, diffuse across the lysosomal membrane to the cytoplasm. The free cholesterol and fatty acids may be re-esterified by ACAT and accumulate in cytoplasmic lipid droplets. Cholesteryl esters may be subsequently hydrolyzed by the neutral CEH. Unesterified cholesterol may be used for membrane formation or excreted from the cell. For excretion to occur, an appropriate acceptor (often HDL) must bind to the cell surface receptors to adsorb unesterified cholesterol from the cell surface. Unesterified cholesterol in the HDL particle may then be esterified by LCAT and transferred by CETP to VLDL. As VLDL acquire more cholesteryl ester, they are converted to LDL, completing the cycle.

Cells also synthesize cholesterol by a complex biosynthetic pathway. Although cholesterol synthesis is particularly associated with the liver, most animal tissues are capable of synthesizing cholesterol (Dietschy and Siperstein, 1967; Clinkenbeard et al., 1975a; 1975b). The major regulatory control point in cholesterol biosynthesis exists near the beginning of the pathway; HMG-CoA reductase is generally considered to be the major regulatory enzyme (Schroepfer, 1981). There is evidence, however, that other enzymes also exert control over mevalonate formation and sterol synthesis. In rat liver homogenates, it has been found that HMG-CoA reductase and to a lesser extent, HMG-CoA synthase are inhibited by cholesterol feeding (White and Rudney, 1970). In avian liver preparations, similar reductions in HMG-CoA synthase were observed following cholesterol feeding (Sugiyama et al., 1972; Clinkenbeard et al., 1975b). Further, studies
of both chicken and rat liver implicated acetoacetyl-CoA thiolase in the regulation of cholesterogenesis following a significant reduction in the activity of this enzyme after cholesterol feeding (Clinkenbeard et al., 1973; 1975b).

Studies of cultured cells have demonstrated that upon transferring L cells from growth medium containing serum to medium containing lipid-deficient serum, incorporation of acetate into cholesterol was stimulated (Howard et al., 1975). The presence of lipid-rich medium markedly reduced the incorporation of acetate into cholesterol in both L cells (Howard et al., 1975) and HeLa cells (Melnykovych et al., 1976). Further, the transfer of CHO cells to lipid-deficient medium resulted in an increase in the activity of HMG-CoA reductase accompanied by concomitant increases in the activities of cytosolic acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase (Chang and Limanek, 1980; Miller et al., 1980; Pajewski et al., 1988). In addition, Chang and Limanek observed the same result with the enzyme mevalonate kinase (1980). Significant increases in the activity of HMG-CoA reductase in HeLa cells have also been observed upon removal of serum from the growth medium (Cavenee and Melnykovych, 1977).

Although it was initially believed that cholesterol itself acted as its own regulator, Kandutsch and Chen reported in 1973 that highly purified cholesterol does not inhibit sterol biosynthesis from labeled acetate nor does it reduce HMG-CoA reductase activity in primary cultures of fetal mouse liver cells. They further demonstrated in L cells, primary liver cells, and established mouse fetal liver cell cultures that unpurified cholesterol caused significant reduction in the activity of HMG-CoA reductase and significantly decreased the metabolism of acetate to sterols (Kandutsch and Chen, 1973). The unpurified cholesterol did not, however affect metabolism of acetate to fatty acids or to carbon dioxide. Neither the rate of RNA synthesis nor rate of general protein synthesis was
affected. It was further shown that these same effects were observed when the cell cultures were treated with 7α-hydroxycholesterol, 7β-hydroxycholesterol, or 7-ketocholesterol, known autoxidation products of cholesterol. Inhibition of HMG-CoA reductase activity by 7-ketocholesterol treatment in human fibroblasts was also reported by Brown and Goldstein (1974). It was suggested at that time that perhaps autoxidation products of cholesterol were the true regulators of cholesterol biosynthesis.

It was further shown that cholesterol derivatives oxygenated in the side chain, particularly 25-hydroxycholesterol, had the same effects in L cells as the sterols oxygenated at the C-7 position (Kandutsch and Chen, 1974). In 1977 it was reported that a number of chemically synthesized 15-oxygenated sterols were found to be very potent inhibitors of both sterol synthesis and HMG-CoA reductase in L cell fibroblast cultures and primary cultures of fetal mouse liver cells (Schroepfer et al., 1977a). In that study, it was found that the concentration of the oxygenated sterols that were required for a 50% reduction in sterol synthesis correlated with the concentration of the oxygenated sterol required for a 50% reduction in the activity of HMG-CoA reductase.

Although oxygenated sterols exhibit inhibitory activity in vivo with respect to sterol synthesis, the mechanism of inhibition has not been fully elucidated. For many oxygenated sterols, the inhibitory action is believed to be associated with the formation and reduction of HMG-CoA by HMG-CoA reductase. It is known, however, that it is not via direct interaction of the inhibitor compound and the HMG-CoA reductase enzyme as no effect on sterol synthesis or HMG-CoA reductase activity was observed when oxygenated sterols were added to cell-free preparations (Kandutsch and Chen, 1974). There is evidence from investigations using mammalian cell culture that regulation of the HMG-CoA reductase may be at the level of both transcriptional (Osborne et al., 1985; Luskey et al., 1983) and translational (Reynolds et al., 1985) control. Mammalian cell culture
studies also support the hypothesis that HMG-CoA reductase activity is decreased by increased degradation of the existing enzyme in the presence of sterols (Gil et al., 1985; Faust et al., 1982; Chang et al., 1981; Chen et al., 1975b; Breslow et al., 1975; Brown et al., 1974; Kandutsch et al., 1974, 1975).

15-Oxygenated sterols are part of a limited group of sterols that are capable of suppressing sterol synthesis in cultured cells and also exhibit hypocholesterolemic effects in intact animals (Schroepfer, 1981). One of the most potent of the 15-oxygenated sterols with respect to inhibition of sterol synthesis and HMG-CoA reductase activity is 5α-cholest-8(14)-en-3β-ol-15-one (See Figure 1). In L cells, it was shown to inhibit sterol synthesis by 50% at a concentration of 0.1 μM and to inhibit HMG-CoA reductase activity by 50% at a concentration of 0.3 μM (Schroepfer et al., 1977a). In cultured CHO K-1 cells, incubated in medium supplemented with lipid-deficient serum, the increased activities of the early synthetic enzymes in the cholesterol biosynthetic pathway, cytosolic acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase, were all suppressed by 5α-cholest-8(14)-en-3β-ol-15-one (Miller et al., 1980; Pajewski et al., 1988). Further, addition of 5α-cholest-8(14)-en-3β-ol-15-one to the growth medium 24 hours prior to the removal of serum lipids prevented the expected increase in these enzymes.

5α-Cholest-8(14)-en-3β-ol-15-one has a marked hypocholesterolemic action in rats, mice (Schroepfer et al., 1977b), baboons (Schroepfer et al., 1982), and Rhesus monkeys (Schroepfer et al., 1984). In addition, this lowering of total serum cholesterol in primates is associated with a decrease in levels of low density lipoprotein plus very low density lipoprotein cholesterol and an increase in high density lipoprotein cholesterol (Schroepfer et al., 1982; Schroepfer, et al., 1984). 5α-Cholest-8(14)-en-3β-ol-15-one has also been
Figure 1. 5α-Cholesten-8(14)en-3β-ol-15-one
shown to significantly decrease intestinal cholesterol absorption in the rat following dietary administration of the compound (Schroepfer et al., 1987a).

This compound has been found to be convertible to cholesterol upon incubation with rat liver homogenate preparations (Monger and Schroepfer, 1988; Monger et al., 1980), and in vivo conversion of the 15-ketosterol to cholesterol has been observed in the rat upon oral (Brabson and Schroepfer, 1988; Schroepfer et al., 1988c) or intravenous (Schroepfer et al., 1988b) administration. The 15-ketosterol has also been shown to be rapidly converted to polar biliary metabolites after intravenous administration of the compound to rats (Schroepfer et al., 1988b). Further, in vivo conversion of the 15-ketosterol to cholesterol has been observed in the baboon upon oral (Schroepfer et al., 1987b; Pajewski et al., 1989) or intravenous (Schroepfer et al., 1988a) administration.

The CHO K-1 cell line has been used to characterize the metabolic effects of the 15-ketosterol on cholesterol biosynthesis. Upon transferring CHO K-1 cells to culture medium containing lipid-deficient fetal calf serum, significant increases in the activities of cytosolic acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase were observed (Miller et al., 1980; Pajewski et al., 1988). At a concentration of $1 \times 10^{-6}$ M, the 15-ketosterol blocked this increase in activities. The oxysterol also reduced the induced elevated levels of these enzymes when the cells were incubated in lipid-deficient medium followed by incubation in medium supplemented with the 15-ketosterol.

Treatment of CHO K-1 cells with the 15-ketosterol significantly reduces de novo synthesis of cholesterol by inhibiting the synthetic enzymes in the pre-mevalonate segment of the pathway. As a result, for normal growth to occur in these cells, an exogenous source of cholesterol must be provided. Even if exogenous cholesterol is present, usually provided in the serum supplement to the growth medium, if the cells are
incubated with concentrations exceeding 10 µM 5α-cholest-8(14)-en-3β-ol-15-one, cell growth as measured by total cellular protein is significantly inhibited. It has been shown, however, that the addition of fatty acids, particularly oleic acid, dramatically reduces the extent to which cell growth is inhibited by the 15-ketosterol (Pinkerton et al., 1988).

Incubation of CHO K-1 cells in a range of concentrations of the 15-ketosterol resulted in a 50% decrease in growth, as measured by total cellular protein (from the control, 0 µM 15-ketosterol in lipid-rich medium), when the concentration reached about 12.5 µM 5α-cholest-8(14)-en-3β-ol-15-one in lipid-rich medium. Upon incubation in the same concentrations of the 15-ketosterol with the addition of 25 µg/ml sodium oleate, a 50% decrease in growth was not observed until the concentration reached approximately 25 µM 5α-cholest-8(14)-en-3β-ol-15-one. Upon the addition of oleate to the growth medium, about twice the amount of the 15-ketosterol was required to affect the cells in the same manner (i.e. 50% decrease in cell growth) as when the cells were incubated in the presence of the 15-ketosterol alone.

In 1985, Dr. F. D. Pinkerton was interested in determining if the CHO K-1 wild type cells could be adapted to continual growth in unusually high concentrations of the 5α-cholest-8(14)-en-3β-ol-15-one that would not normally allow growth of the wild type cells. To accomplish this, wild type cells were subcultured in growth medium supplemented with 10 µM 5α-cholest-8(14)-en-3β-ol-15-one. Surviving colonies were selected and the 15-keto-sterol concentration was increased by 0.5 µM. By repeatedly selecting surviving colonies and incrementally increasing the concentration of the 15-ketosterol by 0.5 µM, an adapted cell line was produced that could be continually maintained in 15 µM 5α-cholest-8(14)-en-3β-ol-15-one. The resultant 15-ketosterol-adapted cell line was designated K(15). Although the K(15) cells were isolated based on their ability to be maintained at a level of 15 µM 5α-cholest-8(14)-en-3β-ol-15-one, it
was found that cell growth continued at a normal rate upon incubation at a level of up to 40 μM 5α-cholest-8(14)-en-3β-ol-15-one. A maximum concentration of 40 μM 15-ketosterol was used in the experiments described in this thesis, as above this concentration, insolubility of the 15-ketosterol becomes a consideration.

The routine growth conditions of the K(15) cells was Ham's F12 growth medium + 5% NCS + 15 μM 5α-cholest-8(14)-en-3β-ol-15-one. One of the most outstanding features of these cells was the appearance of distinct cytoplasmic structures that were vacuole-like in appearance. The number and size of these vacuole-like structures seemed to be related to the concentration of the 15-ketosterol present in the growth medium. Qualitative microscopic observation indicated that the higher the concentration of the 15-ketosterol, the greater the number and larger the size of these structures. It was also an intriguing fact that these cells appeared to have normal growth rates even at a concentration of 40 μM 5α-cholest-8(14)-en-3β-ol-15-one compared to an almost complete cessation of growth in the CHO K-1 wild type cells at this concentration. Because of these unusual features, characterization of this cell line was undertaken.

The effect of 5α-cholest-8(14)-en-3β-ol-15-one on the enzymes in the cholesterol biosynthetic pathway in cultured cells and its effect on the lipoprotein profile in Rhesus monkeys provide more than ample reason for detailed studies of this oxygenated sterol. Cell culture studies are particularly convenient because they allow for a relatively isolated system and a high level of control of experimental conditions. The formation of cytoplasmic vacuole-like structures in a cell line adapted to growth in unusually high concentrations of the 15-keto-sterol makes this newly developed cell line a unique system for the study of the metabolism of the 15-ketosterol. The studies presented in this thesis were designed to provide data concerning the basic characterization of this cell line. Metabolic studies were also designed to investigate 1) the incorporation of the 15-ketosterol into the
K(15) cells, 2) the distribution of sterols in the K(15) cells, and 3) the subsequent excretion of the 15-ketosterol from the K(15) cells.
Chapter II

Materials and Methods
CELL CULTURE

CHO K-1 Cells

Chinese hamster ovary cells (CHO K-1) were originally purchased from the American Type Culture Collection (Rockville, Maryland). These cells were subcloned and maintained in growth conditions which included incubation in Ham's F12 medium (Ham, 1965) supplemented with 5% NCS at 37 °C in a 5% CO2-95% air environment (Forma Scientific model 3014 dual chamber incubator).

15-Ketosterol-Adapted Cells, K(15)

Dr. F. D. Pinkerton isolated a cell line derived from CHO-K1 cells that was adapted to grow in Ham's F12 growth medium + 5% NCS containing 15 μM 5α-cholesterol-8(14)-en-3β-ol-15-one. The results of previous experiments demonstrated that the 15-ketosterol inhibited growth of CHO K-1 cells cultured in the presence of fetal calf serum (Pinkerton et al., 1988). The concentration of the 15-ketosterol required to inhibit cell growth by 50% was 13 μM. The addition of sodium oleate (82 μM) to the culture medium reduced the inhibitory effect of the sterol, and increased the concentration of the 15-ketosterol required to inhibit cell growth by 50% to 25 μM. In an effort to elucidate the mechanism of growth inhibition by the oxysterol, and the mechanism by which the sodium oleate reduced the sterol's inhibitory effect, CHO K-1 cells were isolated that were capable of prolonged growth in the presence of 15 μM 5α-cholesterol-8(14)-en-3β-ol-15-one.

The routine maintenance growth condition for these adapted cells was Ham's F12 medium supplemented with 5% NCS + 15 μM 5α-cholesterol-8(14)-en-3β-ol-15-one. These cells were isolated because of their resistance to growth inhibition of the 15-ketosterol at a concentration of 15 μM. However, it was determined that these cells could be maintained
in concentrations up to 40 $\mu$M 5$\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one. These cells were named the 15-ketosterol-adapted cells and were designated as K(15) cells. Although these cells were originally isolated in FCS-supplemented medium, all characterization presented in the following studies were performed using Ham's F12 medium supplemented with newborn calf serum.

**Subcloning**

To subclone cultures from 100 x 15 mm plates, the medium was aspirated and the cells were rinsed with trypsin (0.05%; 5.0 ml) in Hank's balanced salt solution without magnesium or calcium, previously warmed to 37 °C. An aliquot of the trypsin solution (5 ml) was added and the cells incubated at room temperature for 3 - 5 min for the CHO K-1 or K(15) cells. For routine subculturing, the cells were suspended in the trypsin solution with a Pasteur pipette and aliquots were added to plates containing fresh medium. For experimental plating, an aliquot of the cell suspension was first counted using a hemacytometer and the appropriate volume was then transferred to either 100 or 150 x 15 mm plates containing fresh medium.

**Preparation of Sterol-supplemented Medium**

Sterol-supplemented growth medium was prepared by dissolving the sterol in absolute ethanol (10 mg/ml) and adding an appropriate volume to the medium which was allowed to equilibrate at room temperature for a minimum of 12 hours.

**15-Ketosterol Esterification in the Medium**

Since the esterification process of free 5$\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one was of particular interest in the studies presented, it was important to show that esterification of exogenous 5$\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one provided in the free form as a supplement
in the medium was not occurring. The possibility of this occurring was considered due to the potential presence of the plasma enzyme, lecithin:cholesterol acyl transferase (LCAT) in the newborn calf serum supplements. This enzyme transfers the number two acyl group from phosphatidylcholine to free cholesterol to form cholesteryl esters (reference). Previous work (unpublished, Kirkpatrick, 1986) indicated that esterification of 5α-cholest-8(14)-en-3β-ol-15-one occurred under certain conditions. The addition of DTNB (5,5'-dithiobis- (2-nitrobenzoic acid)), an LCAT inhibitor, reduced this esterification to less than 0.5%. Based on these data, it was presumed that the esterification was probably due to LCAT activity. However, since under routine experimental conditions, sterol esterification in the medium was less than 1%, further experimentation to determine the cause was not pursued.

The possibility of esterification of the sterol supplement was investigated during the studies presented here to verify that esterification of the free 15-ketosterol was not occurring. In every experiment, it was determined that upon 5α-cholest-8(14)-en-3β-ol-15-one or cholesterol supplementation, less than 1% of the supplement was recovered in the esterified form following pre-equilibration of the medium prior to its addition to the cell cultures.

Freezing Procedure

To freeze cell cultures, cells were harvested in the usual manner by trypsinization. Following trypsinization, the cells were collected by low speed centrifugation (1500 rpm). The cells were suspended in NCS containing 15% DMSO and subsequently transferred to 2.5 ml conical freezing vials. The vials were placed on dry ice for transport and storage in the -76 °C freezer.
MATERIALS

Sterols

5α-Cholest-8(14)-en-3β-ol-15-one was prepared according to the method described previously (Wilson et al., 1988; Parish et al., 1977; Schroepfer, 1977a). The purity was determined to be >99% by TLC in hexane: ethyl acetate (70:30).

[2,4-3H]5α-Cholest-8(14)-en-3β-ol-15-one (13.45 mCi per mmol) was prepared according to the method described previously (Monger et al., 1980 and Parish and Schroepfer, 1981). The purity was determined to be >99% by TLC in hexane: ethyl acetate (70:30).

Cholesterol was purchased from U. S. Biochemical Corporation and subsequently purified by the dibromide derivatization procedure according to Fieser (1953) and recrystallized from hexane:methanol. The melting point was determined to be 148.5-149.5°C. The final purity was determined to be >99% by TLC in hexane: ethyl acetate (80:20).

[4-14C]Cholesterol (55 mCi per mmol) was purchased from Amersham Corporation and was subsequently purified by Unisil column chromatography. The final purity was determined to be >99% by TLC in hexane:ethyl acetate (80:20).

Other Chemicals and Supplies

Powdered Ham's F12 growth medium (Ham, 1965), Dulbecco's modified phosphate buffered saline (PBS; KCl, 2.7 mM; KH2PO4, 1.2 mM; NaCl, 137 mM, and Na2HPO4, 8.1 mM), newborn calf serum and fetal calf serum were obtained from M. A. Bioproducts (Walkersville, Maryland) or from Irvine Scientific (Santa Ana, California). Lux plastic petri plates (60,100, or 150 x 15 mm) were from Nunc, Inc. (Naperville,
Illinois). Trypsin was obtained from Gibco Laboratories (Grand Island, NY). Soybean trypsin inhibitor, sodium oleate, EDTA, and 5,5'-dithiobis-(2-nitro-benzoic acid) were purchased from Sigma (St. Louis, Missouri).

The following reagents were obtained from the Sigma Chemical Company: HMG-CoA, Na+ salt, grade II; 90-95%, NADP, Na+ salt, 98%; glucose-6-phosphate, Na+ salt, 98%; glucose-6-phosphate dehydrogenase, type XV; and dithiothreitol (DTT), Sigma grade.

The following radioactive substances were purchased from Amersham Corporation (Arlington Heights, Illinois): [3-14C]3-hydroxy-3-methylglutaryl-CoA (26.2 mCi per mmol) and [2-3H]mevalonic acid (176 mCi per mmol). Sodium [2-3H]acetate (6 Ci per mmol) was purchased from ICN Chemical and Radioisotopes Division (Irvine, California). [1-14C]Oleic acid (56 mCi per mmol) was purchased from Amersham Corporation. The purity (>98%) was verified by radio-TLC in hexane: ethyl acetate: acetic acid (65:36:5).

Acetic anhydride, glacial acetic acid, 2,5-diphenyloxazole, silicic acid (100 mesh), and phosphorus pentoxide were purchased from Mallinckrodt, Inc. (Paris, Kentucky). Sudan Black B, Folin-Ciocalteu's phenol reagent, and cupric sulfate were purchased from Sigma (St. Louis, Missouri) and sodium potassium tartrate was obtained from Fisher Scientific Company (Fairlawn, New Jersey). Unisil (100-200) mesh was obtained from Clarkson Chemical Company, Inc. (Williamsport, Pennsylvania), and Hyflo Super-Cel was obtained from Johns-Manville Products Corporation (Lompoc, California).

Unless otherwise stated, all organic solvents and organic and inorganic reagents were of analytical reagent grade and were obtained from either Mallinckrodt, Inc. (Paris,
Kentucky) or American Burdick & Jackson, a subsidiary of American Hospital Supply Corporation (Muskegon, Michigan). When mixed solvent systems were employed, they were expressed as the ratio of volumes of the individual components. Water used in cell culture was deionized and glass distilled. For other purposes, water purified by a NANOPURE water purifying system, SYBRON/Barnstead (Boston, Massachusetts), was used.

PROCEDURES

Measurement of Radioactivity

Radioactivity was measured in a Packard 1500 liquid scintillation counter. Non-aqueous samples were assayed using a scintillation fluid composed of 2,5-diphenyloxazole (0.4%) in toluene. Radioactivity in scrapings from silica gel TLC plates was assayed using a scintillation fluid composed of 2,5-diphenyloxazole (0.4%) in a mixture of toluene: absolute ethanol (2:1). Aqueous samples were assayed using Scintisol high efficiency scintillation cocktail (Isolab, Inc., Akron, Ohio).

Thin-Layer Chromatography

Thin-layer chromatographic (TLC) analyses were performed on precoated plates of silica gel G (250 μm; Analtech, Inc., Newark, Delaware). Compounds were visualized by exposure to iodine vapor. Alternatively, compounds were visualized by spraying the plate with a solution of molybdic acid and heating the plate in an oven at 130 °C. The molybdic acid solution (Knapp et al., 1975) was prepared as follows. Ammonium molybdate (20 g) were dissolved in concentrated sulfuric acid (25 ml) by heating. The solution was allowed to cool to room temperature, then it was diluted to 400 ml with water.
Determination of Serum Cholesterol

Total cholesterol in NCS was assayed using a modification of the cholesterol Auto-Test (CHOD-PAP Enzymatic Method, catalog #148393; Bio-Dynamics; BMC Division, Boehringer Mannheim, Indianapolis, Indiana). For total cholesterol, the assay reagent was prepared by mixing together component 1 (buffer/4-aminophenzone solution; 250 ml), component 2 (cholesterol esterase/horseradish peroxidase solution; 8 ml), component 3 (cholesterol oxidase solution; 4 ml), and component 4 (phenol solution; 5 ml). The total volume of the solution was adjusted to 500 ml by the addition of deionized water. Duplicate aliquots (50 µl) were taken from each serum sample and diluted with water 0.20 ml. Assay reagent (1.8 ml) was added to each sample, and the solutions were incubated in a water bath at 37 °C for 30 minutes. Absorbance was measured and recorded at 500 nm using a Beckman DB-GT grating spectrophotometer. Cholesterol concentrations were determined using a standard curve.

The free cholesterol in the NCS was determined using the colorimetric assay for sterols described later in this chapter.

Total Lipid Extraction

Total lipids were extracted using a modification of the method of Folch et al. (1957). A mixture of chloroform and methanol (2:1) was added to the aqueous sample, generally a cell homogenate, in a ratio of solvent to sample of 4 to 1. The sample was then vortexed vigorously and the layers were allowed to separate. The lower organic phase contained the extracted lipids. The aqueous and organic phases were separated using a Pasteur pipette.
Delipidization of Serum

Whole NCS was delipidized (Cham et al., 1976) in batches of no larger than 200 ml. Isopropyl ether: n-butanol (3:2) was added to the serum at a ratio of solvent to serum 2:1 in a 3 liter round bottom flask. The mixture was stirred vigorously for 45 minutes, transferred to 6-100 ml glass centrifuge tubes, and centrifuged at 2,000 rpm for 20 minutes in a GSA rotor (Sorvall Superspeed RC-2B refrigerated centrifuge). The bottom serum layers were pooled in a 1 liter round bottom flask. The serum was rotovapped briefly to remove any remaining ether and then dialyzed in cellulose dialysis tubing (M. W. cutoff: 12-14,000; Spectrapor) against running tap water overnight to remove any residual butanol. The material was vacuum filtered through 1.2 μm, 0.45 μm, and 0.22 μm Millipore filters. Subsequently, the delipidized serum was filtered through a Nalgene 0.20 μm sterilization filtering unit and stored at 4 ºC.

Staining and Photography of Lipid-Rich Regions

To visualize the formation of lipid-rich regions in the cytoplasm of the 15-ketosterol-adapted cells, cell preparations were fixed, stained with Sudan Black B (Emmel and Cowdry, 1964; Ruthman, 1970; Troyer, 1980), and subsequently photographed.

15-Ketosterol-adapted cells were grown in F12 growth medium + 5% NCS supplemented with either 15 μM or 40 μM 5α-cholest-8(14)-en-3β-ol-15-one. CHO K-1 cells were grown in F12 medium + 5% NCS. Prior to inoculating the cells, glass slides were sterilized and positioned in the 150 mm culture plate. After 48 hours growth, the growth medium was removed and the cells were rinsed three times with 25 ml PBS. The slides were then fixed by dipping in 10% aqueous formalin (~38% formaldehyde) solution and subsequently stained by dipping in a saturated ethanol solution of Sudan Black B. The slides were then rinsed and covered in 50% glycerol in water. Cover slips were positioned over the cells. The cells were then photographed using a Zeiss photomicroscope 3
(courtesy of Dr. Kate Beckingham), using Pan-X film under oil immersion with a 50X objective.

ASSAYS

Lowry's Method of Protein Determination

The method of Lowry et al. (1951) as modified by Patterson (1979) was used to assay the protein content of cell samples. Lowry's solution C was prepared by mixing 20 g sodium carbonate and 4 g sodium hydroxide in 1 liter water. Lowry's solution was prepared by first mixing 1 part 4% sodium potassium tartrate with 100 parts Lowry's C than adding 1 part 2% cupric sulfate. Aliquots of the protein samples were transferred to triplicate sample tubes (13 x 100 mm); Lowry's solution was added to each tube and vortexed. After at least 10 minutes, 125 µl Folin-Ciocalteu's phenol reagent was added to each tube and vortexed immediately. After 30 minutes, the absorbance was read at 650 nm (VARIAN series 634) in quartz cuvettes. Duplicate bovine albumin standards (20, 40, 60, 80, and 100 µg) were assayed to provide a standard curve.

Colorimetric Assay of Sterols

The reagent for colorimetric assay of sterols (Abell et al., 1952) was made fresh before each assay. Acetic anhydride was mixed in a dry brown bottle with concentrated sulfuric acid in a ratio of 20:1 and cooled on ice for 9 minutes. Glacial acetic acid was added such that the resulting solution was 2:1 acetic anhydride: glacial acetic acid. This mixture was allowed to warm to room temperature. Aliquots (200 µl for cholesterol, 100 µl for lanosterol) from column fractions were added to colorimetric assay tubes (10 x 75 mm), and the solvent was evaporated under nitrogen. Liebermann-Burchard reagent (1.8 ml) was added to each sample and vortexed vigorously. After 30 minutes, cholesterol
was read at 620 nm and lanosterol was read at 420 nm in a Coleman Junior Spectrophotometer.

**Hydroxymethylglutaryl CoA Reductase Assay**

HMG-CoA reductase activity in cell extracts, prepared as described by Brown _et al._ (1976) was assayed in triplicate according to the procedure of Sinensky _et al._ (1979) with slight modifications. The HMG-CoA reductase assay is based on a coupled enzyme reaction. Radiolabeled HMG-CoA is reduced to mevalonic acid by HMG-CoA reductase. The reducing equivalents required for the conversion of HMG-CoA to mevalonic acid are produced by the reaction of glucose-6-phosphate with nicotinamide adenine dinucleotide phosphate (NADP) catalyzed by glucose-6-phosphate dehydrogenase. The mevalonic acid is subsequently converted to mevalonolactone which is readily separated from the reaction mixture and quantified by radio-TLC.

In summary, cells were grown under specified assay conditions, harvested, and assayed. Aliquots of the reaction mixture were separated by thin-layer chromatography and the radioactivity was determined. Finally, the protein content of each cell sample was determined and the HMG-CoA reductase activity was expressed as pmoles/min/mg protein.

Routinely, 5.0 x 10^5 cells per 100 mm culture dish were inoculated into 10 ml Ham's F12 +5% NCS 48 hours in advance of the assay. Three plates were prepared for each assay point. The plates were maintained in F12/NCS at 37 °C until the assay. At the time of harvest, the medium was aspirated, and the plates were rinsed with ice cold PBS + 5 mM DTT. The rinsing solution was added (15 ml per 150 x 15 mm plate) and aspirated. The cells were harvested in PBS/DTT (5.0 ml) by scraping with a rubber policeman. The cell suspensions from three plates were combined in a 15 ml conical cen-
trifuge tube and centrifuged (DYNAC tabletop centrifuge) at 1500 rpm for 5 minutes. The supernatant was aspirated as completely as possible without disturbing the cell pellet. The pellet was frozen on dry ice.

Buffer A was prepared with the following components: 50 mM potassium phosphate, 5 mM EDTA, 200 mM KCl, pH 7.4. Buffer B was prepared by adding 0.25% Kyro EOB detergent to buffer A. The cofactor-substrate solution consisted of: glucose-6-phosphate (40 mM); glucose-6-phosphate dehydrogenase (12 I.U./ml); NADP (5.0 mM); DL-3-hydroxy-3-methyl-[3-14C]glutaryl-CoA (11 dpm per pmol) in buffer A.

The cell pellets were thawed at room temperature, and an appropriate volume of buffer B was added (approximately 0.5 ml per 10^6 cells) to solubilize the microsomal membranes and release the HMG-CoA reductase. Each cell pellet was suspended with a Pasteur pipette, incubated for 10 minutes in a 37 °C water bath, and centrifuged (Beckman TJ-6) at 28,000 rpm for 10 minutes at room temperature to pellet insoluble material. Aliquots (50 μl) of the cell-free supernatant were added to triplicate 6 x 50 mm assay tubes and pre-incubated for 20 minutes at 37 °C. It was convenient to aliquot one sample every 15 seconds. To prepare blanks for the assay, 50 μl of any sample was aliquoted into duplicate assay tubes and 10 N HCl (25 μl) was added immediately prior to incubation to destroy any enzyme activity. The remaining cell-free preparations were frozen for later protein analysis. The cofactor-substrate solution was incubated for 20 minutes prior to adding to the samples to generate reducing equivalents for the reaction. After the cofactor-substrate solution (50 μl) was added, the tubes were vortexed. The samples were incubated for an additional 40 minutes and the reaction terminated by the addition of 10 N HCl (25 μl).

Aliquots (50 μl) of [3H]mevalonolactone (30,000 dpm per 50 μl) were added as an internal standard. The tubes were vortexed and the incubation was continued for 30
minutes to insure complete lactonization of the [3-14C]mevalonate. Prior to TLC, the assay tubes were centrifuged (Beckman TJ-6) at 28,000 rpm at room temperature for 10 minutes to pellet the protein precipitated by the HCl. Approximately 75 µl of the supernatant was spotted on a 5 x 20 cm silica gel G TLC plate (0.25 mm, Analtech). The plates were first dried under a warm stream of air then baked for 3 minutes under a hot stream of air. After cooling to room temperature, the plates were chromatographed in acetone:benzene (1:1) until the solvent had ascended 10 cm from the origin. The region corresponding to Rf=0.40 - 0.70 was scraped into a scintillation vial containing toluene:ethanol (2:1) scintillation fluid and the radioactivity was counted.

Prior to protein assays, the cell-free preparations were subjected to a trichloroacetic acid precipitation to rid the sample of interfering detergent. The samples were thawed and centrifuged at room temperature at 28,000 rpm for 10 minutes. Aliquots (250-500 µl) from each sample were transferred to a 13 x 100 mm sample tube. The samples were diluted to a final volume of 1.0 ml with water. After adding 10% TCA (w/v; 1.09 ml), the samples were allowed to stand on ice for 30 minutes-1 hour, then centrifuged at 28,000 rpm at 4 °C for 20 minutes. The supernatant was drained, the pellet was dissolved in 10% NaOH (400 µl), and H2O (2.1 ml) was added. Aliquots (250-500 µl) were transferred to triplicate sample tubes and assayed for protein following the procedure of Lowry et al. described previously in this section.

Isolation of a Floating Lipid Layer Derived from Cells in Culture

To develop a procedure for analyzing the lipids accumulated in cultured cells, modifications of protocols developed in two laboratories were used (MacKenzie et al., 1966; Rothblat, 1974; Glick et al., 1983). Routinely, K(15) cells (1.0 x 10^6) were inoculated into 150 x 15 mm culture plates containing 25 ml Ham's F12 medium + 5% NCS con-
taining 15 μM 5α-cholest-8(14)-en-3β-ol-15-one. After a 48-hour growth period, six plates for each incubation condition were changed to the experimental media as specified in each experiment. After a 48-hour incubation period, the cells were harvested in the following manner. The six plates for each condition were paired, resulting in triplicate samples of two plates per sample. Aliquots (2.0 ml) of the medium from each of the two culture plates were pooled to comprise a 4.0 ml aliquot of medium from each sample at the time of harvest. These samples were frozen until the radioactivity was determined.

The remainder of the medium was removed by aspiration, and the plates were rinsed three times with 10 ml PBS. The cells were then treated with trypsin (0.05%; 4.0 ml) containing 2% w/v EDTA which caused the cells to detach from the plate. After 1.5-2.0 minutes at 37 °C, soybean trypsin inhibitor (5.0 mg/ml; 1.0 ml) was added to each plate. The cells were suspended in the trypsin solution and transferred by Pasteur pipette to a conical 15 ml centrifuge tube on ice. The two plates were further rinsed with 2.5 ml of trypsin (0.05%) each, and the cell suspensions were pooled and transferred to the same centrifuge tube.

It should be noted that the method of harvesting is a critical step in this procedure. In establishing this protocol, it was determined that harvesting the cells by scraping with a rubber policeman, the method of choice when harvesting cells for the HMG-CoA reductase assay, was an unacceptable harvesting method for this assay. Results indicated that following harvesting by scraping, over half of the incorporated radiolabeled sterols were recovered in the supernatant following centrifugation to collect the harvested cells. Further, failure to include soybean trypsin inhibitor results in some cell lysis also leading to the recovery of significant levels of the incorporated radio-labeled sterols in the supernatant following centrifugation to collect the cells.
The 15-ketosterol-adapted cells were collected by low speed centrifugation (1500 rpm) for 5 minutes at room temperature. After carefully aspirating the supernatant, the cells were resuspended in ice cold distilled water (2.0 ml) using a Pasteur pipette and allowed to stand at room temperature for 5 - 10 minutes. The cell suspensions were then disrupted by dispersion through a 23 gauge needle attached to a 3 ml syringe until no evidence of unbroken cells was observed by microscopic inspection. Four to six dispersions through the needle were required for complete cell disruption. Homogenization appeared most efficient when the samples were allowed to stand at room temperature for about 5 minutes prior to homogenization. After homogenization, the samples were again maintained on ice.

Of the 2.0 ml homogenized samples, 1.250 ml were transferred to 11 x 34 mm polycarbonate centrifuge tubes. The remainder of the sample was reserved for determination of radioactivity and protein content. The samples were centrifuged at 100,000 x g for 1 hr at 5 °C with a slow deceleration rate for the last nine minutes in the Beckman TL-100 ultracentrifuge (TLA 100.2 rotor). The resultant sample consisted of an upper opaque floating lipid layer, a clear intermediate zone, and a pellet. The samples were then separated as follows. The distinct milky white material concentrated at the top of the tube was collected very carefully with a 1000 μl automatic pipetter set at 250 μl; 500 μl of the sample was collected. Based on visual observation, this procedure resulted in separation and collection of an estimated ≥90% of the lipid layer.

Although the interface between the lipid layer and clear supernatant/intermediate zone was very distinct, it was easily perturbed. Even careful removal of the lipid layer occasionally resulted in some dispersion of the lipid material into the upper layer of clear intermediate zone. For this reason, the intermediate zone was divided into two portions: the upper and lower fraction. The upper 500 μl of intermediate zone (mixed with some
of the disturbed floating lipid layer) was collected and designated as the upper intermediate zone. The remaining 250 μl of the intermediate zone was collected and designated the lower intermediate zone. Finally, the remaining pellet was suspended in 500 μl water and collected.

Each of the four fractions was diluted with water to 1.5 ml in 16 x 125 mm glass tubes. Three 50 μl aliquots were removed for the assay of protein and two 25 μl aliquots were removed for analysis of radioactivity. The remainder of each sample was extracted for total lipids using the method of Folch et al. (1957). A mixture (6.0 ml) of chloroform and methanol (2:1) was added and each sample was vortexed vigorously twice for 20 seconds. The samples were refrigerated overnight, and the organic phase was carefully separated from the aqueous phase and precipitated protein interphase. As much of the organic phase as possible was first collected using a Pasteur pipette. Subsequently, 4.0 ml of chloroform were added, and the samples were vortexed. The layers were allowed to separate, and the chloroform was collected. This process was repeated three times for each sample. The aqueous layers were all saved, and aliquots were counted to determine the content of radioactivity. The organic phases were combined and evaporated under nitrogen. A mixture (1.5 ml) of chloroform and methanol (2:1) was added to each of the sample tubes for transfer of the sample to 15 x 45 mm glass screw cap vials. The tubes were then rinsed twice with a mixture (1.5 ml) of chloroform and methanol (2:1). After evaporation, the samples were dissolved in 500 μl chloroform and stored in a freezer at 0 °C for subsequent analysis by radio-TLC.

Analysis of the Centrifuged Fractions

The TLC system used to analyze these samples included development in two solvent systems. First, Baker silica gel, 250 μm, hard-surfaced, 10 x 20 cm TLC plates, previ-
ously marked into four lanes, were developed in benzene containing methanol (0.4-
0.7%). The exact proportion of methanol required for optimum separation was believed
to be related to the humidity of the laboratory environment. A test plate of unlabeled
standards was routinely developed and visualized in an iodine vapor tank each day before
analysis of the experimental samples.

After development of the TLC plates in the benzene solvent system, the plates were
allowed to dry. Each plate was developed a second time in hexane: ethyl acetate:
isopropyl ether: acetic acid (60:30:20:5) to a distance corresponding to \( R_f = 0.4 \). The
plates were subsequently scraped in 0.5 cm increments. This system resulted in separa-
tion of the esters of cholesterol and 5α-cholest-8(14)-en-3β-ol-15-one, free 5α-cholest-
8(14)-en-3β-ol-15-one and cholesterol as well as free fatty acids. One of the advantages
of this system was that both polar and nonpolar lipids could be separated on one plate,
eliminatn the need to separate the esters of cholesterol and the 15-ketosterol on one plate
using benzene or toluene and the free sterols on another using a hexane/ethyl acetate
system. In addition, this system resulted in the separation of triacylglycerols and
15-ketosteryl esters, the two classes that proved to be the most difficult to separate from
one another and still maintain separation of the other compounds.

Using the TLC system described above, approximately 10 μl of each sample was
spotted onto the TLC plate accompanied by unlabeled standards of 5α-cholest-8(14)-en-
3β-ol-15-one, cholesterol, cholesteryl ester (oleate), 5α-cholest-8(14)-en-3β-ol-15-one
esters (palmitate or oleate), triacylglycerol (triolein), and fatty acids (palmitic acid or oleic
acid). After completely developing the plates and scraping, the samples were counted for
5 minutes in 5 ml toluene:ethanol (2:1) scintillation cocktail. Protein analyses were per-
formed on the aliquots described above using the method of Lowry et al. (1951).
To summarize, first the cells were plated, incubated under different conditions, and harvested. The cells were then homogenized and separated into fractions by centrifugation. The lipids were then extracted from each of the fractions. The extraction was followed by radio-TLC to separate the following six compounds: cholesterol, 5α-cholesterol-8(14)-en-3β-ol-15-one, free fatty acids, 5α-cholesterol-8(14)-en-3β-ol-15-one esters, triacylglycerols, and cholesteryl esters. Aliquots of the total sample and the individual fractions are taken for assays of protein and radioactivity.

**High Performance Liquid Chromatography**

Aliquots of the lipid extracts of cell samples were dissolved in CHCl₃ and subjected to HPLC (Chu et al., 1988) on a 5-μm C₁₈ Microsorb column (4.6 mm x 25 cm; Rainin Instrument Co., Inc., Woburn, MA) coupled to an RP-18 NewGuard cartridge (3.2 mm x 1.5 cm; Brownlee Labs, Inc., Santa Clara, CA). The column was then eluted in a gradient manner with two solvent mixtures. Solvent mixture A was composed of isopropyl alcohol/methanol/water (5:4:1), and solvent mixture B was composed of isopropyl alcohol/methanol (4:1). Initially, a mixture of solvent A and solvent B (3:2) was pumped under isocratic conditions for 15 min. The mobile phase was then linearly programmed to a ratio of solvent A to solvent B of 1:3 using a Waters model 680 automated gradient controller (Waters Associates, Milford, MA) for the second 15 min and then maintained at this ratio thereafter. The flow rate was increased in a linear fashion for the first 5 min from 1.0 ml/min to 1.25 ml/min and, in the next 10 min, from 1.25 ml/min to 1.45 ml/min. Thereafter, the flow rate was maintained at 1.45 ml/min. All solvents were of analytical grade (Burdick and Jackson, Inc., Muskegon, MI), and solvent mixtures were filtered through a 0.45-μm Nylon-66 membrane filter (Rainin Instrument Co., Inc.) and degassed prior to use. The analyses were carried out using pumps (model 510), an ultraviolet detection unit (model 481), and a U6K injector unit which were obtained from
Waters Associates. Ultraviolet absorbance was monitored at 210 nm, and eluted fractions (0.45 ml in volume) were collected using an automated fraction collector via Teflon tubing connected to the outlet of the detector unit. This chromatographic system permits resolution of 5α-cholest-8(14)-en-3β-ol-15-one from cholesterol and from fatty acid esters of the two sterols. In addition, this reverse phase HPLC method permits the resolution of the major fatty acid esters of the 15-ketosterol and of cholesterol. However, the oleate and palmitate esters of the 15-ketosterol and of cholesterol are not resolved in this chromatographic system.

Isolation of Nonsaponifiable Lipids

The nonsaponifiable lipids from cell culture incubations were isolated by the following previously described (Pinkerton et al., 1982) procedure. To each sample, 1 ml 15% ethanolic potassium hydroxide (w/v, 95% ethanol) was added. The samples were suspended with a Pasteur pipette, transferred to a 15 ml glass tube, and subsequently hydrolyzed by incubating for three hours in a water bath (70-80 °C). After cooling to room temperature, 1 ml water was added to the samples. The samples were extracted three times with 3 volumes petroleum ether and the combined petroleum ether extracts were backwashed twice with an equal volume of distilled water. The ether was evaporated under nitrogen and the sample was dessicated in vacuo over phosphorus pentoxide overnight to remove any remaining traces of water.

Separation of Nonsaponifiable Lipids

Silicic acid-Super Cel columns were used for the analysis of nonsaponifiable lipids (Schroepfer, 1961 and Pinkerton et al., 1982) from cell culture incubations. The packing material was prepared by mixing silicic acid (100 mesh) and Hy-Flo Super Cel in a 1:1 ratio (w/w) and adding toluene to form a slurry. The slurry was poured into a 1 x 100
cm column and packed under 10-12 psi nitrogen pressure. The nonsaponifiable lipid sample was applied under pressure in 2-3 ml toluene. Before application of the sample to the column, cholesterol (10 mg) and lanosterol (2 mg) standards were added to the sample. The sample was eluted using three solvent systems. Fractions (32 minutes) were collected using an automatic fraction collector (ISCO Model 273):

1) toluene; 120 fractions
2) toluene: ether (4:1); 40 fractions
3) chloroform:methanol (2:1); 40 fractions

To determine the total radioactivity of an applied sample, a 20 µl aliquot was reserved and counted.
Chapter III.

Characterization of the 15-Ketosterol-Adapted Cells
A. Background

5α-Cholest-8(14)-en-3β-ol-15-one is a potent regulator of cholesterol metabolism. In cultured mammalian cells, this 15-ketosterol inhibits sterol biosynthesis (Schroepfer et al., 1977a) and reduces the levels of activity of the enzymes involved in the synthesis and reduction of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) (Schroepfer et al., 1977a; Miller et al., 1980; Pajewski et al., 1988). It has been shown in this laboratory that the 15-ketosterol inhibits growth of CHO K-1 cells incubated in medium containing fetal calf serum (Pinkerton et al., 1988). When CHO K-1 cells were incubated in medium containing fetal calf serum and supplemented with increasing concentrations of the 15-ketosterol, cell growth, as measured by protein accumulation, was inhibited in a concentration-dependent fashion (see Figure 2). The concentration of the 15-ketosterol required to inhibit growth by 50% was approximately 13 μM. Almost complete inhibition of growth was observed at 25 μM 5α-cholest-8(14)-en-3β-ol-15-one.

Sodium oleate, added to the incubation medium, partially overcame this inhibitory effect (Pinkerton et al., 1988). The presence of 25 μg/ml (82 μM) sodium oleate in the incubation medium caused significant reduction of the inhibitory effect of the 15-ketosterol at all inhibitory concentrations except 50 μM (See Figure 2). With the addition of sodium oleate to the growth medium, the concentration of the 15-ketosterol required to inhibit growth by 50% was increased to approximately 25 μM.

Interest in establishing the mechanism by which the addition of sodium oleate reduced the inhibitory effects of the 15-ketosterol with respect to cell growth led to the isolation of a cell line adapted to grow in unusually high concentrations of the 15-keto-sterol. This cell line was designated as the 15-ketosterol-adapted cell, denoted as the K(15) cell. Routine maintenance conditions for this adapted cell were F12 growth medium + 5% newborn calf serum + 15 μM 5α-cholest-8(14)-en-3β-ol-15-one.
Figure 2. The inhibition of growth of CHO K-1 cells by 5α-cholest-8(14)-en-3β-ol-15-one in the absence ( ●--●) or presence of ( ○--○) of 82 μM sodium oleate. Experimental details are presented in the text. Data are represented as Mean ± S.D. (n=8)
B. Isolation of the 15-Ketosterol-Adapted Cells

A CHO K-1-derived cell line resistant to the inhibitory effects of 15 μM 15-ketosterol was isolated by Dr. F. D. Pinkerton by exposing cells to incrementally higher concentrations of the 15-ketosterol, beginning at 10 μM 15-ketosterol in lipid containing medium. At each step in the selection process, the concentration of oxygenated sterol in the medium was increased by 0.5 μM.

Approximately 1 x 10⁷ cells were inoculated into 150 mm culture dishes containing 25 ml F12 growth medium supplemented with fetal calf serum (8% final concentration). After a 48-hour incubation period the medium was changed to experimental medium supplemented with 10 μM 5α-cholest-8(14)-en-3β-ol-15-one. The cells were incubated in the medium until a number of colonies of resistant cells remained on the plates. The medium was changed at two day intervals during this period. Approximately 25 of the largest colonies were selected using cloning cylinders at the time of trypsinization; these colonies were either subcultured into either normal growth medium (F12 + 8% FCS) or experimental medium (+10 μM 15-ketosterol). The former populations were expanded and frozen at 76°C. The latter populations were expanded and passaged in the presence of the 15-oxysterol (10 μM) until populations of resistant cells exhibiting normal growth rates (doubling times of 12-16 hours) were obtained. One population of cells was arbitrarily chosen for further selection, and the remainder of the populations were frozen in medium containing the 15-ketosterol.

The process described above was repeated for each incremental increase of 0.5 μM 15-ketosterol concentration in the medium. During the selection process, no effort was made to determine the actual total number of resistant colonies obtained from a given concentration of the 15-ketosterol. Furthermore, because of the labor intensive nature of the
selection process, only one of the 25 separate populations resistant to a given 15-oxysterol concentration was used for the next step in the selection sequence.
C. Determination of the Average Generation Time

As part of a series of experiments designed to characterize the 15-ketosterol-adapted cells, the average generation time for these cells was determined during the exponential phase of cell growth and compared to the value obtained for the CHO K-1 wild type cell line.

The two cell types, CHO K-1 and K(15), were subcultured into 150 x 15 mm tissue culture plates at the following densities:

1) \(1.0 \times 10^5\) cells per plate,
2) \(2.5 \times 10^5\) cells per plate, and
3) \(5.0 \times 10^5\) cells per plate.

The CHO K-1 cells were maintained in Ham's F12 medium + 5% NCS. The K(15) cells were maintained in Ham's F12 medium + 5% NCS supplemented with 15 \(\mu\)M 5\(\alpha\)-cholest-8(14)-en-3\(\beta\)-ol-15-one. At 24, 48, and 72 hours after subculturing, the cells were harvested by trypsinization, collected by centrifugation, and resuspended in a known volume of growth medium. The cells were counted using a hemacytometer. Duplicate plates were counted two times each. The average generation time was then calculated for each cell type using the formula (Kuchler, 1977):

\[
g = \frac{t_2 - t_1}{\log_2 y - \log_2 x}
\]

where \(g = \text{average generation time},\)
\(t_2 = \text{time of second cell counting},\)
\(t_1 = \text{time of first cell counting},\)
\(y = \text{number of cells at } t_2,\)
\(x = \text{number of cells at } t_1.\)
Figure 3 illustrates the growth of the two cell types over the time period observed. The average generation time, based on the data gathered at 24 hours and 48 hours after subculturing, for the 15-ketosterol-adapted cells was calculated to be $14.0 \pm 0.6$ hours ($n=3$) compared to an average generation time of $11.6 \pm 1.5$ hours ($n=3$) for the wild type CHO cell line. There was no significant statistical difference ($p=0.06$) between the average generation time of the 15-ketosterol-adapted cells incubated in lipid-rich medium containing 15 $\mu$M 15-ketosterol and the CHO K-1 wild type cells incubated in lipid-rich growth medium containing no 15-ketosterol.
Figure 3. Growth comparison of the 15-ketosterol-adapted cells and the CHO K-1 cells.

Open ovals represent the growth of the 15-ketosterol-adapted cells
Closed ovals represent the growth of the CHO K-1 cells
D. Stability of the 15-Ketosterol Resistance Adaptation

Introduction

Another aspect of characterizing the 15-ketosterol-adapted cells was to determine the stability of these cells' ability to be maintained in medium supplemented with 15 \( \mu \text{M} \) 5\( \alpha \)-cholesterol-8(14)-en-3\( \beta \)-ol-15-one. The purpose of this experiment was to determine if the K(15) cells would retain the ability to grow in medium supplemented with high concentrations of the 15-ketosterol after removal of the 15-ketosterol supplement from the growth medium for an extended period of time.

In general, K(15) cells were removed from the routine 15-ketosterol supplemented growth conditions and plated into growth medium supplemented only with NCS, grown for 20 generations, then subcultured directly into 15 \( \mu \text{M} \) 5\( \alpha \)-cholesterol-8(14)-en-3\( \beta \)-ol-15-one-supplemented medium. The growth of these cells, as measured by cell protein, was then compared to control cells that had been continuously maintained at a level of 15 \( \mu \text{M} \) 5\( \alpha \)-cholesterol-8(14)-en-3\( \beta \)-ol-15-one.

Method

Initially, K(15) cells (1 \( \times \) 10\(^6\)) were subcultured from a growth condition of Ham's F12 medium + 5% NCS + 15 \( \mu \text{M} \) 5\( \alpha \)-cholesterol-8(14)-en-3\( \beta \)-ol-15-one into a 150 x 15 mm culture plate containing Ham's F12 growth medium + 5% NCS. After two days, fresh medium containing only 5% NCS was added. After an additional three days, the cells were subcultured into 8-150 x 15 mm culture plates, again containing medium + NCS only. After three days, fresh growth medium was added. After another four days, the cells were subcultured into two sets of 12 plates (2.5 \( \times \) 10\(^6\) cells per plate).

The first set of plates contained Ham's F12 growth medium + 5% NCS supplemented with 15 \( \mu \text{M} \) 5\( \alpha \)-cholesterol-8(14)-en-3\( \beta \)-ol-15-one and the second set contained Ham's F12 medium + 5% NCS only. Further, a third set of cells were plated: K(15) cells that not been
removed from growth medium supplemented with the 15-ketosterol. The third population of K(15) cells had been continually maintained in Ham's F12 medium + 5% NCS + 15 μM 5α-cholest-8(14)-en-3β-ol-15-one.

At 24 hour intervals for 4 days, the cells were harvested by trypsinization and collected by centrifugation 5 minutes at 1500 rpm. At each time point, triplicate plates for each of the three sets of cells were harvested. The samples were frozen until the samples were assayed for protein. Protein was assayed following the procedure of Lowry et al. (1951) as described in the Materials and Methods chapter.

Results

The results indicated that there was no difference in the growth rate of the K(15) cells that were removed from the 15-ketosterol-containing medium for twenty generations then incubated again at a level of 15 μM 5α-cholest-8(14)-en-3β-ol-15-one compared to the growth of K(15) cells that never been removed from the 15-ketosterol-containing medium or K(15) cells that continued to be grown in medium supplemented with NCS only. Figure 4 illustrates the growth of these three cell populations for a period of 72 hours. The results indicate that the ability of the cells to grow in 15 μM 5α-cholest-8(14)-en-3β-ol-15-one is not dependent upon continuous incubation in the presence of the 15-ketosterol.

Studies previously presented demonstrated that incubation of CHO K-1 wild type cells at a level of 13 μM 5α-cholest-8(14)-en-3β-ol-15-one resulted in a 50% reduction of cell growth as measured by total cell protein. The K(15) cells were derived from CHO K-1 cells that were incrementally adapted to growth in increasing concentrations of the 15-ketosterol. The K(15) cells had been continuously maintained in growth medium supplemented with 15 μM 5α-cholest-8(14)-en-3β-ol-15-one for a period of almost two years. The reason for the adaptation to growth in unusually high concentrations of the 15-ketosterol has not been established. This experiment was performed to determine if this adaptation was a permanent
change or one that could be maintained only as long as the cells were maintained under
conditions of selective pressure. It was considered likely that upon removal of the
15-ketosterol supplement from the K(15) cells for an extended period of time, the cells
would lose their ability to grow under these conditions. The results of this experiment,
however, demonstrated that the cells did not lose the ability to grow at unusually high
concentrations of the 15-ketosterol. After 20 generations removed from the 15-ketosterol-
containing medium, the cells were again subcultured in medium supplemented with 15 μM
5α-cholest-8(14)-en-3β-ol-15-one. The growth rate of these cells was consistent with the
growth rate of control cells. Although the mechanism responsible for the adaptation has not
been determined, the results of this experiment indicate that the adaptation is stable.
E. Staining and Photography of 15-Ketosterol-Adapted Cells

Introduction

The purpose of this study was to illustrate the accumulation of lipid-rich regions present in the cytoplasm of the 15-ketosterol-adapted cells when incubated at increasing concentrations of 5α-cholest-8(14)-en-3β-ol-15-one. K(15) cell preparations were fixed, stained with Sudan Black B (Emmel and Cowdry 1964; Ruthman, 1970; Troyer, 1980) and subsequently photographed.

Method

15-Ketosterol-adapted cells were grown in F12 growth medium + 5% NCS supplemented with either 15 μM or 40 μM 5α-cholest-8(14)-en-3β-ol-15-one. CHO K-1 cells were grown in F12 medium + 5% NCS. Prior to inoculating the cells, glass slides were sterilized and positioned in the 150 mm culture plate. After 48 hours growth, the growth medium was removed and the cells were rinsed three times with 25 ml PBS. The slides were then fixed by dipping in a 10% aqueous formalin (~38% formaldehyde) solution and stained by dipping in a saturated ethanol solution of Sudan Black B. The slides were subsequently rinsed in PBS and covered in 50% glycerol in water. Cover slips were positioned over the cells.

The cells were then photographed using a Zeiss photomicroscope 3 (courtesy of Dr. Kate Beckingham) using Pan-X film under oil immersion with a 50X objective.

Results

Figures 5, 6, and 7 illustrate the results. In these photographs, the lipid-rich regions appear as black spots. As the cell membranes are not distinguishable under these conditions, only shadows of the cells are detectable. Figure 5 is a photograph of the CHO K-1 cells after incubation in Ham's F12 medium + 5% NCS. In the CHO cells, there is evi-
Figure 5. CHO K-1 cells (Ham's F12NCS5) stained with Sudan Black B. The cells were photographed using a Zeiss photomicroscope 3 (50X objective) under oil immersion.
dence of limited numbers of lipid-rich regions. The stained regions are small and not abundant.

In Figure 6, the K(15) cells incubated in 15 μM 5α-cholest-8(14)-en-3β-ol-15-one demonstrate a greater number of lipid-rich regions. Though the membranes of the cells are not clearly distinguishable, it is observable that the size of the 15-ketosterol-adapted cells is larger than the wild type cells. Visual estimation indicates that the CHO cells have an approximate length of 60 μm while the 15-ketosterol adapted cells have a length of approximately 80-160 μm.

Figure 7 clearly shows the dramatic accumulation of lipid-rich regions particularly concentrated in the cytoplasm surrounding the nucleus when K(15) cells are incubated in growth medium supplemented with 40 μM 5α-cholest-8(14)-en-3β-ol-15-one. Not only are the regions much more numerous, but the size of these Sudan Black-staining regions appear to be significantly increased. It was this phenomenon that created such an interest in investigating these cells, particularly with respect to the composition of these perinuclear lipid-rich regions and the metabolism of the 15-ketosterol.
Figure 6. 15-Ketosterol-adapted cells (Hams F12NCS5 + 15 μM 15-ketosterol) stained with Sudan Black B. The cells were photographed using a Zeiss photomicroscope 3 (50X objective) under oil immersion.
Figure 7. 15-Ketosterol-adapted cells (Hams F12NCS5 + 40 μM 15-ketosterol) stained with Sudan Black B. The cells were photographed using a Zeiss photomicroscope 3 (50X objective) under oil immersion.
F. HMG-CoA Reductase Activity in the 15-Ketosterol-Adapted Cells

Introduction

5α-Cholest-8(14)-en-3β-ol-15-one is a potent inhibitor of sterol synthesis and causes a reduction in the level of HMG-CoA reductase in animal cells in culture (Pajewski et al., 1988; Pinkerton et al., 1982; Miller et al., 1980; Schroepfer et al., 1977; 1976). HMG-CoA reductase is commonly considered to be a key regulatory enzyme in sterol biosynthesis. This study was performed to determine the HMG-CoA reductase activity in the 15-ketosterol-adapted cells.

Method

HMG-CoA reductase activity was assayed following the procedure described in the Materials and Methods section. The levels of the reductase activity of the K(15) cells were assayed under the following incubation conditions:

1) Ham's F12 + 5% NCS supplemented with 40 μM 5α-cholest-8(14)-en-3β-ol-15-one,

2) Ham's F12 + 5% NCS supplemented with 40 μM 5α-cholest-8(14)-en-3β-ol-15-one + 25 μg/ml oleate, and

3) Ham's F12 + 5% NCS supplemented with 40 μM cholesterol.

The levels of the HMG-CoA reductase activity were also determined for the wild type CHO K-1 cells following incubation in F12 medium + 5% delipidized NCS. It has been shown in other studies that incubation under these conditions induces markedly elevated levels of HMG-CoA reductase activity.

K(15) cells (1 x 10^6) cells were inoculated into 150 x 15 mm culture plates containing F12 growth medium + 5% NCS supplemented with 15 μM 5α-cholest-8(14)-en-3β-ol-15-one. The cells were incubated at 37 °C for 48 hours. Subsequently the medium was replaced with the experimental medium as described above, and the cells were allowed to
incubate for an additional 48 hours. For the assay, two plates were combined per sample, and triplicate samples were assayed in triplicate for HMG-CoA reductase activity.

Results

The HMG-CoA reductase activities, expressed as pmoles mevalonic acid per min per mg protein, are presented in numerical form in Table 1 and graphically in Figure 8. The HMG-CoA reductase activity in the 15-ketosterol-adapted cells incubated at 40 μM 5α-cholesterol-8(14)-en-3β-ol-15-one was 44± 6.2 pmols per min per mg protein. The HMG-CoA reductase activity in the 15-ketosterol-adapted cells incubated at 40 μM 5α-cholesterol-8(14)-en-3β-ol-15-one + 25 μg/ml olate was 59± 3.2 pmols per min per mg protein. The HMG-CoA reductase activity in the 15-ketosterol-adapted cells incubated at 40 μM cholesterol was 28 ± 6.7 pmols per min per mg protein. The HMG-CoA reductase activity in the CHO K-1 wild type cells incubated under delipidized conditions was 809 ± 41.4 pmols per min per mg protein.

Previous studies have shown that CHO K-1 wild type cells when incubated in lipid-rich medium (F12FCS8) have an HMG-CoA reductase activity of <100 pmoles mevalonic acid per min per mg protein (Miller et al., 1980). Previous experiments, as well as the assay presented here, indicate that the transfer of CHO K-1 cells to growth medium containing delipidized serum results in a tremendous increase in the level of HMG-CoA reductase activity (Pajewski et al., 1988; Chang and Limanek, 1980; Miller et al., 1980). In the experiment presented here, the activity was determined to be 809 ± 41 pmoles mevalonic acid per min per mg protein. Further, studies in this laboratory have also shown that in the CHO K-1 cells, the addition of 1 μM 5α-cholesterol-8(14)-en-3β-ol-15-one to the growth medium of cells growing under delipidized conditions results in dramatic decreases in the high levels of the reductase enzymes (Pajewski et al., 1988; Miller et al., 1980).
Table 1. HMG-CoA reductase activity in the K(15) cells and the CHO K-1 cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Incubation Condition</th>
<th>Activity ± S. D. (pmols / min / mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K(15)</td>
<td>40 µM 15-ketosterol (5% NCS)</td>
<td>44 ± 6.2</td>
</tr>
<tr>
<td>K(15)</td>
<td>40 µM 15-ketosterol + 25 µg/ml oleate (5% NCS)</td>
<td>59 ± 3.2</td>
</tr>
<tr>
<td>K(15)</td>
<td>40 µM cholesterol (5% NCS)</td>
<td>28 ± 6.7</td>
</tr>
<tr>
<td>K-1</td>
<td>5% lipid-deficient NCS, 16 hr incubation</td>
<td>809 ± 41.4</td>
</tr>
</tbody>
</table>
Figure 8. HMG-CoA reductase activity measured in the 15-ketosterol-adapted cells and the CHO K-1 cells.

1 = K(15) cells: 40 μM 15-ketosterol; F12NCS5
2 = K(15) cells: 40 μM 15-ketosterol + 25 μg/ml oleate; F12NCS5
3 = K(15) cells: 40 μM cholesterol; F12NCS5
4 = CHO K-1 cells: F12 + 5% delipidized NCS

Note: The inset graph illustrates the reductase activities measured from the K(15) cells (conditions 1, 2, and 3) on a different scale.
In this experiment, it was shown that under all conditions assayed, the activity of HMG-CoA reductase in the K(15) cells remained very low. It is important to note, however, that measurable levels of HMG-CoA reductase were consistently detected and that marked increases in the reductase activity in the adapted cells were not observed. Further, these results rule against the possibility that the adaptation of the K(15) cells is due to a loss of the ability to regulate the HMG-CoA reductase enzyme. In the case of UT-1 cells, a CHO-derived cell line adapted to growth in high concentrations of compactin, a competitive inhibitor of the reductase, a gene amplification allows the cells to grow under conditions inhibitory to the parent cell line. The increased concentration of compactin in the growth medium resulted in increased reductase activity (Luskey et al., 1983). This is clearly not the case with the K(15) cells.
Chapter IV.

Metabolism of 5α-Cholest-8(14)-en-3β-ol-15-one in the 15-Ketosterol-Adapted Cells
A. Incorporation of 5α-Cholest-8(14)-en-3β-ol-15-one and Cholesterol in the K(15) and CHO K-1 Cells
Incorporation of $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one and cholesterol in the K(15) cells

Introduction

In the series of experiments described in this section, the incorporation of $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one and cholesterol in the 15-ketosterol-adapted cells was investigated. The purpose of these experiments was to monitor 15-ketosterol incorporation as a function of time in the K(15) cells when incubated in growth medium containing two different concentrations of the 15-ketosterol. Incorporation of cholesterol into the K(15) cells was also investigated. Further, the relative esterification of the incorporated sterols was determined by radio-TLC analysis.

Method

15-Ketosterol-adapted cells (1.0 x 10$^6$ cells/plate) were plated into 150 mm culture plates containing Ham's F12 growth medium + 5% NCS (F12NCS5) containing 15 $\mu$M $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one. After a 48-hour growth period at 37 °C, the culture plates were changed to the experimental medium described below:

Experiment 1: Ham's F12 medium + 5% NCS containing 15 $\mu$M [2,4-3H] $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one (3.6 $\mu$Ci 3H/plate) + 2.2 $\mu$M [4-14C]cholesterol (3.1 $\mu$Ci 14C/plate) or

Experiment 2: Ham's F12 medium + 5% NCS containing 40 $\mu$M [2,4-3H] $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one (2.1 $\mu$Ci 3H/plate).

After an additional 48-hour incubation period, the medium was aspirated and the cells were rinsed twice with 10 ml PBS, and 5 ml of 0.05% trypsin in Hank's balanced salt
solution were added. After incubating at 37 °C for 7.5 minutes, 5.0 ml soybean trypsin inhibitor (1.5 mg/ml) were added to the plates. The cells were collected and the plates were rinsed twice with PBS (2.5 ml). After pelleting the cells at 1500 rpm for 5 minutes, the cells were resuspended in 2.0 ml water to promote cell swelling. The cells were homogenized by aspirating four times through a 23 gauge needle fitted to a 3 ml syringe. Aliquots (100 µl) were taken of the medium, the cell homogenate, and the trypsin supernatant. These aliquots were added to Scintisol scintillation cocktail (5.0 ml) and counted in a Packard 1500 scintillation counter. Protein determinations were also performed on the cell homogenates following the procedure of Lowry et al. (1951). Three plates per time point were individually harvested at 2, 4, 6, 12, 24, and 48 hours.

Aliquots (1.0 ml) of the cell homogenates were extracted following the method of Folch et al. (1957). Samples from these total lipid extracts were analyzed by radio-TLC using the solvent system previously described. Selected samples from different time points during the assay were separated by TLC, and the plates were scraped in 1 cm increments to verify that the only regions containing radioactivity were those corresponding to the free and esterified 15-ketosterol. For the remaining samples the regions corresponding to the chromatographic mobility of the free sterols and sterol esters as well as the origin region were scraped from the silica gel TLC plates and were added to Scintisol scintillation cocktail (5.0 ml) and counted in a Packard 1500 scintillation counter.

**Results**

**Experiment 1**

**Incorporation of 5α-cholest-8(14)-en-3β-ol-15-one**

Figure 9 illustrates the incorporation of 5α-cholest-8(14)-en-3β-ol-15-one as a function of time in the K(15) cells when incubated in Ham's F12 medium + 5% NCS containing 15
Figure 9. Incorporation of 15-ketosterol as a function of time in the K(15) cells in Ham's F12NCS5 containing 15 μM $[^3]$H15-ketosterol and 2.2 μM $[^{14}]$Ccholesterol. The data are expressed as mean ± standard deviation for determinations from 3 separate plates. Total 15-ketosterol incorporation [•----•], free15-ketosterol [o----o], and 15-ketosteryl esters [□----□] recovered in the cell.
μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one + 2.2 μM [4-14C]cholesterol. The 15-ketosterol incorporation reached a maximum value of 37.4 ± 0.5 nmoles 15-ketosterol per mg total cellular protein after 12 hours of incubation in the medium containing the radiolabeled 5α-cholest-8(14)-en-3β-ol-15-one. After only 2 hours, 26.2 ± 0.5 nmoles 15-ketosterol per mg protein had been incorporated (70% of the maximum value). The values for each time point are presented in Table 2. In subsequent values presented in this section, "mg total cellular protein" will be noted as simply "mg protein".

Figure 9 also illustrates the relative quantities of free 15-ketosterol and 15-ketosteryl esters as determined by radio-TLC analyses of the lipid extracts obtained from the cell homogenate at each time point. After 2 hours, 22.2 ± 0.2 nmoles free 15-ketosterol/ mg protein were recovered. At the same time period, only 4.0 ± 0.2 nmoles/mg protein (or 15.1% of the total incorporated 15-ketosterol) was recovered in the esterified form. The quantity of free 15-ketosterol remained constant during the 48 hours of the experiment at the level determined for the 2 hour assay. The amount of 15-ketosteryl esters per mg protein increased until 12 hours when the value reached a maximum of 16.8 ± 0.8 nmoles per mg protein or 44.9% of the total incorporated 15-ketosterol. The values for each time point are presented in Table 2.

**Incorporation of Cholesterol**

The newborn calf serum contained 61.1 mg total cholesterol per dl serum and 14.5 mg free cholesterol per dl serum. This translated into 18.8 μM free cholesterol in growth medium containing 5% NCS. The total concentration of free cholesterol in the incubation medium due to the NCS and the addition of [4-14C]cholesterol was 21.0 μM free cholesterol. The calculations used to determine the values presented in the tables and figures
Table 2. Incorporation of 5α-cholesterol-8(14)-en-3β-ol-15-one as a function of time in the K(15) cells\(^{(1)}\).

<table>
<thead>
<tr>
<th>Hours</th>
<th>Incorporated 15-Ketosterol(^{(2)})</th>
<th>Free 15-Ketosterol(^{(3)})</th>
<th>15-Ketosteryl Esters(^{(3)})</th>
<th>% Esters(^{(4)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>26.2 ± 0.5</td>
<td>22.2 ± 0.2</td>
<td>4.0 ± 0.2</td>
<td>15.1%</td>
</tr>
<tr>
<td>4</td>
<td>29.2 ± 1.7</td>
<td>21.6 ± 0.4</td>
<td>7.6 ± 0.4</td>
<td>26.1%</td>
</tr>
<tr>
<td>6</td>
<td>34.2 ± 2.7</td>
<td>22.9 ± 0.5</td>
<td>11.3 ± 0.5</td>
<td>32.9%</td>
</tr>
<tr>
<td>12</td>
<td>37.4 ± 0.5</td>
<td>20.6 ± 0.8</td>
<td>16.8 ± 0.8</td>
<td>44.9%</td>
</tr>
<tr>
<td>24</td>
<td>31.0 ± 2.9</td>
<td>19.7 ± 0.9</td>
<td>11.3 ± 0.9</td>
<td>36.4%</td>
</tr>
<tr>
<td>48</td>
<td>31.4 ± 0.8</td>
<td>21.9 ± 0.6</td>
<td>9.5 ± 0.6</td>
<td>30.1%</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Experiment 1 incubation condition = F12NCS5 containing 15 μM [2,4-\(^{3}\)H]5α-cholesterol-8(14)-en-3β-ol-15-one + 2.2 μM [4-\(^{14}\)C]cholesterol

\(^{(2)}\) nmoles 15-ketosterol incorporated from the growth medium per mg total cellular protein ± S. D. \((N=3)\)

\(^{(3)}\) nmoles free or esterified 15-ketosterol recovered in the cell homogenate per mg total cellular protein ± S. D. \((N=3)\)

\(^{(4)}\) Percent 15-ketosteryl esters of the total incorporated 15-ketosterol
were based on a specific activity that included the cholesterol added via the serum supplement.

Figure 10 illustrates the incorporation of cholesterol as a function of time in the K(15) cells incubated in F12NCS5 containing 15 µM [³H]15-ketosterol + 22 µM [¹⁴C]cholesterol. While the incorporation of cholesterol reached a plateau after 12 hours at 35.8 ± 0.0 nmoles cholesterol per mg protein, the incorporation continued to increase throughout the 48 hours of the experiment, reaching a final value of 58.2 ± 2.0 nmoles cholesterol per mg protein. The values for each time point are presented in Table 3.

Figure 10 also illustrates the relative quantities of free cholesterol and cholesteryl esters recovered in the cell homogenate from each time point. Unlike the incorporation of the 15-ketosterol, the incorporated cholesterol in the cell homogenate was predominantly composed of free cholesterol. Through 24 hours, greater that 95% of the total cholesterol corresponded to free cholesterol. The values for each time point are presented in Table 3.

Comparison of 15-Ketosterol and Cholesterol Incorporation

Figure 11 illustrates a comparison of the incorporation of 5α-cholest-8(14)-en-3β-ol-15-one and cholesterol as a function of time in the K(15) cells. Although the incorporation of cholesterol was less than the incorporation of the 15-ketosterol during the early part of the experiment (up to 6 hours), the values for the cholesterol and the oxygenated sterol were almost identical at 12 hours. In the later hours (24, 48 hours), the incorporation of the 15-ketosterol did not increase further, whereas the incorporation of the cholesterol continued to increase throughout the 48-hour duration of the experiment.
Figure 10. Incorporation of $[^{14}\text{C}]$cholesterol as a function of time in the K(15) cells in Ham's F12NCS5 containing 15 μM $[^{3}\text{H}]$15-ketosterol and 2.2 μM $[^{14}\text{C}]$cholesterol. The data are expressed as mean ± standard deviation for determinations from 3 separate plates. Total cholesterol [●----●] incorporation; free cholesterol [○----○] and cholesteryl esters [□----□] recovered in the cell.
Table 3. Incorporation of cholesterol as a function of time in the K(15) cells\(^{(1)}\).

<table>
<thead>
<tr>
<th>Hours Esters(^{(4)})</th>
<th>Incorporated Cholesterol(^{(2)})</th>
<th>Free Cholesterol(^{(3)})</th>
<th>Cholesteryl Esters(^{(3)})</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>12.8 ± 0.4</td>
<td>12.7 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.6%</td>
</tr>
<tr>
<td>4</td>
<td>18.5 ± 0.6</td>
<td>18.4 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.6%</td>
</tr>
<tr>
<td>6</td>
<td>24.0 ± 2.3</td>
<td>23.8 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>1.0%</td>
</tr>
<tr>
<td>12</td>
<td>35.8 ± 0.0</td>
<td>35.0 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>2.2%</td>
</tr>
<tr>
<td>24</td>
<td>39.9 ± 4.4</td>
<td>37.9 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>4.9%</td>
</tr>
<tr>
<td>48</td>
<td>58.2 ± 2.0</td>
<td>51.8 ± 0.3</td>
<td>6.4 ± 0.3</td>
<td>11.0%</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Experiment 1 incubation condition = F12NCS5 containing 15 μM [2,4-\(^{3}H\)5α-cholest-8(14)-en-3β-ol-15-one + 2.2 μM [4-\(^{14}C\)]cholesterol

\(^{(2)}\) nmole cholesterol incorporated from the growth medium per mg total cellular protein ± S. D. (N=3)

\(^{(3)}\) Nmoles free or esterified cholesterol recovered in the cell homogenate per mg total cellular protein ± S. D. (N=3)

4) Percent cholesteryl esters of the total incorporated cholesterol
Figure 11. Incorporation of 15-ketosterol and cholesterol as a function of time in the K(15) cells in Ham's F12NCS5 containing 15 μM [3H]15-ketosterol and 2.2 μM [14C]cholesterol. The data are expressed as mean ± standard deviation for determinations from 3 separate plates. Total 15-ketosterol [•----•] incorporation; total cholesterol [○-----○] incorporation.
Experiment 2

Incorporation of 5α-cholest-8(14)-en-3β-ol-15-one

Figure 12 illustrates the incorporation of 5α-cholest-8(14)-en-3β-ol-15-one as a function of time in the K(15) cells incubated in Ham's F12 medium + 5% NCS containing 40 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one. The incorporation peaked with a value of 253.0 ± 10.8 nmoles 15-ketosterol per mg protein after 12 hours of incubation in the medium containing the radiolabeled 15-ketosterol. After just 2 hours, 116.7 ± 7.5 nmoles 15-ketosterol per mg protein had been incorporated (46.1% of the 12 hour value). The values for each time point are presented in Table 4.

Figure 12 also illustrates the relative quantities of free 15-ketosterol and 15-ketosteryl esters recovered in the cell homogenate from each time point. After 2 hours, 75.4 ± 1.6 nmoles free 15-ketosterol/ mg protein were recovered. At that same time, 41.3 ± 1.6 nmoles/ mg protein (or 35.4% of the total 15-ketosterol) was recovered in the esterified form. The quantity of free 15-ketosterol remained constant during the 48 hours of the experiment at the level determined for the 2 hour assay. The amount of 15-ketosteryl esters/mg protein, however, continued to increase until 12 hours when the value reached 181.7 ± 1.8 nmoles per mg protein or 71.8% of the total incorporated 15-ketosterol. The values for the incorporation of the 15-ketosterol and the amount of 15-ketosteryl esters recovered at 24 hours were only slightly higher than the values determined for the 12 hour time point. It is clear from the figure, however, that both the incorporation and relative esterification of the 15-ketosterol were reaching plateaus by 12 hours. The values for each time point are presented in Table 4.
Figure 12. Incorporation of 15-ketosterol as a function of time in the K(15) cells in Ham's F12NCS5 containing 40 μM [3H]15-ketosterol. The data are expressed as mean ± standard deviation for determinations from 3 separate plates. Total 15-ketosterol [●-----●] incorporation; free 15-ketosterol [○-----○] and 15-ketosteryl esters [□-----□] recovered in the cell.
Table 4. Incorporation of 5α-cholestan-8(14)-en-3β-ol-15-one as a function of time in the K(15) cells(1).

<table>
<thead>
<tr>
<th>Hours</th>
<th>Incorporated 15-Ketosterol(2)</th>
<th>Free 15-Ketosterol(3)</th>
<th>15-Ketosteryl Esters(3)</th>
<th>% Esters(4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>116.7 ± 7.5</td>
<td>75.4 ± 1.6</td>
<td>41.3 ± 1.6</td>
<td>35.4%</td>
</tr>
<tr>
<td>4</td>
<td>155.3 ± 2.6</td>
<td>71.3 ± 1.6</td>
<td>84.0 ± 1.6</td>
<td>54.1%</td>
</tr>
<tr>
<td>6</td>
<td>198.3 ± 11.0</td>
<td>73.4 ± 1.4</td>
<td>124.9 ± 1.4</td>
<td>63.0%</td>
</tr>
<tr>
<td>12</td>
<td>253.0 ± 11.0</td>
<td>71.3 ± 1.8</td>
<td>181.7 ± 1.8</td>
<td>71.8%</td>
</tr>
<tr>
<td>24</td>
<td>259.2 ± 9.1</td>
<td>69.7 ± 4.1</td>
<td>189.5 ± 4.1</td>
<td>73.1%</td>
</tr>
<tr>
<td>48</td>
<td>239.4 ± 20.0</td>
<td>82.6 ± 2.6</td>
<td>156.8 ± 2.6</td>
<td>65.5%</td>
</tr>
</tbody>
</table>

(1) Experiment 2 incubation condition = F12NC5 containing 40 µM [2,4-3H]5α-cholestan-8(14)-en-3β-ol-15-one

(2) nmoles 15-ketosterol incorporated from the growth medium per mg total cellular protein ± S. D. (N=3)

(3) nmoles free or esterified 15-ketosterol recovered in the cell homogenate per mg total cellular protein ± S. D. (N=3)

(4) Percent 15-ketosteryl esters of the total incorporated 15-ketosterol
Comparison of the incorporation of 5α-cholest-8(14)-en-3β-ol-15-one in K(15) cells incubated in F12NCS5 containing either 15 μM or 40 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one

Figure 13 illustrates a comparison of the incorporation of 5α-cholest-8(14)-en-3β-ol-15-one in K(15) cells incubated in F12NCS5 containing either 15 μM or 40 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one. The upper panel illustrates the incorporation in the K(15) cells incubated in medium containing 15 μM 15-ketosterol; the bottom panel illustrates the incorporation in the K(15) cells incubated in medium containing 40 μM 15-ketosterol. After 12 hours, when incubated in 15 μM 5α-cholest-8(14)-en-3β-ol-15-one, the K(15) cells had incorporated 37.4 ± 0.5 nmoles 15-ketosterol per mg protein. When incubated in 40 μM 5α-cholest-8(14)-en-3β-ol-15-one, the cells incorporated 253.0 ± 11.0 nmoles 15-ketosterol per mg protein. This represents an almost seven-fold increase in 5α-cholest-8(14)-en-3β-ol-15-one incorporation after an increase of less than three-fold in the concentration of the 15-ketosterol in the medium.

The amounts of free and esterified 15-ketosterol contained in the cell homogenates were also determined. In both cases, the level of 15-ketosterol found in the cell homogenate after 2 hours remained constant for the 48-hour duration of the experiment. When incubated at a level of 15 μM 15-ketosterol, 22.2 ± 0.2 nmoles 15-ketosterol per mg protein was recovered in the cell homogenate after 2 hours. When incubated in 40 μM 15-ketosterol, 75.4 ± 1.6 nmoles free 15-ketosterol per mg protein was recovered in the cell homogenate after 2 hours. This represents an increase in the free 15-ketosterol recovered in the cell homogenate of about three-fold, paralleling the increase of the concentration of the 15-ketosterol in the medium by about three-fold.
Figure 13. Incorporation of 15-ketosterol as a function of time in the K(15) cells in Ham's F12NCS5 containing either (A) 15 μM or (B) 40 μM [3H] 15-ketosterol. The data are expressed as the mean ± standard deviation for determinations from 3 separate plates. Total 15-ketosterol [●----● ] incorporation; free 15-ketosterol [○----○] and 15-ketosteryl esters [□----□] recovered in the cell.
The greatest difference between the two experiments appears to be in the relative amount of 15-ketosteryl esters recovered in the cell homogenates. When incubated at a level of 15 µM 15-ketosterol, the 15-ketosteryl esters recovered in the homogenate after 12 hours was 16.8 ± 0.8 nmoles per mg protein; when incubated in 40 µM 15-ketosterol, the 15-ketosteryl esters recovered was 181.7 ± 1.8 nmoles/ mg protein. This represents an increase in the 15-ketosteryl esters recovered in the cell homogenate of almost 11-fold. The intriguing observation is that while in both cases the level of free 15-ketosterol remained constant after 2 hours, in the 15 µM case the amount of 15-ketosteryl esters remained well below that of the free 15-ketosterol, but in the 40 µM case the amount of 15-ketosteryl esters surpassed that of the free 15-ketosterol at 4 hours. The amount of 15-ketosteryl esters continued to increase through 12 hours (and to a lesser extent 24 hours) until there were over twice as much 15-ketosteryl esters recovered in the cell homogenate as free 15-ketosterol.
Incorporation of $5\alpha$-cholest-8(14)-en-5$\beta$-ol-15-one and cholesterol in the CHO K-1 cells

Introduction

In this series of experiments, the incorporation of $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one and cholesterol in the CHO K-1 cells was investigated. The purpose of these experiments was to monitor the amount of $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one and cholesterol incorporation in the CHO K-1 cells as a function of time. Further, the relative esterification of the incorporated sterols within the cell was determined by radio-TLC analysis.

CHO K-1 cells ($1.0 \times 10^6$ cells/plate) were plated into 150 mm culture plates containing Ham's F12 growth medium + 5% NCS. After a 48-hour growth period at 37 °C, the culture plates were changed to the experimental medium described below:

Experiment 3: Ham's F12 medium + 5% NCS containing 5 $\mu$M [2,4-$^3$H]$5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one (1.8 $\mu$Ci $^3$H/plate) or

Experiment 4: Ham's F12 medium + 5% NCS containing 1.5 $\mu$M[2,4-$^{14}$C] cholesterol (2.0 $\mu$Ci $^{14}$C/plate).

After an additional 48-hour incubation period, the medium was aspirated and the cells were rinsed twice with 10 ml PBS and 5 ml of 0.05% trypsin in Hank's balanced salt solution were added. After incubating at 37 °C for 7.5 minutes, 5.0 ml soybean trypsin inhibitor (1.5 mg/ml) were added to the plates. The cells were collected and the plates were rinsed twice with PBS (2.5 ml). After pelleting the cells at 1500 rpm for 5 minutes, the cells were resuspended in 2.0 ml water to promote cell swelling. The cells were homogenized by aspirating four times through a 23 gauge needle fitted to a 3 ml syringe. Aliquots (100 $\mu$l) were taken of the medium, the cell homogenate, and the trypsin super-
natant. These aliquots were added to Scintisol scintillation cocktail (5.0 ml) and counted in a Packard 1500 scintillation counter for 3 minutes. Protein determinations were also performed on the cell homogenates following the procedure of Lowry et al. (1951). Three plates per time point were individually harvested at 2, 4, 6, 12, 24, and 48 hours.

Aliquots (1.0 ml) of the cell homogenates were extracted following the method of Folch et al. (1951). Samples from these total lipid extracts were analyzed by radio-TLC using the solvent systems described previously. The regions corresponding to the chromatographic mobility of the free sterols and sterol esters were scraped from the silica gel TLC plates and were added to Scintisol scintillation cocktail (5.0 ml) and counted in a Packard 1500 scintillation counter.

Results

Experiment 3

Incorporation of 5α-cholest-8(14)-en-3β-ol-15-one

Figure 14 illustrates the incorporation of 5α-cholest-8(14)-en-3β-ol-15-one as a function of time in the CHO K-1 cells when incubated in Ham's F12 medium + 5% NCS containing 5 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one. Incorporation reached a value of 12.3 ± 0.5 nmoles 15-ketosterol per mg total cellular protein after 6 hours of incubation in the medium containing the radiolabeled 15-ketosterol. After 12 hours, the value was slightly higher, 12.9 ± 0.5 nmoles 15-ketosterol per mg total cellular protein, but the figure clearly shows that the incorporation of the 15-ketosterol had reached a plateau by 6 hours. After just 2 hours, 9.4 ± 0.2 nmoles 15-ketosterol per mg total cellular protein had been incorporated (73% of the maximum value). The values for each time
point are presented in Table 5. In subsequent values presented in this section, "mg total cellular protein" will be noted as simply "mg protein".

Figure 14 also illustrates the relative quantities of free 15-ketosterol and 15-ketosteryl esters recovered in the cell homogenate from each time point. After 2 hours, 6.3 ± 0.0 nmoles free 15-ketosterol per mg protein were recovered in the cell homogenate; 3.1 ± 0.0 nmoles per mg protein was recovered in the esterified form. There was twice as much free 15-ketosterol as 15-ketosteryl esters in the cell homogenate at this time point. The quantity of free 15-ketosterol continued to increase only until 4 hours, at which point the amount of free 15-ketosterol reached a plateau of 7.4 ± 0.2 nmoles per mg protein and remained approximately constant during the remainder of 48 hours of the experiment. The amount of 15-ketosteryl esters per mg protein increased until 4-6 hours when the value reached a value of 4.1 ± 0.2 nmoles per mg protein. This value also remained approximately constant during the remainder of 48 hours of the experiment. The values for each time point are presented in Table 4.

**Experiment 4**

Incorporation of Cholesterol

The incorporation of cholesterol was determined in cell cultures of CHO K-1 cells incubated in F12NCS5 + 1.5 μM [4-14C]cholesterol. As has been presented previously, the newborn calf serum contained 14.5 mg free cholesterol per dl serum. This translated into 18.8 μM free cholesterol when the growth medium was supplemented with 5% NCS. The total concentration of free cholesterol in the incubation medium due to the NCS supplement and the addition of [4-14C]cholesterol was 20.3 μM free cholesterol. The calculations used to determine the values presented in the tables and figures were based on a specific activity that included the free cholesterol added via the serum supplement.
Table 5. Incorporation of 5α-cholest-8(14)-en-3β-ol-15-one as a function of time in the CHO K-1 cells(1).

<table>
<thead>
<tr>
<th>Hours</th>
<th>Incorporated 15-Ketosterol(2)</th>
<th>Free 15-Ketosterol(3)</th>
<th>15-Ketosteryl Esters(3)</th>
<th>% Esters(4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>9.4 ± 0.2</td>
<td>6.3 ± 0.0</td>
<td>3.1 ± 0.0</td>
<td>32.9%</td>
</tr>
<tr>
<td>4</td>
<td>11.5 ± 0.6</td>
<td>7.4 ± 0.2</td>
<td>4.1 ± 0.2</td>
<td>35.8%</td>
</tr>
<tr>
<td>6</td>
<td>12.3 ± 0.5</td>
<td>7.7 ± 0.3</td>
<td>4.6 ± 0.3</td>
<td>37.6%</td>
</tr>
<tr>
<td>12</td>
<td>12.9 ± 0.5</td>
<td>7.9 ± 0.3</td>
<td>5.0 ± 0.3</td>
<td>39.1%</td>
</tr>
<tr>
<td>24</td>
<td>12.6 ± 0.8</td>
<td>7.7 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>39.2%</td>
</tr>
<tr>
<td>48</td>
<td>9.2 ± 0.3</td>
<td>6.0 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>34.8%</td>
</tr>
</tbody>
</table>

(1) Experiment 3 incubation condition = F12NCS5 containing 5 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one

(2) nmole 15-ketosterol incorporated from the growth medium per mg total cellular protein ± S. D. (N=3)

(3) nmole free or esterified 15-ketosterol recovered in the cell homogenate per mg total cellular protein ± S. D. (N=3)

(4) Percent 15-ketosteryl esters of the total incorporated 15-ketosterol
Figure 15 illustrates the incorporation of cholesterol as a function of time in the CHO K-1 cells. The incorporation of cholesterol peaked by 24 hours at 55.9 ± 3.0 nmoles cholesterol per mg protein. The values for each time point are presented in Table 6.

Figure 15 also illustrates the relative quantities of free cholesterol and cholesteryl esters recovered in the cell homogenate from each time point. The total cholesterol of the cell homogenate was predominantly composed of free cholesterol. Through the first 24 hours of the experiment, at least 83% of the incorporated cholesterol was identified as free cholesterol. The values for each time point are presented in Table 6.
Figure 15. Incorporation of cholesterol as a function of time in the CHO K-1 cells in Ham's F12NCS5 containing 1.5 μM [\textsuperscript{14}C]cholesterol. The data are expressed as mean ± standard deviation for determinations from 3 separate plates. Total cholesterol [●---●] incorporation; free cholesterol [○---○] and cholesteryl esters [□---□] recovered in the cell.
Table 6. Incorporation of cholesterol as a function of time in the CHO K-1 cells\(^{(1)}\).

<table>
<thead>
<tr>
<th>Hours</th>
<th>Incorporated Cholesterol(^{(2)})</th>
<th>Free Cholesterol(^{(3)})</th>
<th>Cholesteryl Esters(^{(3)})</th>
<th>% Esters(^{(4)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8.2 ± 0.7</td>
<td>7.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>7.1%</td>
</tr>
<tr>
<td>4</td>
<td>15.4 ± 0.8</td>
<td>13.6 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>11.4%</td>
</tr>
<tr>
<td>6</td>
<td>23.3 ± 2.4</td>
<td>19.9 ± 0.1</td>
<td>3.3 ± 0.1</td>
<td>14.2%</td>
</tr>
<tr>
<td>12</td>
<td>40.0 ± 2.6</td>
<td>33.0 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td>17.4%</td>
</tr>
<tr>
<td>24</td>
<td>55.9 ± 3.0</td>
<td>46.2 ± 0.5</td>
<td>9.7 ± 0.5</td>
<td>17.3%</td>
</tr>
<tr>
<td>48</td>
<td>54.0 ± 1.2</td>
<td>35.9 ± 1.0</td>
<td>18.1 ± 1.0</td>
<td>33.6%</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Experiment 4 incubation condition = F12NCS5 containing 1.5 µM [\(^{4-14}\)C]cholesterol

\(^{(2)}\) nmoles cholesterol incorporated from the growth medium per mg total cellular protein ± S. D. (N=3)

\(^{(3)}\) nmoles free or esterified cholesterol recovered in the cell homogenate per mg total cellular protein ± S. D. (N=3)

\(^{(4)}\) Percent cholesteryl esters of the total incorporated cholesterol
Comparison of the incorporation of 5α-cholest-8(14)-en-3β-ol-15-one and cholesterol in CHO K-1 and K(15) cells

Figure 16 compares the incorporation of 5α-cholest-8(14)-en-3β-ol-15-one and cholesterol in the CHO K-1 cells when incubated in F12NCS5 containing either 5 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one or 1.5 μM [4-14C]cholesterol and the K(15) cells when incubated in F12NCS5 containing 15 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one and 2.2 μM [4-14C]cholesterol. The top panel refers to the incorporation in the CHO K-1 cells; the bottom panel refers to the incorporation in the K(15) cells.

In the CHO K-1 cells, the incorporation of cholesterol (8.2 ± 0.7 nmoles per mg protein, Table 6) was only slightly less than the incorporation of the 15-ketosterol (9.4 ± 0.2 nmoles per mg protein, Table 5) after 2 hours (see Figure 16, top panel). By 24 hours, however, over three times as much cholesterol (40.0 ± 2.6 nmoles per mg protein, Table 6) had been incorporated as the 15-ketosterol (12.9 ± 0.5 nmoles per mg protein, Table 5). Further, the incorporation of the 15-ketosterol reached a plateau after just 2-4 hours; cholesterol incorporation reached a maximum at 24 hours.

In the K(15) cells, incorporation of the 15-ketosterol was greater than the incorporation of cholesterol in the early hours (through 6 hours), but by 12 hours, the incorporation of cholesterol and the oxygenated sterol was almost identical (see Figure 16, bottom panel). At the 24- and 48-hour assays, cholesterol incorporation was greater than the incorporation of the 15-ketosterol.

The maximum 15-ketosterol that was incorporated in the CHO K-1 cells was 12.9 ± 0.5 nmoles per mg protein (Table 5) compared to 37.4 ± 0.5 nmoles per mg protein in the K(15) cells (Table 2). The almost three-fold increase in the incorporation of the
Figure 16. Incorporation of 15-ketosterol and cholesterol as a function of time in the (A) CHO K-1 cells in F12NCS5 containing 5 μM [³H]15-ketosterol or 1.5 μM [¹⁴C]cholesterol and (B) K(15) cells in Ham's F12NCS5 containing 15 μM [³H]15-ketosterol and 2.2 μM [¹⁴C]cholesterol. The data are expressed as mean ± standard deviation for determinations from 3 separate plates. Total 15-ketosterol [♦—♦] incorporation; total cholesterol [●—●] incorporation.
15-ketosterol in the K(15) cells paralleled the three-fold increase in the concentration of 5α-cholest-8(14)-en-3β-ol-15-one in the growth medium.

Although the concentrations of free cholesterol in the incubation medium were very similar in both cases (20.3 μM in the case of the CHO K-1 cells and 21.0 μM in the K(15) cells), the incorporation of cholesterol varied between the wild type and the 15-ketosterol-adapted cells. The wild type cells reached a maximum incorporation of cholesterol after 24 hours with 55.9 ± 3.0 nmoles cholesterol per mg protein (Table 6). After 24 hours, the K(15) cells incorporated 39.9 ± 4.4 nmoles cholesterol per mg protein (Table 3). The incorporation of cholesterol continued to increase in the K(15) cells for the 48-hour duration of the experiment, reaching 58.2 ± 2.0 nmoles cholesterol per mg protein, a value very close to that of the CHO K-1 cells at 48 hours, 54.0 ± 1.2 nmoles cholesterol per mg protein.

In experiment 1 (K(15) cells, 15 μM 15-ketosterol) and experiment 3 (CHO K-1 cells, 5 μM 15-ketosterol), the relative esterification of the incorporated 15-ketosterol was very similar in the two cells types (see Figures 9 and 14; Tables 2 and 5). Although the absolute amounts of incorporated 15-ketosterol differed by a factor of approximately three, as described above, the percentages of free and esterified 15-ketosterol were similar. After a 2 hour incubation in the respective [3H]15-ketosterol-containing media, most of the 15-ketosterol was found in the free form in the homogenate: 67.1% free 15-ketosterol in the wild type cells and 84.9% in the K(15) cells. The percent esterification of the incorporated 15-ketosterol reached a maximum in both cell types by 12 hours. Of the incorporated 15-ketosterol in the wild type cells, 39.1% was recovered in the esterified form at 12 hours. Of the incorporated 15-keto sterol in the 15-ketosterol-adapted cells, 44.9% was recovered in the esterified form at 12 hours.
In the K(15) cell, however, the relative esterification of the incorporated 15-ketosterol varied with the concentration of exogenous 15-ketosterol. As was shown in Figure 10 and Table 4, after a 2-hour incubation in growth medium containing 40 μM 15-ketosterol, 35.4% of the incorporated 15-ketosterol was recovered in the esterified form. At the same time point, 15.1% of the 15-ketosterol was recovered in the esterified form upon incubation in 15 μM 15-ketosterol. This two-fold difference in the percent esterification was maintained throughout entire period of the experiments. At 24 hours, 35.4% of the 15-ketosterol was recovered in the esterified form in the cells incubated in an exogenous concentration of 15 μM 15-ketosterol, and 73.1% of the 15-ketosterol was recovered in the esterified form upon incubation in an exogenous concentration of 40 μM 15-ketosterol. Further, it should be noted that no conversion of the 15-ketosterol to cholesterol, cholesteryl esters, or polar metabolites was observed.
B. Distribution of $^3$H and $^{14}$C in K(15) and CHO K-1 Cells

After Incubation With

$[2,4-^3$H]5α-Cholest-8(14)-en-3β-ol-15-one and [4-$^{14}$Cl]Cholesterol
Distribution of $^3$H and $^{14}$C in K(15) cells after incubation with $[2,4-^3$H$]5\alpha$-cholest-8(14)-en-3β-ol-15-one and $[4-^{14}$C$]cholesterol$

**Experiment 1**

15-Ketosterol-adapted cells ($1.0 \times 10^6$) were inoculated into 150 x 15 mm culture plates containing Ham's F12 medium + 5% NCS (F12NCS5) containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one (25 ml). After a 48-hour growth period at 37 °C, six culture plates were changed to the experimental medium described below:

Ham's F12 medium + 5% NCS containing 40 μM $[2,4-^3$H$]5\alpha$-cholest-8(14)-en-3β-ol-15-one (3.5 μCi $[^3$H/sample]), 0.6 μM $[4-^{14}$C$]cholesterol$ (1.7 μCi $[^{14}$C/sample]).

After an additional growth period of 48 hours, the cells were harvested and processed as described in the Materials and Methods chapter. The cell samples were centrifuged and separated into four fractions: the floating lipid layer, the upper and lower intermediate zones and the pellet fraction. Each of the fractions were extracted for total lipids following the procedure of Folch et al. Less than 0.1% of the tritium or $^{14}$C was recovered in the aqueous phases derived from the extraction of the floating lipid layer and the upper and lower intermediate zones. From the pellet fraction, less than 1% of the tritium and less than 0.5% of the $^{14}$C was recovered in the aqueous phase. The organic phases containing the total lipids from each fraction were subsequently analyzed by TLC, as described in the Materials and Methods chapter.
**Distribution of $^3$H**

The overall distribution of tritium derived from [2,4-$^3$H]5α-cholest-8(14)-en-3β-ol-15-one in the centrifuged fractions was as follows. The floating lipid layer contained 41.5 ± 1.8% of the total tritium recovered in all of the fractions (see Table 7). The pellet fraction contained 35.4 ± 2.1% of the total tritium recovered in all of the fractions. The upper and lower intermediate zones contained 6.5 ± 0.6% and 16.5 ± 0.3%, respectively, of the total tritium recovered in all of the fractions.

Figure 17 presents the chromatographic distribution of tritium recovered from each of the centrifuged fractions. As seen in Figure 17, each fraction contained a peak of $^3$H that migrated to a region corresponding to an Rf of 0.31-0.40. This region corresponded to the mobility of an authentic sample of 15-ketosteryl palmitate. Table 8 presents the percentage distribution of tritium in the centrifuged fractions. The radioactivity that co-migrated with the 15-ketosteryl ester standard in the floating lipid layer represented 96.6 ± 0.4% of the radioactivity recovered in this fraction. About 90% of the radioactivity recovered in the upper and lower intermediate zones also co-migrated with the 15-ketosteryl ester standard. In the pellet fraction, 36.9 ± 2.9% of the tritium migrated to the same region corresponding to an Rf of 0.31-0.40.

The greatest amount of tritium that co-migrated with the free 15-ketosterol standard (Rf = 0.06-0.11) was observed in the pellet fraction and represented 61.0 ± 3.2% of the tritium recovered in this fraction. The tritium recovered in the corresponding region in the other three fractions comprised 11% or less of the total tritium recovered in each fraction.
Table 7. Summary of the distribution of $^3$H from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^3$H in fraction (of total $^3$H contained in all fractions)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S. D. (N=3)</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>41.5 ± 1.8%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>6.5 ± 0.6%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>16.5 ± 0.3%</td>
</tr>
<tr>
<td>Pellet</td>
<td>35.4 ± 2.1%</td>
</tr>
</tbody>
</table>

(1) Experiment 1 incubation condition: 40 µM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.6 µM [4-$^{14}$C]cholesterol in F12 NCS5.
Figure 17. Chromatographic distribution of $^3$H in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation conditions (Experiment 1) for the cells and TLC conditions are described in the text.
Table 8. Summary of chromatographic distribution of $^3$H from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
<th>Origin</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>1.8 ± 0.1%</td>
<td>96.6 ± 0.4%</td>
<td>&lt;0.1%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>2.9 ± 0.6%</td>
<td>94.2 ± 0.3%</td>
<td>&lt;0.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>10.8 ± 0.8%</td>
<td>87.4 ± 0.4%</td>
<td>&lt;0.1%</td>
<td>1.8%</td>
</tr>
<tr>
<td>Pellet</td>
<td>61.0 ± 3.2%</td>
<td>36.9 ± 2.9%</td>
<td>0.7 ± 0.1%</td>
<td>1.4%</td>
</tr>
</tbody>
</table>

Mean ± S. D. (N=3)

(1) Experiment 1 incubation condition: 40 μM [2,4-$^3$H]5α-cholestan-8(15)-en-3β-ol-15-one, 0.6 μM [4-14C]cholesterol in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f = 0.06$-0.11 and had the same chromatographic mobility as an authentic sample of the free 15-ketosterol.

(b) Region B corresponds to a scraped region of $R_f = 0.31$-0.40 and had the same chromatographic mobility as an authentic sample of 15-ketosteryl palmitate.
Less than 1% of the total tritium recovered in each fraction was recovered at the origin and less than 3% of the total tritium recovered in each fraction was recovered in regions other than the two regions corresponding to the free and esterified 15-ketosterol. Figure 18 illustrates the distribution of free 15-ketosterol in the centrifuged fractions. A total of $37.6 \pm 4.3$ nmoles free 15-ketosterol per mg total cellular protein was recovered in the sum of the centrifuged fractions. In subsequent values presented in this section, "mg total cellular protein" will be noted as simply "mg protein". The free 15-ketosterol recovered in the pellet fraction accounted for the greatest proportion (88.9%) of the total free 15-ketosterol. The lower and upper intermediate zones accounted for 7.3% and 0.8% of the total free 15-ketosterol, respectively. The floating lipid layer contained 3.0% of the total free 15-ketosterol.

Figure 19 illustrates the distribution of 15-ketosteryl esters in the centrifuged fractions. A total of $113.3 \pm 5.2$ nmoles 15-ketosteryl esters per mg protein was recovered in the sum of the centrifuged fractions. The 15-ketosteryl esters recovered in the floating lipid layer accounted for the greatest proportion ($61.6 \pm 1.8$ nmoles per mg protein; 54.3%) of the total 15-ketosteryl esters. The upper and lower intermediate zones accounted for 8.2% and 19.7% of the total 15-ketosteryl ester, respectively. The pellet fraction contained 17.7% of the total 15-ketosteryl ester.

A total of $150.9 \pm 9.5$ nmoles total 15-ketosterol (free and esterified) were recovered in all of the centrifuged fractions. The distribution of free and esterified 15-ketosterol indicated that the greatest concentration of the 15-ketosterol was in the esterified form in the floating lipid layer (Figure 20). The 15-ketosteryl ester recovered in the floating lipid layer represented 40.8% of the total (free and esterified) 15-ketosterol recovered from all of the centrifuged fractions. The next higher concentration of 15-ketosterol was in the pellet fraction in the free form; the free 15-ketosterol recovered in this fraction accounted
Figure 18. Distribution of free 15-ketosterol in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-³H]5α-cholest-8(14)-en-3β-ol-15-one and 0.6 μM [4-¹⁴C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 19. Distribution of 15-ketosteryl esters in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one and 0.6 μM [4-14C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 20. Distribution of free 15-ketosterol (striped bars) and 15-ketosteryl esters (solid bars) in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-^3^H]5α-cholest-8(14)-en-3β-ol-15-one and 0.6 μM [4-^14^C]cholesterol for 48 hours. The bars represent the mean value (N=3); the white bars represent the standard deviation.
for 22.1% of the total 15-ketosterol recovered in all of the fractions. The 15-ketosteryl ester recovered in the lower intermediate zone and the pellet accounted for 14.8% and 13.3%, respectively, of the total free and esterified 15-ketosterol recovered in all of the centrifuged fractions.

**Distribution of \(^{14}\text{C}\)**

The overall distribution of \(^{14}\text{C}\) in the centrifuged fractions was as follows. The floating lipid layer contained 9.8 ± 1.2% of the total \(^{14}\text{C}\) recovered in all of the fractions (see Table 9). The upper and lower intermediate zones contained 1.8 ± 0.3% and 10.0 ± 1.1%, respectively, of the total \(^{14}\text{C}\) recovered in all of the fractions. The pellet fraction contained 78.4 ± 2.7% of the total \(^{14}\text{C}\) recovered in all of the fractions.

Figure 21 presents the chromatographic distribution of \(^{14}\text{C}\) recovered in each of the centrifuged fractions. Each of the fractions contained a peak of \(^{14}\text{C}\) that migrated to a region corresponding to an \(R_f\) of 0.14-0.20. This region corresponded to the mobility of an authentic sample of free cholesterol. The pellet fraction contained the greatest amount of \(^{14}\text{C}\) that co-migrated with the free cholesterol standard; the radioactivity contained in this region represented 92.4 ± 0.9% of the \(^{14}\text{C}\) recovered in the fraction (see Table 10). As the pellet fraction contained almost 80% of the total \(^{14}\text{C}\) recovered in all of the centrifuged fractions, this indicates that most of the [\(^{14}\text{C}\)]cholesterol incorporated into the cells from the growth medium remained in the free form and was recovered in the pellet fraction. Although there was much less radioactivity recovered in the intermediate zones (<12% of the total), 63.0 ± 1.2% of the radioactivity recovered in the lower intermediate zone co-migrated with the free cholesterol standard. In the floating lipid layer, only 12.8 ± 0.3% of the \(^{14}\text{C}\) migrated to the region corresponding to the migration of free cholesterol.
Table 9. Summary of the distribution of $^{14}$C from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^{14}$C in fraction (of total $^{14}$C contained in all fractions)</th>
<th>Mean ± S. D. (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td></td>
<td>9.8 ± 1.2%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td></td>
<td>1.8 ± 0.3%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td></td>
<td>10.0 ± 1.1%</td>
</tr>
<tr>
<td>Pellet</td>
<td></td>
<td>78.4 ± 2.7%</td>
</tr>
</tbody>
</table>

(1) Experiment 1 incubation condition: 40 µM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.6 µM [4-$^{14}$C]cholesterol in F12NCS5.
Figure 21. Chromatographic distribution of $^{14}$C in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation condition (Experiment 1) for the cells and TLC conditions are described in the text.
Table 10. Summary of chromatographic distribution of $^{14}$C from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
<th>Origin</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>12.8 ± 0.3%</td>
<td>81.4 ± 1.4%</td>
<td>0.4 ± 0.1%</td>
<td>5.3%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>18.9 ± 5.0%</td>
<td>73.6 ± 5.3%</td>
<td>1.1 ± 1.9%</td>
<td>6.4%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>63.0 ± 1.2%</td>
<td>31.2 ± 1.7%</td>
<td>0.4 ± 0.3%</td>
<td>5.4%</td>
</tr>
<tr>
<td>Pellet</td>
<td>92.4 ± 0.9%</td>
<td>3.6 ± 0.1%</td>
<td>0.9 ± 0.3%</td>
<td>3.1%</td>
</tr>
</tbody>
</table>

Mean ± S. D. (N=3)

(1) Experiment 1 incubation condition: 40 µM [2,4-3H]5α-cholest-8(15)-en-3β-ol-15-one, 0.6 µM [4-$^{14}$C]cholesterol in F12NCSS.

(a) Region A corresponds to a scraped region of $R_f$ = 0.14-0.20 and had the same chromatographic mobility as an authentic sample of free cholesterol.

(b) Region B corresponds to a scraped region of $R_f$ = 0.69-0.74 and had the same chromatographic mobility as an authentic sample of cholesteryl oleate.
As seen in Figure 21, the floating lipid layer and the upper and lower intermediate zones each contained a peak of $^{14}$C that migrated to a region corresponding to an Rf of 0.69-0.74. This region corresponded to the mobility of an authentic sample of cholesteryl oleate. The radioactivity that co-migrated with the cholesteryl ester standard in the floating lipid layer represented 81.4 ± 1.4% of the radioactivity recovered in this fraction. In the pellet fraction, only 3.6 ± 0.1% of the $^{14}$C co-migrated with the cholesteryl ester standard (Table 10).

No more than 1.1 ± 1.9% of the total $^{14}$C recovered in each fraction was recovered at the origin of the corresponding TLC plate, and no more than 6.4% of the total $^{14}$C recovered in each fraction was recovered in regions other than the two regions corresponding to the free and esterified cholesterol.

Figure 22 illustrates the distribution of free cholesterol in the centrifuged fractions. A total of 15.3 ± 1.8 nmoles free cholesterol per mg protein was recovered in the sum of the centrifuged fractions. The free cholesterol recovered in the pellet fraction (13.8 ± 1.6 nmoles/mg protein) accounted for the greatest proportion (90.4%) of the total free cholesterol. The floating lipid layer contained only 1.5% of the total free cholesterol.

Figure 23 illustrates the distribution of cholesteryl esters in the centrifuged fractions. A total of only 2.90 ± 0.08 nmoles cholesteryl esters per mg protein was recovered in the sum of the centrifuged fractions. The cholesteryl esters recovered in the floating lipid layer (1.48 ± 0.02 nmoles cholesteryl ester per mg protein) accounted for the greatest proportion (51.0%) of the total cholesteryl esters. The lower intermediate zone accounted for 20.4% of the total cholesteryl ester and the pellet fraction contained 20.2% of the cholesteryl ester.
Figure 22. Distribution of free cholesterol in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-\textsuperscript{3}H]5α-cholest- 8(14)-en-3β-ol-15-one and 0.6 μM [4-\textsuperscript{14}C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 23. Distribution of cholesteryl esters in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-³H]5α-cholesterol-8(14)-en-3β-ol-15-one and 0.6 μM [4-¹⁴C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
A total of $18.2 \pm 1.8$ nmoles total cholesterol (free and esterified) per mg protein were recovered in all of the centrifuged fractions (see Figure 24). The distribution of free and esterified cholesterol indicated that the greatest concentration of cholesterol was in the free form in the pellet. The cholesterol recovered in this fraction represented 76.0% of the total (free and esterified) cholesterol recovered from all of the centrifuged fractions. The cholesteryl ester recovered in the floating lipid layer accounted for only 8.1% of the total cholesterol (free and esterified) recovered in all fractions. The remaining 15.9% of the total cholesterol was distributed in the free and esterified forms in the remaining centrifuged fractions.

**Distribution of Protein**

Aliquots from each of the centrifuged fractions as well as the cell homogenate before centrifugation, were assayed for protein following the procedure of Lowry et al. Triplicate aliquots of each of the three sample were assayed; the values were averaged and the standard deviation was calculated. It was determined from an aliquot of the cell homogenate that the total sample contained $3.80 \pm 0.21$ mg protein. The average recovery of protein was determined by summing the protein values obtained from aliquots assayed from each of the fractions. The average recovery of protein was calculated to be $86.0 \pm 7.1\%$. The percent of protein in each fraction was calculated based on the summed value of the total recovered protein. The greatest amount of protein (57.7%) was recovered in the pellet fraction. The floating lipid layer contained 7.7% of the recovered protein. The upper and lower intermediate zones contained 19.3% and 15.3% of the recovered protein, respectively.
Figure 24. Overall distribution of free cholesterol (striped bars) and cholesteryl esters (solid bars) in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-^3^H]5α-cholest-8(14)-en-3β-ol-15-one and 0.6 μM [4-^14^C]cholesterol for 48 hours. The bars represent the mean value (N=3); the white bars represent the standard deviation.
Distribution of $^3$H and $^{14}$C in K(15) cells after incubation with [2,4-$^3$H]5α-cholest-8(14)-en-3β-ol-15-one and [4-$^{14}$C]oleate

Experiment 2

15-Ketosterol-adapted cells (1.0 x 10^6) were inoculated into 150 x 15 mm culture plates containing Ham's F12 medium + 5% NCS (F12NCS5) containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one (25 ml). After a 48-hour growth period at 37 °C, six culture plates were changed to the experimental medium described below:

Ham's F12 medium + 5% NCS containing 40 μM [2,4-$^3$H]5α-cholest-8(14)-en-3β-ol-15-one (3.5 μCi $[^3]$H/sample), 0.33 μM [1-$^{14}$C]oleate (0.90 μCi $[^{14}$C]/sample).

After an additional growth period of 48 hours, the cells were harvested and processed as described in the Materials and Methods chapter. The cell samples were centrifuged and separated into four fractions: the floating lipid layer, the upper and lower intermediate zones and the pellet fraction. Each of the fractions were extracted for total lipids following the procedure of Folch et al. Less than 0.1% of the tritium or $^{14}$C was recovered in the aqueous phases derived from the extraction of the floating lipid layer and the upper and lower intermediate zones. From the pellet fraction, less than 1% of the tritium and 3.5% of the $^{14}$C was recovered in the aqueous phase. The organic phases containing the total lipids from each fraction were subsequently analyzed by TLC, as described in the Materials and Methods chapter.
Distribution of $^3$H

A summary of the overall distribution of tritium in the centrifuged fractions is presented in Table 11. The floating lipid layer contained 45.6 ± 1.9 % of the total tritium recovered in all of the fractions. The pellet fraction contained 36.4 ± 0.6% of the total tritium recovered in all of the fractions. The upper and lower intermediate zones contained 6.1 ± 1.1% and 12.0 ± 2.2%, respectively, of the total tritium recovered in all of the fractions.

Figure 25 presents the chromatographic distribution of tritium recovered from each of the centrifuged fractions. As seen in Figure 25, each fraction contained a peak of $^3$H that migrated to a region corresponding to an $R_f$ of 0.31-0.40. This region corresponded to the mobility of an authentic sample of 15-ketosteryl palmitate. The radioactivity that comigrated with the 15-ketosteryl ester standard in the floating lipid layer represented 96.4 ± 0.3% of the radioactivity recovered in this fraction (see Table 12). About 90% of the radioactivity recovered in the upper and lower intermediate zones also co-migrated with the 15-ketosteryl ester standard. In the pellet fraction 27.5 ± 1.2% of the tritium migrated to the region corresponding to an $R_f$ of 0.31-0.40.

Each of the fractions also contained a peak that co-migrated with free 15-ketosterol standard ($R_f = 0.06-0.11$). The greatest amount of tritium that co-migrated with the free 15-ketosterol standard was observed in the pellet fraction; the radioactivity contained in this region represented 57.0 ± 0.9% of the tritium recovered in the pellet fraction. The tritium recovered in the corresponding region in the other three fractions comprised 10% or less of the total tritium recovered in each fraction. The only fraction in which significant tritium was recovered at the origin was the pellet fraction (representing ~5% of the total tritium recovered in all the fractions). No effort was made to further characterize this
Table 11. Summary of the distribution of $^3$H from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^3$H in fraction (of total $^3$H contained in all fractions)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S. D. (N=3)</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>45.6 ± 1.9%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>6.1 ± 1.1%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>12.0 ± 2.2%</td>
</tr>
<tr>
<td>Pellet</td>
<td>36.4 ± 0.6%</td>
</tr>
</tbody>
</table>

(1) Experiment 2 incubation condition: 40 μM [2,4-$^3$H]5α-cholesterol-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.
Figure 25. Chromatographic distribution of $^3$H in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation condition (Experiment 2) for the cells and TLC conditions are described in the text.
Table 12. Summary of chromatographic distribution of $^3$H from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
<th>Origin</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>1.9 ± 0.1%</td>
<td>96.4 ± 0.3%</td>
<td>&lt;0.1%</td>
<td>2.0%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>2.2 ± 0.5%</td>
<td>95.0 ± 2.0%</td>
<td>&lt;0.1%</td>
<td>8.3%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>10.5 ± 1.4%</td>
<td>86.2 ± 0.9%</td>
<td>0.8 ± 0.4%</td>
<td>4.2%</td>
</tr>
<tr>
<td>Pellet</td>
<td>57.0 ± 0.9%</td>
<td>27.5 ± 1.2%</td>
<td>13.5 ± 1.9%</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

(1) Experiment 2 incubation condition: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f = 0.06$-0.11 and had the same chromatographic mobility as an authentic sample of the free 15-ketosterol.

(b) Region B corresponds to a scraped region of $R_f = 0.31$-0.40 and had the same chromatographic mobility as an authentic sample of 15-ketosteryl palmitate.
polar material recovered in the pellet fractions. It should be noted that the recovery of tritium (derived from \(^{3}\text{H}\)15-ketosterol) at the origin was an inconsistent finding. Small amounts of tritium were recovered at the origin only in the distribution experiments in which \(^{14}\text{C}\)oleate was used as a substrate. In these experiments (distribution experiments 2 and 3), between 25% and 40% of the \(^{14}\text{C}\) was recovered at the origin. Further, \(^{3}\text{H}\) polar material was not recovered at the origin in previously presented experiments investigating the incorporation of the \(^{3}\text{H}\)15-ketosterol in the K(15) cells.

Less than 1% of the tritium from the other three fractions was recovered at the origin. No more than 8.3% of the total tritium recovered in each fraction was recovered in regions other than the origin or the two regions corresponding to the free and esterified 15-ketosterol (see Table 12).

Figure 26 illustrates the distribution of free 15-ketosterol in the centrifuged fractions. A total of 37.8 ± 2.1 nmols free 15-ketosterol per mg total cellular protein was recovered in the sum of the centrifuged fractions. In subsequent values presented in this section, "mg total cellular protein" will be noted as simply "mg protein". The free 15-ketosterol recovered in the pellet fraction accounted for the greatest proportion (90.1%) of the total free 15-ketosterol. The lower and upper intermediate zones accounted for 5.6% and 0.6% of the total free 15-ketosterol, respectively. The floating lipid layer contained 3.7% of the total free 15-ketosterol.

Figure 27 illustrates the distribution of 15-ketosteryl esters in the centrifuged fractions. A total of 115.2 ± 5.1 nmols 15-ketosteryl esters per mg protein was recovered in the sum of the centrifuged fractions. The 15-ketosterol esters recovered in the floating lipid layer accounted for the greatest proportion (62.8%) of the total 15-ketosterol esters. The upper and lower intermediate zones accounted for 8.1% and 14.8% of the total 15-ketosterol ester, respectively. The pellet fraction contained 14.3% of the total 15-ketosterol ester.
Figure 26. Distribution of free 15-ketosterol in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-³H]5α-cholest-8(14)-en-3β-ol-15-one and 0.33 μM [1-¹⁴C]oleate for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 27. Distribution of 15-ketosteryl esters in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-^3^H]5α-chol- est-8(14)-en-3β-ol-15-one and 0.33μM [1-^1^4^C]oleate for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
The overall distribution of total 15-ketosterol (free and esterified) is presented in Figure 28. A total of 153.2 ± 7.2 nmol total 15-ketosterol per mg protein were recovered in all of the centrifuged fractions. The distribution of free and esterified 15-ketosterol indicates that the greatest concentration of the 15-ketosterol was in the esterified form in the floating lipid layer. The 15-ketosterol ester recovered in this fraction represented 47.2% of the total 15-ketosterol recovered from all of the centrifuged fractions. The next higher concentration of the 15-ketosterol was in the pellet fraction in the free form; the free 15-ketosterol recovered in the pellet fraction accounted for 22.3% of the total 15-ketosterol recovered in all of the fractions. The 15-ketosteryl ester recovered in the lower intermediate zone and the pellet accounted for 11.2% and 10.8%, respectively, of the total free and esterified 15-ketosterol recovered in all of the centrifuged fractions.

**Distribution of $^{14}$C**

A summary of the overall distribution of $^{14}$C in the centrifuged fractions is presented in Table 13. The floating lipid layer contained 34.2 ± 0.4% of the total $^{14}$C recovered in all of the fractions. The upper and lower intermediate zones contained 4.5 ± 0.8% and 9.1 ± 2.0%, respectively, of the total $^{14}$C recovered in all of the fractions. The pellet fraction contained 52.3 ± 1.8% of the total $^{14}$C recovered in all of the fractions.

Figure 29 presents the chromatographic distribution of $^{14}$C recovered in each of the centrifuged fractions. The region containing the greatest amount of $^{14}$C was the origin region of the pellet fraction. The radioactivity contained in this region represented 78.2% of the $^{14}$C recovered in the fraction (see also Table 14). As seen in Figure 29, the floating lipid layer and the upper and lower intermediate zones each contained a peak of $^{14}$C that migrated to a region corresponding to an Rf of 0.31-0.37. This region corresponded to the mobility of an authentic sample of 15-ketosteryl palmitate. The radioactivity that co-
Figure 28. Overall distribution of free 15-ketosterol (striped bars) and 15-ketosteryl esters (solid bars) in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one and 0.33 μM [1-14C]oleate for 48 hours. The bars represent the mean value (N=3); the white bars represent the standard deviation.
Table 13. Summary of the distribution of $^{14}$C from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^{14}$C in fraction (of total $^{14}$C contained in all fractions)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S. D. (N=3)</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>34.2 ± 0.4%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>4.5 ± 0.8%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>9.1 ± 2.0%</td>
</tr>
<tr>
<td>Pellet</td>
<td>52.3 ± 1.8%</td>
</tr>
</tbody>
</table>

(1) Experiment 2 incubation condition: 40 μM [2,4-3H]5α-cholesterol-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.
Figure 29. Chromatographic distribution of $^{14}$C in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation condition (Experiment 2) for the cells and TLC conditions are described in the text.
Table 14. Summary of chromatographic distribution of $^{14}$C from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

Percent $^{14}$C recovered in scraped regions (of total $^{14}$C recovered in fraction):

Mean ± S. D. (N=3)

<table>
<thead>
<tr>
<th>REGION:</th>
<th>Origin</th>
<th>A (a)</th>
<th>B (b)</th>
<th>C (c)</th>
<th>D (d)</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>1.5 ±1.5%</td>
<td>1.2 ±0.1%</td>
<td>87.7 ±0.9%</td>
<td>3.5 ± 0.5%</td>
<td>2.9 ± 0.1%</td>
<td>3.2 ± 2.0%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>0.2 ± 0.2%</td>
<td>1.6 ± 0.1%</td>
<td>86.3 ± 1.9%</td>
<td>3.4 ± 0.9%</td>
<td>2.6 ± 0.3%</td>
<td>5.9 ± 0.8%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>11.7 ± 2.7%</td>
<td>3.0 ± 0.4%</td>
<td>76.9 ± 3.3%</td>
<td>1.9 ± 0.8%</td>
<td>2.9 ± 0.2%</td>
<td>4.6 ± 3.2%</td>
</tr>
<tr>
<td>Pellet</td>
<td>78.2 ± 1.3%</td>
<td>5.3 ± 0.4%</td>
<td>11.9 ± 0.6%</td>
<td>0.7 ± 0.3%</td>
<td>0.6 ± 0.1%</td>
<td>3.5 ± 0.9%</td>
</tr>
</tbody>
</table>

(1) Experiment 2 incubation condition: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f$ = 0.17-0.23 and had the same chromatographic mobility as an authentic sample of oleic acid.

(b) Region B corresponds to a scraped region of $R_f$ = 0.31-0.40 and had the same chromatographic mobility as an authentic sample of 15-ketosteryl palmitate.

(c) Region C corresponds to a scraped region of $R_f$ = 0.40-0.46 and had the same chromatographic mobility as an authentic sample of triolein.

(d) Region D corresponds to a scraped region of $R_f$ = 0.71-0.77 and had the same chromatographic mobility as an authentic sample of cholesteryl oleate.
migrated with the 15-ketosteryl ester standard in the floating lipid layer represented 87.7 ± 0.9% of the radioactivity recovered in this fraction (see Table 14, region B). Although there was much less radioactivity recovered in the intermediate zones (<15% of the total) than in the floating lipid layer, 86.3 ± 1.9% and 76.9 ± 3.3% of the radioactivity recovered in the upper and lower intermediate zones, respectively, co-migrated with the 15-ketosteryl ester standard. In the pellet fraction, only 11.9 ± 0.6% of the 14C co-migrated with the 15-ketosteryl ester standard.

Other regions containing radioactivity were the regions corresponding to the mobility of free fatty acids (Rf= 0.17-0.23), triacylglycerols (Rf=0.40-0.46), and cholesteryl oleate (Rf=0.71-0.77). The radioactivity recovered in these regions was generally <3% of the radioactivity recovered in each centrifuged fraction (see Table 14; regions A, C and D).

Less than 6% of the total 14C recovered in each fraction was recovered in regions other than the regions corresponding to the [14C]oleate-derived lipids discussed above (see Table 14).

The overall percent distribution of 14C is presented in Table 15. This analysis revealed that there were two distinct regions of migration of 14C in this chromatographic analysis of the centrifuged fractions. Firstly, 39.6% of the total 14C recovered in all of the fractions combined remained at the origin in the pellet fraction; secondly, 29.0% of the total 14C co-migrated with 15-ketosteryl palmitate standard in the floating lipid layer. In the upper and lower intermediate zones, the only significant amount of 14C was also found in the region corresponding to the migration of 15-ketosteryl palmitate standard; these regions represented <7.0% of the total radioactivity recovered in all of the fractions combined.
Table 15. Percent overall distribution of [14C]oleate-derived lipids recovered in centrifuged fractions of 15-ketosterol-adapted cells(1).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>FFA</th>
<th>15-Ketosteryl Esters</th>
<th>Triglycerides</th>
<th>Cholesteryl Esters</th>
<th>Origin</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>&lt;1%</td>
<td>29.0%</td>
<td>1.2%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
<td>1.9%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>&lt;1%</td>
<td>6.3%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>&lt;1%</td>
<td>6.7%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
<td>1.1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Pellet Fraction</td>
<td>2.7%</td>
<td>6.0%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
<td>39.6%</td>
<td>1.8%</td>
</tr>
</tbody>
</table>

(1) Experiment 2 incubation condition: 40 μM [2,4-3H]5α-cholest-8(15)-en-3β-ol-15-one, 0.33 μM [1-14C]oleate in F12NCS5.
**Distribution of Protein**

Aliquots from each of the centrifuged fractions as well as the cell homogenate before centrifugation, were assayed for protein following the procedure of Lowry et al. Triplicate aliquots of each of the three sample were assayed; the values were averaged and the standard deviation was calculated. It was determined that the total sample contained $3.90 \pm 0.21$ mg protein. The average recovery of protein was determined by summing the protein values obtained from aliquots assayed from each of the fractions. The average recovery of protein was calculated to be $86.0 \pm 7.1\%$. The percent of protein in each fraction was calculated based on the summed value of the total recovered protein. The greatest amount of protein (58.0%) was recovered in the pellet fraction. The floating lipid layer contained 8.5% of the recovered protein. The upper and lower intermediate zones contained 12.5% and 21.0% of the recovered protein, respectively.
Distribution of $^3\text{H}$ and $^{14}\text{C}$ in K(15) cells after incubation with [2,4-$^3\text{H}$]5α-cholest-8(14)-en-3β-ol-15-one and [4-$^{14}\text{C}$]oleate

**Experiment 3**

15-Ketosterol-adapted cells (1.0 x 10$^6$) were inoculated into 150 x 15 mm culture plates containing Ham's F12 medium + 5% NCS (F12NCS5) containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one (25 ml). After a 48-hour growth period at 37 °C, six culture plates were changed to the experimental medium described below:

Ham's F12 medium + 5% NCS containing 40 μM [2,4-$^3\text{H}$]5α-cholest-8(14)-en-3β-ol-15-one (3.5 μCi $[^3\text{H}]$/sample), 88 μM [1-$^{14}\text{C}$]oleate (0.91 μCi $[^{14}\text{C}]$/sample).

After an additional growth period of 48 hours, the cells were harvested and processed as described in the *Materials and Methods* chapter. The cell samples were centrifuged and separated into four fractions: the floating lipid layer, the upper and lower intermediate zones and the pellet fraction. Each of the fractions were extracted for total lipids following the procedure of Folch *et al.* Less than 0.1% of the tritium or $^{14}\text{C}$ was recovered in the aqueous phases derived from the extraction of the floating lipid layer and the upper and lower intermediate zones. From the pellet fraction, less than 1% of the tritium and less than 0.5% of the $^{14}\text{C}$ was recovered in the aqueous phase. The organic phases containing the total lipids from each fraction were subsequently analyzed by TLC, as described in the *Materials and Methods* chapter.
Distribution of $^3\text{H}$

A summary of the overall distribution of tritium in the centrifuged fractions is presented in Table 16. The floating lipid layer contained $61.3 \pm 2.6\%$ of the total tritium recovered in all of the fractions. The pellet fraction contained $22.4 \pm 2.4\%$ of the total tritium recovered in all of the fractions. The upper and lower intermediate zones contained $10.8 \pm 4.8\%$ and $5.2 \pm 1.0\%$, respectively, of the total tritium recovered in all of the fractions.

Figure 30 presents the chromatographic distribution of tritium recovered from each of the centrifuged fractions. As seen in Figure 30, each fraction contained a peak of $^3\text{H}$ that migrated to a region corresponding to an $R_f$ of 0.34-0.43. This region corresponded to the mobility of an authentic sample of 15-ketosteryl palmitate. The radioactivity that co-migrated with the 15-ketosteryl ester standard in the floating lipid layer represented $95.7 \pm 0.4\%$ of the tritium recovered in this fraction (see Table 17). The radioactivity recovered in the upper and lower intermediate zones that co-migrated with the 15-ketosteryl ester standard accounted for $86.5 \pm 7.5\%$ and $70.0 \pm 4.3\%$, respectively. In the pellet fraction $13.3 \pm 3.5\%$ of the tritium migrated to the region corresponding to an $R_f$ of 0.34-0.43.

The greatest amount of tritium that co-migrated with the free 15-ketosterol standard ($R_f = 0.09-0.14$) was observed in the pellet fraction. The radioactivity contained in this region represented $70.9 \pm 2.2\%$ of the tritium recovered in this fraction. The tritium recovered in the corresponding region in the lower intermediate zone accounted for $22.4 \pm 6.5\%$ of the tritium recovered in that fraction. Only $2.5 \pm 0.1\%$ and $3.3 \pm 0.7\%$ of the tritium in the floating lipid layer and the upper intermediate zone, respec-
Table 16. Summary of the distribution of $^3$H from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^3$H in fraction (of total $^3$H contained in all fractions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>61.3 ± 2.6%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>10.8 ± 4.8%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>5.2 ± 1.0%</td>
</tr>
<tr>
<td>Pellet</td>
<td>22.4 ± 2.4%</td>
</tr>
</tbody>
</table>

(1) Experiment 3 incubation condition: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 88 μM [1-$^{14}$C]oleate in F12NCS5.
Figure 30. Chromatographic distribution of $^3$H in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation conditions (Experiment 3) for the cells and TLC conditions are described in the text.
Table 17. Summary of chromatographic distribution of $^3$H from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
<th>Origin</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>2.5 ± 0.1%</td>
<td>95.7 ± 0.4%</td>
<td>0.3 ± 0.01%</td>
<td>1.8%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>3.3 ± 0.7%</td>
<td>86.5 ± 7.5%</td>
<td>0.9 ± 1.5%</td>
<td>9.3%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>22.4 ± 6.5%</td>
<td>70.0 ± 4.3%</td>
<td>1.3 ± 0.8%</td>
<td>6.3%</td>
</tr>
<tr>
<td>Pellet</td>
<td>70.9 ± 2.2%</td>
<td>13.3 ± 3.5%</td>
<td>11.1 ± 2.9%</td>
<td>4.7%</td>
</tr>
</tbody>
</table>

Mean ± S. D. (N=3)

(1) Experiment 3 incubation condition: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 88 μM [1-14C]oleate in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f = 0.06-0.11$ and had the same chromatographic mobility as an authentic sample of free 15-ketosterol.

(b) Region B corresponds to a scraped region of $R_f = 0.31-0.40$ and had the same chromatographic mobility as an authentic sample of 15-ketosteryl palmitate.
tively, was found in the region having the same mobility as an authentic sample of free 15-ketosterol.

The only fraction in which a significant amount of tritium was recovered was the pellet fraction (representing ~2.5% of the total tritium recovered in all the fractions). No effort was made to further characterize this polar material recovered in the pellet fractions. It should be noted that the recovery of tritium (derived from $[^{3}\text{H}]15$-ketosterol) at the origin was an inconsistent finding. Small amounts of tritium were recovered at the origin only in the distribution experiments in which $[^{14}\text{C}]$oleate was used as a substrate. In these experiments (distribution experiments 2 and 3), between 25% and 40% of the $^{14}\text{C}$ was recovered at the origin. Further, $[^{3}\text{H}]$polar material was not recovered at the origin in previously presented experiments investigating the incorporation of the $[^{3}\text{H}15$-ketosterol in the K(15) cells.

Less than 10% of the total tritium recovered in each fraction was recovered in regions other than the origin and the two regions corresponding to the free and esterified 15-ketosterol (see Table 17).

Figure 31 illustrates the distribution of free 15-ketosterol in the centrifuged fractions. A total of $30.8 \pm 4.7$ nmoles free 15-ketosterol per mg total cellular protein was recovered in the sum of the centrifuged fractions. In subsequent values presented in this section, "mg total cellular protein" will be noted as simply "mg protein". The free 15-ketosterol recovered in the pellet fraction accounted for the greatest proportion (84.4%) of the total free 15-ketosterol. The lower and upper intermediate zones accounted for 5.6% and 1.9% of the total free 15-ketosterol, respectively. The floating lipid layer contained 8.1% of the total free 15-ketosterol.
Figure 31. Distribution of free 15-ketosterol in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one and 88 μM [1-14C]oleate for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 32 illustrates the distribution of 15-ketosteryl esters in the centrifuged fractions. A total of 121.3 ± 14.6 nmoles 15-ketosteryl esters per mg protein was recovered in the sum of the centrifuged fractions. The 15-ketosterol esters recovered in the floating lipid layer accounted for the greatest proportion (78.8%) of the total 15-ketosteryl esters. The upper and lower intermediate zones accounted for 12.8% and 4.2% of the total 15-ketosteryl ester, respectively. The pellet fraction contained only 4.1% of the total 15-ketosterol ester.

Figure 33 presents an overall summary of the distribution of total 15-ketosterol (free and esterified). A total of 152.1 ± 19.3 nmoles total 15-ketosterol per mg protein were recovered in all of the centrifuged fractions. The distribution of free and esterified 15-ketosterol indicated that the greatest concentration of the 15-ketosterol was in the esterified form in the floating lipid layer. The 15-ketosteryl ester recovered in this fraction represented 62.9% of the total (free and esterified) 15-ketosterol recovered from all of the centrifuged fractions. The next higher concentration of the 15-ketosterol was in the pellet fraction in the free form; the free 15-ketosterol recovered in this fraction accounted for 17.1% of the total 15-ketosterol recovered in all of the fractions. The 15-ketosteryl ester recovered in the upper intermediate zone accounted for 10.4% of the total free and esterified 15-ketosterol recovered in all of the centrifuged fractions.
Figure 32. Distribution of 15-ketosteryl esters in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 µM [2,4-^3H]5α-cholest-8(14)-en-3β-ol-15-one and 88 µM [1-^14C]oleate for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 33. Overall distribution of free 15-ketosterol (striped bars) and 15-ketosteryl esters (solid bars) in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 40 μM [2,4-³H]5α-cholest-8(14)-en-3β-ol-15-one and 88 μM [1-¹⁴C]oleate for 48 hours. The bars represent the mean value (N=3); the white bars represent the standard deviation.
Distribution of $^{14}$C

A summary of the overall distribution of $^{14}$C in the centrifuged fraction is presented in Table 18. The floating lipid layer contained $53.0 \pm 1.2\%$ of the total $^{14}$C recovered in all of the fractions. The upper and lower intermediate zones accounted for $9.1 \pm 3.2\%$ and $4.9 \pm 1.0\%$, respectively, of the total $^{14}$C recovered in all of the fractions. The pellet fraction contained $33.0 \pm 2.4\%$ of the total $^{14}$C recovered in all of the fractions.

Figure 34 presents the chromatographic distribution of $^{14}$C recovered in each of the centrifuged fractions. The region containing the greatest amount of $^{14}$C was the origin region of the pellet fraction. The radioactivity contained in this region represented $77.3 \pm 2.4\%$ of the $^{14}$C recovered in the pellet fraction (see also Table 19). In the lower intermediate zone, a significant amount of $^{14}$C ($17.1 \pm 8.3\%$) was recovered at the origin as well.

As seen in Figure 34, the floating lipid layer and the upper and lower intermediate zones each contained a peak of $^{14}$C that migrated to a region corresponding to an Rf of 0.37-0.46. This region corresponded to the mobility of an authentic sample of 15-ketosteryl palmitate. The radioactivity that co-migrated with the 15-ketosteryl ester standard in the floating lipid layer represented $54.7 \pm 1.6\%$. The radioactivity that co-migrated with the triacylglycerol standard (Rf = 0.49-0.57) in the floating lipid layer represented $35.1 \pm 2.2\%$ of the radioactivity recovered in this fraction (see Table 19). Although there was much less radioactivity recovered in the intermediate zones (<14% of the total) than in the floating lipid layer, $51.7\% \pm 4.6\%$ and $36.6 \pm 4.0\%$ of the radioactivity recovered in the upper and lower intermediate zones, respectively, co-migrated with the 15-ketosteryl ester standard; $32.3 \pm 6.5\%$ and $26.5 \pm 2.8\%$ of the
Table 18. Summary of the distribution of $^{14}$C from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^{14}$C in fraction (of total $^{14}$C contained in all fractions)</th>
<th>Mean ± S. D. (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>53.0 ± 1.2%</td>
<td></td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>9.1 ± 3.2%</td>
<td></td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>4.9 ± 1.0%</td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>33.0 ± 2.4%</td>
<td></td>
</tr>
</tbody>
</table>

(1) Experiment 3 incubation condition: 40 μM [2,4-3H]5α-cholesten-8(15)-en-3β-ol-15-one, 88 μM [1-14C]oleate in F12NCS5.
Figure 34. Chromatographic distribution of $^{14}$C in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation conditions (Experiment 3) for the cells and TLC conditions are described in the text.
Table 19. Summary of chromatographic distribution of $^{14}$C from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Origin</th>
<th>A (a)</th>
<th>B (b)</th>
<th>C (c)</th>
<th>D (d)</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>0.1 ±0.0%</td>
<td>1.6 ±0.1%</td>
<td>54.7 ±1.6%</td>
<td>35.1 ± 2.2%</td>
<td>2.6 ± 0.7%</td>
<td>6.0 ± 2.1%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>0.3 ± 0.2%</td>
<td>3.8 ± 1.8%</td>
<td>54.7 ± 4.6%</td>
<td>32.3 ± 6.5%</td>
<td>2.7 ± 0.5%</td>
<td>9.1 ± 2.0%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>17.1 ± 8.3%</td>
<td>7.3 ± 1.3%</td>
<td>36.6 ± 4.0%</td>
<td>26.5 ± 2.8%</td>
<td>2.5 ± 1.4%</td>
<td>10.0 ± 6.0%</td>
</tr>
<tr>
<td>Pellet</td>
<td>77.3 ± 2.4%</td>
<td>8.2 ± 0.1%</td>
<td>4.9 ± 0.7%</td>
<td>3.5 ± 0.6%</td>
<td>0.3 ± 0.1%</td>
<td>5.9 ± 1.3%</td>
</tr>
</tbody>
</table>


(a) Region A corresponds to a scraped region of $R_f = 0.17-0.23$ and had the same chromatographic mobility as an authentic sample of oleic acid.

(b) Region B corresponds to a scraped region of $R_f = 0.37-0.46$ and had the same chromatographic mobility as an authentic sample of 15-ketosteryl palmitate.

(c) Region C corresponds to a scraped region of $R_f = 0.49-0.57$ and had the same chromatographic mobility as an authentic sample of triolein.

(d) Region D corresponds to a scraped region of $R_f = 0.69-0.77$ and had the same chromatographic mobility as an authentic sample of cholesteryl oleate.
radioactivity recovered in the upper and lower intermediate zones, respectively, co-
migrated with the triacylglycerol standard. In the pellet fraction, only 4.9 ± 0.7% of
the $^{14}C$ co-migrated with the 15-ketosteryl ester standard.

The $^{14}C$ recovered in the region of $R_f= 0.46-0.49$ was believed to be derived
from minor overlapping of both [$^{14}C$]15-ketosteryl esters and [$^{14}C$]triacylglycerols.
The $^{14}C$ recovered in this region accounted for only 2.3 ± 1.4% of the total $^{14}C$ of
each fraction and was not included in any calculations of either 15-ketosteryl esters or
triacylglycerols. This radioactivity was included in the "all other regions" calculations
(see Table 19).

Other regions containing radioactivity were the regions corresponding to the
mobility of free fatty acids ( $R_f= 0.17-0.23$) and cholesteryl esters ( $R_f =0.69-0.77$).
The radioactivity recovered in these regions was generally <5% of the radioactivity
recovered in each centrifuged fraction (see Table 19).

Ten percent or less of the total $^{14}C$ recovered in each fraction was recovered in
regions other than the regions corresponding to the [$^{14}C$]oleate-derived lipids
discussed above (see Table 19).

Table 20 presents a summary of the overall percent distribution of $^{14}C$. This
analysis showed that there were two distinct regions of migration of $^{14}C$ in this
chromatographic analysis of the centrifuged fractions. Firstly, 28.4% of the total $^{14}C$
co-migrated with 15-ketosteryl palmitate standard in the floating lipid layer; secondly,
25.2% of the total $^{14}C$ recovered in all of the fractions combined remained at the
origin in the pellet fraction. Further 18.2% of the total $^{14}C$ was recovered in the
floating lipid layer in the region corresponding to triacylglycerols. The three regions
combined accounted for 71.8% of the total $^{14}C$ recovered in all of the fractions.
Table 20. Percent overall distribution of $[^{14}$C]oleate-derived lipids recovered in centrifuged fractions from 15-ketosterol-adapted cells(1).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>FFA</th>
<th>15-Ketosteryl Esters</th>
<th>Triglycerides</th>
<th>Cholesteryl Esters</th>
<th>Origin</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid</td>
<td>&lt;1%</td>
<td>28.4%</td>
<td>18.2%</td>
<td>1.3%</td>
<td>&lt;1%</td>
<td>3.6%</td>
</tr>
<tr>
<td>Layer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Intermediate</td>
<td>&lt;1%</td>
<td>4.6%</td>
<td>2.9%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
<td>2.2%</td>
</tr>
<tr>
<td>Zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower Intermediate</td>
<td>&lt;1%</td>
<td>1.7%</td>
<td>1.2%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>2.7%</td>
<td>1.6%</td>
<td>1.1%</td>
<td>&lt;1%</td>
<td>25.2%</td>
<td>1.9%</td>
</tr>
</tbody>
</table>

(1) Experiment 3 incubation condition: 40 μM [2,4-3H]5α-cholest-8(15)-en-3β-ol-15-one, 88 μM [1-14C]oleate in F12 NCS5.
Distribution of Protein

Aliquots from each of the centrifuged fractions as well as the cell homogenate before centrifugation, were assayed for protein following the procedure of Lowry et al. Triplicate aliquots of each of the three sample were assayed; the values were averaged and the standard deviation was calculated. It was determined that the total sample contained 3.61 ± 0.14 mg protein. The average recovery of protein was determined by summing the protein values obtained from aliquots assayed from each of the fractions. The average recovery of protein was calculated to be 86.0 ± 7.1%. The percent of protein in each fraction was calculated based on the summed value of the total recovered protein. The greatest amount of protein (59.1%) was recovered in the pellet fraction. The floating lipid layer contained 9.4% of the recovered protein. The upper and lower intermediate zones contained 14.4% and 17.2% of the recovered protein, respectively.
Reverse phase HPLC analysis of the putative 15-ketosteryl ester region resulting from TLC separation

This section describes the reverse phase HPLC analysis of lipids recovered in the floating lipid layer derived from 15-ketosterol-adapted cells incubated in 40 μM [2,4-\textsuperscript{3}H] 5α-cholesterol-8(14)-en-3β-ol-15-one + 0.6 μM [4-\textsuperscript{14}C]oleate in F12NCS5. The K(15) cell cultures were prepared and the floating lipid layer was isolated as described in the Materials and Methods chapter. The lipids from the floating lipid layer were extracted and subsequently separated by TLC also described in the Materials and Methods chapter. TLC analysis of the lipids recovered in the floating lipid layer revealed that 96.4 ± 0.3% of the [\textsuperscript{3}H]15-ketosterol-derived lipids and 87.7 ± 0.9% of the [\textsuperscript{14}C]oleate-derived lipids co-migrated with an authentic sample of the palmitate ester of 5α-cholest-8(14)-en-3β-ol-15-one. This section describes the subsequent reverse phase HPLC analysis of the lipids recovered in that region to verify that the [\textsuperscript{3}H]15-ketosterol-derived lipids were in fact the esters of the 15-ketosterol.

Following TLC separation, the region corresponding to the putative 15-ketosteryl ester region was scraped and eluted with chloroform:methanol:H\textsubscript{2}O (5:5:1). The sample was subsequently analyzed using reversed-phase high performance liquid chromatography. Because of the small amount of mass present in the sample, non-radiolabeled standards of the 15-ketosterol esters were simultaneously injected with the sample.

The HPLC system used a 5-μm C\textsubscript{18} Microsorb column and a gradient system of solvents using two solvent mixtures (solvent A; isopropanol: methanol: water and solvent B; isopropanol: methanol). The system is described in further detail in the Materials and Methods chapter. In this system 100 fractions were collected; 15-ketosteryl esters elute in the region of fractions 30-65. The absorbance profile of cold standards, shown in Figure 35, illustrates the
Figure 35. Reverse phase HPLC separation of 5α-cholest-8(14)-en-3α-ol-15-one ester standards. The indicated peaks correspond to: linolenic (18:3), arachidonic (20:4), linoleic (18:2), oleic (18:1) or palmitic (16:0), stearic (18:0), and arachidic (20:0) esters of the 15-ketosterol.
separation of a series of esters of 5α-cholest-8(14)-en-3β-ol-15-one. 15-Ketosteryl esters separate based on chain length and degree of saturation of the fatty acid moieties. In general, the 15-ketosteryl esters having the greatest mobilities are those with fatty acyl moieties of shorter chain lengths and multiple unsaturations. It should be noted also that the oleate (18:1) and palmitate (16:0) esters of the 15-ketosterol are not resolved in this chromatographic system.

Figure 36 illustrates the distribution of $^3$H in the 15-ketosteryl ester region derived from the floating lipid layer of the 15-ketosterol-adapted cells after a 48-hour incubation in F12NCS5 containing 40 $\mu$M [2,4-$^3$H]5α-cholest-8(14)-en-3β-ol-15-one + 0.6 $\mu$M [4-$^{14}$C]oleate that resulted from HPLC analysis. The majority of the $^3$H (66%) corresponded to the region having the chromatographic mobility of the oleate or palmitate esters of the 15-ketosterol (peak B). The other peaks, corresponding to the linoleate, stearate, and arachidate esters of the 15-ketosterol accounted for a total of 22% of the recovered tritium. These minor peaks represented low levels of radioactivity and their further identification was not pursued.

The radioactivity profile from this analysis verifies that the isolated material that has the same chromatographic mobility as 15-ketosterol esters by radio-TLC also co-migrate with authentic samples of 5α-cholest-8(14)-en-3β-ol-15-one esters by HPLC separation.
Figure 36. Reverse phase HPLC analysis of the distribution of $^{3}$H in the 15-ketosteryl ester region from TLC separation of the lipid extract of the floating lipid layer of K(15) cells after a 48-hour incubation in 40 μM [2,4-$^{3}$H]5α-cholest-8(14)-en-3β-ol-15-one and 0.6 μM [1-$^{14}$C]oleate. The radioactive components corresponded in their chromatographic mobilities to those of: A- linoleic (18:2), B- oleic (18:1) or palmitic (16:0), C-stearic (18:0), and D- arachidic (20:0) esters of the 15-ketosterol. Peak E was not identified.
Distribution of $^{14}$C in K(15) cells after incubation with 5α-cholest-8(14)-en-3β-ol-15-one and [4-$^{14}$C]cholesterol

Experiment 4

15-Ketosterol-adapted cells (1.0 x 10⁶) were inoculated into 150 x 15 mm culture plates containing Ham's F12 medium + 5% NCS (F12NCS5) containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one (25 ml). After a 48-hour growth period at 37 °C, six culture plates were changed to the experimental medium described below:

Ham's F12 medium + 5% NCS containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one + 20.7 μM [4-$^{14}$C]cholesterol (2.4 μCi [$^{14}$C/sample).

After an additional growth period of 48 hours, the cells were harvested and processed as described in the Materials and Methods chapter. The cell samples were centrifuged and separated into four fractions: the floating lipid layer, the upper and lower intermediate zones and the pellet fraction. Each of the fractions were extracted for total lipids following the procedure of Folch et al. Less than 0.1% of the $^{14}$C was recovered in the aqueous phases derived from the extraction of the floating lipid layer and the upper and lower intermediate zones. From the pellet fraction, less than 0.5% of the $^{14}$C was recovered in the aqueous phase. The organic phases containing the total lipids from each fraction were subsequently analyzed by TLC, as described in the Materials and Methods chapter.

Distribution of $^{14}$C

A summary of the overall distribution of $^{14}$C in the centrifuged fractions is presented in Table 21. The floating lipid layer contained 27.1 ± 9.0% of the the total $^{14}$C recovered in all of the fractions. The upper and lower intermediate zones contained $1.2 \pm 0.6$% and $5.2 \pm 0.3$%,
Table 21. Summary of the distribution of $^{14}$C from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^{14}$C in fraction (of total $^{14}$C contained in all fractions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>27.1 $\pm$ 9.0%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>1.2 $\pm$ 0.6%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>5.2 $\pm$ 0.3%</td>
</tr>
<tr>
<td>Pellet</td>
<td>66.5 $\pm$ 9.3%</td>
</tr>
</tbody>
</table>

(1) Experiment 4 incubation condition: 15 $\mu$M 5α-cholest-8(15)-en-3β-ol-15-one, 0.19 $\mu$M [4-$^{14}$C]cholesterol in F12NCS5.
respectively, of the total $^{14}$C recovered in all of the fractions. The pellet fraction contained 66.5±9.3% of the total $^{14}$C recovered in all of the fractions.

Figure 37 presents the chromatographic distribution of $^{14}$C recovered from each of the centrifuged fractions. The pellet fraction contained the greatest amount of $^{14}$C that co-migrated with the free cholesterol standard ($R_f = 0.23-0.29$). The radioactivity contained in this region represented 94.9 ± 0.3% of the $^{14}$C recovered in the fraction (see also Table 22). As the pellet fraction contained 66.5 ± 9.3% of the total $^{14}$C recovered in all of the centrifuged fractions, this indicates that the majority of [$^{14}$C]cholesterol incorporated into the cells from the growth medium remained in the free form and was recovered in this fraction. In the lower intermediate zone, 70.5 ± 2.4% of the $^{14}$C was recovered in the region corresponding to the mobility of an authentic sample of cholesterol.

As seen in Figure 37, the floating lipid layer and the upper and lower intermediate zones each contained a peak of $^{14}$C that migrated to a region corresponding to an $R_f$ of 0.69-0.77. This region corresponded to the mobility of an authentic sample of cholesteryl oleate. The radioactivity that co-migrated with the cholesteryl ester standard in the floating lipid layer represented 97.8 ± 0.9% of the radioactivity recovered in this fraction. In the pellet fraction, only 3.1 ± 0.2% of the $^{14}$C co-migrated with the cholesteryl ester standard (see Table 22).

Very little $^{14}$C recovered in each fraction was recovered in regions other than the two regions corresponding to the free and esterified cholesterol (see Table 22).

Figure 38 illustrates the distribution of free cholesterol in the centrifuged fractions. A total of 12.6 ± 1.2 nmoles free cholesterol per mg total cellular protein was recovered in the sum of the centrifuged fractions. The free cholesterol recovered in the pellet fraction (11.8 ± 1.2 nmoles per mg total cellular protein) accounted for the greatest proportion (93.7%) of the total free cholesterol. The floating lipid layer contained less than 1% of the total free cholesterol.
Figure 37. Chromatographic distribution of $^{14}$C in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation conditions (Experiment 4) for the cells and TLC conditions are described in the text.
Table 22. Summary of chromatographic distribution of $^{14}$C from centrifuged fractions derived from 15-ketosterol-adapted cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
<th>Origin</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>1.8 ± 0.2%</td>
<td>97.8 ± 0.9%</td>
<td>0.0 ± 0.0%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>5.0 ± 2.8%</td>
<td>86.1 ± 9.4%</td>
<td>0.8 ± 0.8%</td>
<td>8.1%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>70.5 ± 2.4%</td>
<td>26.1 ± 2.0%</td>
<td>0.7 ± 0.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Pellet</td>
<td>94.9 ± 0.3%</td>
<td>3.1 ± 0.2%</td>
<td>0.4 ± 0.1%</td>
<td>1.7%</td>
</tr>
</tbody>
</table>

Percent $^{14}$C recovered in scraped regions (of the total $^{14}$C recovered in fraction):

Mean ± S. D. (N=3)

(1) Experiment 4 incubation condition: 15 μM 5α-cholest-8(15)-en-3β-ol-15-one, 19 μM [4-$^{14}$C] cholesterol in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f$ = 0.23-0.29 and had the same chromatographic mobility as an authentic sample of free cholesterol.

(b) Region B corresponds to a scraped region of $R_f$ = 0.69-0.77 and had the same chromatographic mobility as an authentic sample of cholesteryl oleate.
Figure 38. Distribution of free cholesterol in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one and 20.7 μM [4-14C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 39 illustrates the distribution of cholesteryl esters in the centrifuged fractions. A total of 5.7 ± 1.1 nmoles cholesteryl esters per mg total cellular protein was recovered in the sum of the centrifuged fractions. The cholesteryl esters recovered in the floating lipid layer (4.9 ± 1.0 nmoles cholesteryl ester per mg total cellular protein) accounted for the greatest proportion (85.1%) of the total cholesteryl esters. The pellet fraction contained only 6.8% of the recovered cholesteryl esters.

Figure 40 presents a summary of the overall distribution of cholesterol (free and esterified) recovered in the centrifuged fractions. A total of 18.3 ± 2.3 nmoles total cholesterol (free and esterified) per mg total cellular protein were recovered in all of the centrifuged fractions. The distribution of free and esterified cholesterol indicated that the greatest concentration of cholesterol was in the free form in the pellet. The cholesteryl ester recovered in this fraction represented 64.5% of the total (free and esterified) cholesterol recovered from all of the centrifuged fractions. The cholesteryl ester recovered in the floating lipid layer accounted for 26.5% of the total cholesterol (free and esterified) recovered in all fractions. The remaining 9.0% of the total cholesterol was distributed in the free and esterified forms in the remaining centrifuged fractions.

**Distribution of Protein**

Aliquots from each of the centrifuged fractions as well as the cell homogenate before centrifugation, were assayed for protein following the procedure of Lowry et al. Triplicate aliquots of each of the three sample were assayed; the values were averaged and the standard deviation was calculated. It was determined that the total sample contained 4.39 ± 0.17 mg protein. The average recovery of protein was determined by summing the protein values obtained from aliquots assayed from each of the fractions. The average recovery of protein was calculated to be 86.0 ± 7.1%. The percent of protein in each fraction
Figure 39. Distribution of cholesteryl esters in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12NCS5 containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one and 20.7 μM [4-14C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 40. Overall distribution of free cholesterol and cholesteryl esters in the floating lipid layer, upper and lower intermediate zones and pellet fraction derived from K(15) cells incubated in F12 NCS5 containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one and 20.7 μM [4-14C]cholesterol for 48 hours. The black and striped bars represent the mean value (N=3); the white bars represent the standard deviation.
was calculated based on the summed value of the total recovered protein. The greatest amount of protein (60.6%) was recovered in the pellet fraction. The floating lipid layer contained 7.5% of the recovered protein. The upper and lower intermediate zones contained 9.4% and 22.6% of the recovered protein, respectively.
Distribution of $^{14}$C in CHO K-1 cells after incubation with [4-$^{14}$C]cholesterol

Experiment 5

Chinese hamster ovary (CHO) cells ($6.0 \times 10^5$) were inoculated into 150 x 15 mm culture plates containing Ham's F12 medium containing 5% NCS (F12NCS5; 25 ml). After a 48-hour growth period at 37 °C, six culture plates were changed to the experimental medium described below:

Ham's F12 medium + 5% NCS containing 2.7 µM [4-$^{14}$C]cholesterol (3.65 µCi [$^{14}$C]/sample).

After an additional growth period of 48 hours, the cells were harvested and processed as described in the Materials and Methods chapter. The cell samples were centrifuged and separated into four fractions: the floating lipid layer, the upper and lower intermediate zones and the pellet fraction. Each of the fractions were extracted for lipids; the lipids from each fraction were subsequently analyzed by TLC, as described in the Materials and Methods chapter.

Distribution of $^{14}$C

A summary of the overall distribution of $^{14}$C in the centrifuged fractions is presented in Table 23. The floating lipid layer contained $17.2 \pm 1.3\%$ of the total $^{14}$C recovered in all of the fractions. The upper and lower intermediate zones contained $1.2 \pm 0.3\%$ and $8.1 \pm 1.9\%$, respectively, of the total $^{14}$C recovered in all of the fractions. The pellet fraction contained $73.4 \pm 9.7\%$ of the total $^{14}$C recovered in all of the fractions.
Table 23. Summary of the distribution of $^{14}$C from centrifuged fractions derived from CHO K-1 cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^{14}$C in fraction (of total $^{14}$C contained in all fractions)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S. D. (N=3)</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>17.2 ± 1.3%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>1.2 ± 0.3%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>8.1 ± 1.9%</td>
</tr>
<tr>
<td>Pellet</td>
<td>73.4 ± 9.7%</td>
</tr>
</tbody>
</table>

(1) Experiment 5 incubation condition: Ham's F12 growth medium containing 5% NCS, 2.7 μM [4-$^{14}$C]cholesterol.
Figure 41 presents the chromatographic distribution of $^{14}$C recovered from each of the centrifuged fractions. The pellet fraction contained the greatest amount of $^{14}$C that co-migrated with the free cholesterol standard ($R_f = 0.18-0.24$). The radioactivity contained in this region represented $91.1 \pm 1.2\%$ of the $^{14}$C recovered in the fraction (see also Table 24). As the pellet fraction contained $73.4 \pm 9.7\%$ of the total $^{14}$C recovered in all of the centrifuged fractions, this indicates that the majority of [$^{14}$C]cholesterol incorporated into the cells from the growth medium remained in the free form and was recovered in the pellet fraction. In the lower intermediate zone, $32.0 \pm 5.0\%$ of the $^{14}$C was recovered in the region corresponding to the mobility of an authentic sample of free cholesterol.

As seen in Figure 41, each of the fractions contained a peak of $^{14}$C that migrated to a region corresponding to an $R_f$ of 0.68-0.79. This region corresponded to the mobility of an authentic sample of cholesteryl oleate. The radioactivity that co-migrated with the cholesteryl ester standard in the floating lipid layer represented $97.1 \pm 0.2\%$ of the radioactivity recovered in this fraction. In the pellet fraction, $8.1 \pm 1.3\%$ of the $^{14}$C co-migrated with the cholesteryl ester standard (Table 24). In the upper and lower intermediate zones, $93.0 \pm 3.0\%$ and $66.9 \pm 4.9\%$, respectively, of the $^{14}$C recovered co-migrated with the cholesteryl ester standard.

Of the $^{14}$C recovered in each fraction, $0.2 \pm 0.1\%$ or less was recovered at the origin. Less than $1\%$ of the $^{14}$C recovered in each fraction was recovered at the origin and in regions other than the two regions corresponding to the free and esterified cholesterol (see Table 24).

Figure 42 illustrates the distribution of free cholesterol in the centrifuged fractions. A total of $39.2 \pm 5.1$ nmoles free cholesterol per mg total cellular protein was recovered in the sum of the centrifuged fractions. The free cholesterol recovered in the pellet fraction ($37.5 \pm 4.9$ nmoles per mg protein) accounted for the greatest proportion (95.7\%) of the total free cholesterol. The floating lipid layer contained less than $1\%$ of the total free cholesterol.
Figure 41. Chromatographic distribution of $^{14}$C in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation conditions (Experiment 5) for the cells and TLC conditions are described in the text.
Table 24. Summary of chromatographic distribution of \(^{14}\text{C}\) from centrifuged fractions derived from CHO K-1 cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
<th>Origin</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>2.1 ± 0.1%</td>
<td>97.1 ± 0.2%</td>
<td>&lt;0.1%</td>
<td>0.7 ± 0.1%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>6.7 ± 3.2%</td>
<td>93.0 ± 3.0%</td>
<td>&lt;0.1%</td>
<td>0.3 ± 0.5%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>32.0 ± 5.0%</td>
<td>66.9 ± 4.9%</td>
<td>0.2 ± 0.1%</td>
<td>0.9 ± 0.2%</td>
</tr>
<tr>
<td>Pellet</td>
<td>91.1 ± 1.2%</td>
<td>8.1 ± 1.3%</td>
<td>0.1 ± 0.0%</td>
<td>0.7 ± 0.1%</td>
</tr>
</tbody>
</table>

Mean ± S. D. (N=3)

(1) Experiment 5 incubation condition: Ham's F12 growth medium containing 5% NCS, 2.7 µM [4-\(^{14}\text{C}\)]cholesterol.

(a) Region A corresponds to a scraped region of \(R_f = 0.18-0.24\) and had the same chromatographic mobility as an authentic sample of free cholesterol.

(b) Region B corresponds to a scraped region of \(R_f = 0.68-0.79\) and had the same chromatographic mobility as an authentic sample of cholesteryl oleate.
Figure 42. Distribution of free cholesterol in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from CHO cells incubated in F12NCS5 containing 2.7 μM [4-\(^{14}\)C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 43 illustrates the distribution of cholesteryl esters in the centrifuged fractions. A total of $16.4 \pm 2.1$ nmoles cholesteryl esters per mg total cellular protein was recovered in the sum of the centrifuged fractions. The cholesteryl esters recovered in the floating lipid layer ($9.4 \pm 0.7$ nmoles cholesteryl ester per mg protein) accounted for the greatest proportion (57.1%) of the total cholesteryl esters. The pellet fraction and the lower intermediate zone contained 19.2% and 19.8, respectively, of the recovered cholesteryl esters.

Figure 44 presents a summary of the overall distribution of cholesterol (free and esterified) recovered in the centrifuged fractions. A total of $55.6 \pm 7.2$ nmoles total cholesterol per mg total cellular protein were recovered in all of the centrifuged fractions. The distribution of free and esterified cholesterol indicated that the greatest concentration of cholesterol was in the free form in the pellet. The cholesteryl ester recovered in this fraction represented 67.4% of the total cholesterol recovered from all of the centrifuged fractions. The cholesteryl ester recovered in the floating lipid layer accounted for 16.9% of the total cholesterol recovered in all fractions. The remaining 15.7% of the total cholesterol was distributed in the free and esterified forms in the remaining centrifuged fractions.

**Distribution of Protein**

Aliquots from each of the centrifuged fractions as well as the cell homogenate before centrifugation, were assayed for protein following the procedure of Lowry *et al.*. Triplicate aliquots of each of the three sample were assayed; the values were averaged and the standard deviation was calculated.

It was determined that the total sample contained $2.35 \pm 0.12$ mg protein. The greatest amount of protein (59.1%) was recovered in the pellet fraction. The floating lipid layer contained 10.2% of the recovered protein. The upper and lower intermediate zones contained 4.7% and 26.0% of the recovered protein, respectively.
Figure 43. Distribution of cholesteryl esters in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from CHO cells incubated in F12NCS5 containing 2.7 μM [4-\textsuperscript{14}C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 44. Overall distribution of free cholesterol (striped bars) and cholesteryl esters (solid bars) in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from CHO cells incubated in F12NCS5 containing 2.7 μM [4-\(^{14}\)C]cholesterol (Experiment 5) for 48 hours. The bars represent the mean value (N=3); the white bars represent the standard deviation.
**Distribution of $^3$H and $^{14}$C in CHO K-1 cells after incubation with [2,4-$^3$H]5α-cholest-8(14)-en-3β-ol-15-one and [4-$^{14}$C]cholesterol**

**Experiment 6**

Chinese hamster ovary (CHO) cells ($6.0 \times 10^5$) were inoculated into 150 x 15 mm culture plates containing Ham's F12 medium containing 5% NCS (F12NCS5; 25 ml). After a 48-hour growth period at 37 °C, six culture plates were changed to the experimental medium described below:

- Ham's F12 medium + 5% NCS containing 5.8 μM [2,4-$^3$H]5α-cholest-8(14)-en-3β-ol-15-one (1.95 μCi [$^3$H]/sample), 0.7 μM [4-$^{14}$C]cholesterol (0.95 μCi [$^{14}$C]/sample).

After an additional growth period of 48 hours, the cells were harvested and processed as described in the Materials and Methods chapter. The cell samples were centrifuged and separated into four fractions: the floating lipid layer, the upper and lower intermediate zones and the pellet fraction. Each of the fractions were extracted for lipids; the lipids from each fraction were subsequently analyzed by TLC, as described in the Materials and Methods chapter.

**Distribution of $^3$H**

The overall distribution of $^3$H in the centrifuged fractions was as follows. The floating lipid layer contained 11.2 ± 0.7% of the total tritium recovered in all of the fractions (see Table 25). The pellet fraction contained 81.6 ± 7.9% of the total tritium recovered in all of the fractions. The upper and lower intermediate zones contained 0.8 ± 0.1% and 6.4 ± 3.1%, respectively, of the total tritium recovered in all of the fractions.
Table 25. Summary of the distribution of $^3$H from centrifuged fractions derived from CHO K-1 cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^3$H in fraction (of total $^3$H contained in all fractions) Mean ± S. D. (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>11.2 ± 0.7%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>0.8 ± 0.1%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>6.4 ± 3.1%</td>
</tr>
<tr>
<td>Pellet</td>
<td>81.6 ± 7.9%</td>
</tr>
</tbody>
</table>

(1) Experiment 6 incubation condition: 5 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.7 μM [4-$^14$C]cholesterol in F12NCS5.
Figure 45 presents the chromatographic distribution of tritium recovered from each of the centrifuged fractions. As seen in Figure 45, each fraction contained a peak of $^3$H that migrated to a region corresponding to an $R_f$ of 0.44–0.56. This region corresponded to the mobility of an authentic sample of 15-ketosteryl palmitate. Table 26 presents the percentage distribution of tritium in the centrifuged fractions. The radioactivity that co-migrated with the 15-ketosteryl ester standard in the floating lipid layer represented 93.8 ± 0.8% of the radioactivity recovered in this fraction. In the upper and lower intermediate zones, 87.4 ± 3.1% and 70.0 ± 12.0% of the tritium, respectively, was recovered in the region corresponding to the mobility of an authentic sample of 15-ketosteryl palmitate. In the pellet fraction, only 8.9 ± 0.4% of the tritium migrated to the same region corresponding to an $R_f$ of 0.44–0.56.

The greatest amount of tritium that co-migrated with the free 15-ketosterol standard ($R_f$ = 0.06-0.12) was observed in the pellet fraction and represented 90.6 ± 0.6% of the tritium recovered in this fraction. The tritium recovered in the corresponding region in the floating lipid layer comprised only 4.3 ± 0.4% of the total tritium recovered in this fraction.

Less than 0.1% of the total tritium recovered in each fraction was recovered at the origin and less than 2% of the total tritium recovered in each fraction was recovered in regions other than the two regions corresponding to the free and esterified 15-ketosterol.

Figure 46 illustrates the distribution of free 15-ketosterol in the centrifuged fractions. A total of 7.7 ± 0.9 nmoles free 15-ketosterol per mg total cellular protein was recovered in the sum of the centrifuged fractions. The free 15-ketosterol recovered in the pellet fraction accounted for the greatest proportion (95.6%) of the total free 15-ketosterol. The floating lipid layer contained less than 1% of the total free 15-ketosterol.
Figure 45. Chromatographic distribution of $^3$H in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation conditions (Experiment 6) for the cells and TLC conditions are described in the text.
Table 26. Summary of chromatographic distribution of \(^3\)H from centrifuged fractions derived from CHO K-1 cells \(^{(1)}\).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (^{(a)})</th>
<th>Region B (^{(b)})</th>
<th>Origin</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>4.3 ± 0.4%</td>
<td>93.8 ± 0.8%</td>
<td>0.0%</td>
<td>1.9 ± 0.6%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>12.4 ± 3.4%</td>
<td>87.4 ± 3.1%</td>
<td>0.0%</td>
<td>0.2 ± 0.3%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>28.1 ± 13.3%</td>
<td>70.0 ± 12.0%</td>
<td>0.0%</td>
<td>1.9 ± 1.8%</td>
</tr>
<tr>
<td>Pellet</td>
<td>90.6 ± 0.6%</td>
<td>8.9 ± 0.4%</td>
<td>&lt;0.1%</td>
<td>0.4 ± 0.6%</td>
</tr>
</tbody>
</table>

Mean ± S. D. (N=3)

\(^{(1)}\) Experiment 6 incubation condition: 5 \(\mu\)M \([2,4-\text{H}]\)5\(\alpha\)-cholest-8(15)-en-3\(\beta\)-ol-15-one, 0.7 \(\mu\)M \([4,14\text{C}]\)cholesterol in F12NCS5.

\(^{(a)}\) Region A corresponds to a scraped region of \(R_f = 0.06-0.12\) and had the same chromatographic mobility as an authentic sample of the free 15-ketosterol.

\(^{(b)}\) Region B corresponds to a scraped region of \(R_f = 0.44-0.56\) and had the same chromatographic mobility as an authentic sample of 15-ketosteryl palmitate.
Figure 46. Distribution of free 15-ketosterol in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from CHO cells incubated in F12NCS5 containing 5 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one and 0.7 μM [4-14C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 47 illustrates the distribution of 15-ketosteryl esters in the centrifuged fractions. A total of 2.3 ± 0.3 nmoles 15-ketosteryl esters per mg protein was recovered in the sum of the centrifuged fractions. The 15-ketosteryl esters recovered in the floating lipid layer accounted for the greatest proportion (47.6%) of the total 15-ketosteryl esters. The upper and lower intermediate zones accounted for 3.1% and 16.9% of the total 15-ketosteryl ester, respectively. The pellet fraction contained 32.4% of the total 15-ketosteryl ester.

A total of 10.0 ± 1.2 nmoles total 15-ketosterol (free and esterified) were recovered in all of the centrifuged fractions. The distribution of free and esterified 15-ketosterol indicates that the greatest concentration of 15-ketosterol was in the free form in the pellet fraction (see Figure 48). The 15-ketosteryl ester recovered in the pellet fraction represented 74.0% of the total (free and esterified) 15-ketosterol recovered from all of the centrifuged fractions. The next higher concentration of 15-ketosterol was in the floating lipid layer in the esterified form; the 15-ketosteryl ester recovered in the floating lipid layer accounted for 10.7% of the total 15-ketosterol recovered in all of the fractions. The 15-ketosteryl ester recovered in the pellet fraction accounted for 7.3% of the total free and esterified 15-ketosterol recovered in all of the centrifuged fractions.

**Distribution of $^{14}$C**

The overall distribution of $^{14}$C in the centrifuged fractions was as follows. The floating lipid layer contained 12.2 ± 1.3% of the total $^{14}$C recovered in all of the fractions (see Table 27). The upper and lower intermediate zones contained 0.9 ± 0.2% and 6.0 ± 2.7%, respectively, of the total $^{14}$C recovered in all of the fractions. The pellet fraction contained 80.9 ± 8.3% of the total $^{14}$C recovered in all of the fractions.
Figure 47. Distribution of 15-ketosteryl esters in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from CHO cells incubated in F12NCS5 containing 5 μM [2,4- 3H]5α-cholesta-8(14)-en-3β-ol-15-one and 0.7 μM [4- 14C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 48. Overall distribution of free 15-ketosterol (striped bars) and 15-ketosteryl esters (solid bars) in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from CHO cells incubated in F12NCS5 containing 5 µM [2,4-^3^H]5α-cholest-8(14)-en-3β-ol-15-one and 0.7 µM [4-^14^C]cholesterol (Experiment 6) for 48 hours. The bars represent the mean value (N=3); the white bars represent the standard deviation.
Table 27. Summary of the distribution of $^{14}$C from centrifuged fractions derived from CHO K-1 cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^{14}$C in fraction (of total $^{14}$C contained in all fractions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>12.2 ± 1.3%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>0.9 ± 0.2%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>6.0 ± 2.7%</td>
</tr>
<tr>
<td>Pellet</td>
<td>80.9 ± 8.3%</td>
</tr>
</tbody>
</table>

(1) Experiment 6 incubation condition: 5 $\mu$M $[2,4,3H]5\alpha$-choleste-
8(15)-en-3\beta-ol-15-one, 0.7 $\mu$M [4-$^{14}$C]cholesterol in F12NCS5.
Figure 49 presents the chromatographic distribution of $^{14}$C recovered in each of the centrifuged fractions. Each of the fractions contained a peak of $^{14}$C that migrated to a region corresponding to an Rf of 0.18-0.24. This region corresponded to the mobility of an authentic sample of free cholesterol. The pellet fraction contained the greatest amount of $^{14}$C that co-migrated with the free cholesterol standard; the radioactivity contained in this region represented 89.0 $\pm$ 0.2% of the $^{14}$C recovered in the fraction (see Table 28). As the pellet fraction contained 81% of the total $^{14}$C recovered in all of the centrifuged fractions, this indicates that most of the $[^{14}C]$cholesterol incorporated into the cells from the growth medium remained in the free form and was recovered in the pellet fraction. In the floating lipid layer, only 1.8 $\pm$ 0.1% of the $^{14}$C migrated to the region corresponding to the migration of free cholesterol.

As seen in Figure 49, the floating lipid layer and the upper and lower intermediate zones each contained a peak of $^{14}$C that migrated to a region corresponding to an Rf of 0.68-0.79. This region corresponded to the mobility of an authentic sample of cholesteryl oleate. The radioactivity that co-migrated with the cholesteryl ester standard in the floating lipid layer represented 97.8 $\pm$ 0.1% of the radioactivity recovered in this fraction. In the pellet fraction, only 10.2 $\pm$ 0.2% of the $^{14}$C co-migrated with the cholesteryl ester standard (Table 28).

Less than 0.1% of the total $^{14}$C recovered in each fraction was recovered at the origin of the corresponding TLC plate and less than 2% of the total $^{14}$C recovered in each fraction was recovered in regions other than the two regions corresponding to the free and esterified cholesterol.

Figure 50 illustrates the distribution of free cholesterol in the centrifuged fractions. A total of 20.1$\pm$ 2.3 nmole free cholesterol per mg protein was recovered in the sum of the
Figure 49. Chromatographic distribution of $^{14}$C in the floating lipid layer (A), upper (B) and lower (C) intermediate zones, and pellet (D) fraction derived from K(15) cells. The incubation conditions (Experiment 6) for the cells and TLC conditions are described in the text.
Table 28. Summary of chromatographic distribution of $^{14}$C from centrifuged fractions derived from CHO K-1 cells (1).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
<th>Origin</th>
<th>All others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Lipid Layer</td>
<td>1.8 ± 0.1%</td>
<td>97.8 ± 0.1%</td>
<td>0.0%</td>
<td>0.3 ± 0.2%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>7.1 ± 1.4%</td>
<td>91.7 ± 3.0%</td>
<td>0.0%</td>
<td>1.2 ± 1.5%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>26.9 ± 5.1%</td>
<td>71.3 ± 6.4%</td>
<td>0.0%</td>
<td>1.8 ± 1.4%</td>
</tr>
<tr>
<td>Pellet</td>
<td>89.0 ± 0.2%</td>
<td>10.2 ± 0.2%</td>
<td>&lt;0.1</td>
<td>0.8 ± 0.1%</td>
</tr>
</tbody>
</table>

(1) Experiment 6 incubation condition: 5 μM [2,4,3H]5α-cholest-8(15)-en-3β-ol-15-one, 0.7 μM [4-$^{14}$C]cholesterol in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f = 0.18-0.24$ and had the same chromatographic mobility as an authentic sample of free cholesterol.

(b) Region B corresponds to a scraped region of $R_f = 0.68-0.79$ and had the same chromatographic mobility as an authentic sample of cholesteryl oleate.
Figure 50. Distribution of free cholesterol in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from CHO cells incubated in F12NCS5 containing 5 μM [2,4-\(^3\)H]5α-cholest-8(14)-en-3β-ol-15-one and 0.7 μM [4-\(^{14}\)C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
centrifuged fractions. The free cholesterol recovered in the pellet fraction (19.5 ± 2.0 nmoles/mg protein) accounted for the greatest proportion (97.1%) of the total free cholesterol. The floating lipid layer contained only 0.3% of the total free cholesterol.

Figure 51 illustrates the distribution of cholesteryl esters in the centrifuged fractions. A total of only 7.0 ± 1.1 nmoles cholesteryl esters per mg protein was recovered in the sum of the centrifuged fractions. The cholesteryl esters recovered in the floating lipid layer (3.3 ± 0.4 nmoles cholesteryl ester per mg protein) accounted for the greatest proportion (46.4%) of the total cholesteryl esters. The lower intermediate zone accounted for 17.7% of the total cholesteryl ester and the pellet fraction contained 32.0% of the cholesteryl ester.

A total of 27.1 ± 3.4 nmoles total cholesterol (free and esterified) were recovered in all of the centrifuged fractions (Figure 52). The distribution of free and esterified cholesterol indicated that the greatest concentration of cholesterol was in the free form in the pellet. The cholesterol recovered in this fraction represented 71.9% of the total (free and esterified) cholesterol recovered from all of the centrifuged fractions. The cholesteryl ester recovered in the floating lipid layer and the pellet fraction accounted for 12.0% and 8.3% of the total cholesterol recovered in all fractions. The remaining 7.8% of the total cholesterol was distributed in the free and esterified forms in the remaining centrifuged fractions.

**Distribution of Protein**

Aliquots from each of the centrifuged fractions as well as the cell homogenate before centrifugation, were assayed for protein following the procedure of Lowry et al. Triplicate aliquots of each of the three sample were assayed; the values were averaged and
Figure 51. Distribution of cholesteryl esters in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from CHO cells incubated in F12NCS5 containing 5 μM [2,4-3H]5α-cholesta-8(14)-en-3β-ol-15-one and 0.7 μM [4-14C]cholesterol for 48 hours. The black bars represent the mean value (N=3); the white bars represent the standard deviation.
Figure 52. Overall distribution of free cholesterol (striped bars) and cholesteryl esters (solid bars) in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from CHO cells incubated in F12NCS5 containing 5 µM [2,4-^3^H]5α-cholest-8(14)-en-3β-ol-15-one and 0.7 µM [4-^14^C]cholesterol (Experiment 6) for 48 hours. The bars represent the mean value (N=3); the white bars represent the standard deviation.
the standard deviation was calculated. It was determined that the total sample contained 1.90± 0.17 mg protein. The greatest amount of protein (60.9%) was recovered in the pellet fraction. The floating lipid layer contained 8.3% of the recovered protein. The upper and lower intermediate zones contained 4.8% and 26.0% of the recovered protein, respectively.
Comparison of the distribution of $^3$H in the centrifuged fractions following analysis of K(15) cells incubated in experiment 1 and experiment 2.

In the 15-ketosterol-adapted cells, the distribution of [2,4-$^3$H]5α-cholesta-
8(14)-en-3β-ol-15-one-derived lipids was compared between experiment 1 (40 μM
[2,4-$^3$H]5α-cholesta-8(14)-en-3β-ol-15-one + 0.6 μM [4-$^{14}$C]cholesterol in
F12NCS5 medium) and experiment 2 (40 μM [2,4-$^3$H]5α-cholesta-8(14)-en-3β-ol-
15-one + 0.33 μM [1-$^{14}$C]oleate in F12NCS5 medium). The only difference between
the two incubation conditions was that either [$^{14}$C]cholesterol or [$^{14}$C]oleate were
present in trace concentrations.

Table 29 presents a comparison of experiment 1 and experiment 2 with respect to
the overall distribution of tritium in the four centrifuged fractions. Table 30 presents a
comparison of the percent tritium of each fraction that was recovered in the regions
corresponding to the chromatographic mobilities of free 15-ketosterol ($R_f$=0.06-0.11)
and 15-ketosteryl esters ($R_f$=0.31-0.40). The purpose of these tables is to illustrate the
similarity between the results of the two experiments to demonstrate the reproducibility
of the experimental protocol.

The overall distribution of tritium in the centrifuged fractions was almost identical
in both sets of experiments (see Table 29). Statistical comparison of these results
indicated that there was no significant difference between the two sets of values. The
distribution of tritium in each of the centrifuged fractions in the regions corresponding
to the chromatographic mobilities of free 15-ketosterol and 15-ketosteryl esters was
also extremely similar (see Table 30). In the floating lipid layer and the upper and
Table 29. Comparison of the distribution of $^3$H from the centrifuged fractions derived from the 15-ketosterol-adapted cells of experiment 1(1) and experiment 2(2) .

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^3$H in fraction (of total $^3$H contained in all fractions)</th>
<th>Mean ± S. D. (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>41.5 ± 1.8%</td>
<td>45.6 ± 1.9%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>6.5 ± 0.6%</td>
<td>6.1 ± 1.1%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>16.5 ± 0.3%</td>
<td>12.0 ± 2.2%</td>
</tr>
<tr>
<td>Pellet</td>
<td>35.4 ± 2.1%</td>
<td>36.4 ± 0.6%</td>
</tr>
</tbody>
</table>

(1) Experiment 1 incubation condition 1: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.6 μM [4-$^{14}$C]cholesterol in F12NCS5.

(2) Experiment 2 incubation condition 2: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.
Table 30. Comparison of chromatographic distribution of $^3$H from the centrifuged fractions derived from 15-ketosterol-adapted cells from experiment 1(1) and experiment 2(2).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>1.8 ± 0.1%</td>
<td>1.9 ± 0.1%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>2.9 ± 0.6%</td>
<td>2.2 ± 0.5%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>10.8 ± 0.8%</td>
<td>10.5 ± 1.4%</td>
</tr>
<tr>
<td>Pellet</td>
<td>61.0 ± 3.2%</td>
<td>57.0 ± 0.9%</td>
</tr>
</tbody>
</table>

Mean ± S.D. (N=3)

(1) Experiment 1 incubation condition 1: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.6 μM [4-$^{14}$C] cholesterol in F12NCS5.

(2) Experiment 2 incubation condition 2: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f = 0.06-0.11$ and had the same chromatographic mobility as an authentic sample of the free 15-ketosterol.

(b) Region B corresponds to a scraped region of $R_f = 0.31-0.40$ and had the same chromatographic mobility as an authentic sample of 15-ketosteryl palmitate.
lower intermediate zones, the corresponding values from the two experiments were within 1% of each other. The only significant difference between the two experiments was that in the pellet fraction of the samples from experiment 1, 36.9 ± 2.9% of the tritium was recovered in the region corresponding to the 15-ketosteryl esters, while in the pellet fractions from experiment 2, 27.5 ± 1.2% of the tritium was recovered in that same region (p=0.007). The free 15-ketosterol recovered in the pellet fractions from the two experiments were not significantly different from one another (p>0.1).
Comparison of the distribution of $^3$H and $^{14}$C in the centrifuged fractions following analysis of K(15) cells incubated in experiment 2 and experiment 3.

Distribution of $^3$H

The distribution of $^3$H between samples from experiment 2 (40 µM [2,4-$^3$H]5α-cholest-8(14)-en-3β-ol-15-one + 0.33 µM [$^{14}$C]oleate in F12NCS5 medium) and experiment 3 (40 µM [2,4-$^3$H]5α-cholest-8(14)-en-3β-ol-15-one + 88 µM [$^{14}$C]oleate in F12NCS5 medium) is compared in this section. The difference between the two experiments was that the growth medium of experiment 2 contained a trace addition of [$^{14}$C]oleate while in experiment 3, the [$^{14}$C]oleate was added in a significant concentration (88 µM). Previous experiments in CHO K-1 cells have indicated that the addition of 88 µM oleate significantly reduces the decrease in cell growth observed when the wild type cells are incubated in concentrations exceeding 10 µM 5α-cholest-8(14)-en-3β-ol-15-one (Pinkerton et al., 1988). Analysis of the K(15) cell under similar conditions of oleate supplementation was pursued to gather data that would lead to an explanation of how the adapted cells are able to grow at normal rates in the presence of high concentrations of the 15-ketosterol (40 µM). It was also hoped that the information from the experiments with the K(15) cells would lead to an explanation of the effects that oleate supplementation has in the wild type cells also regarding cell growth at higher than normal concentrations of the 15-ketosterol.

The most notable difference between the two analyses was that the overall distribution of tritium among the centrifuged fractions was shifted in the samples from experiment 3 such that more tritium was recovered in the floating lipid layer (see Table 31). Significantly more tritium was recovered in the floating lipid layer from the
Table 31. Comparison of the distribution of $^3$H from the centrifuged fractions derived from 15-ketosterol-adapted cells from experiment 2(1) and experiment 3(2).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^3$H in fraction (of total $^3$H contained in all fractions)</th>
<th>Mean ± S. D. (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 2</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>45.6 ± 1.9%</td>
<td>61.3 ± 2.6%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>6.1 ± 1.1%</td>
<td>10.8 ± 4.8%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>12.0 ± 2.2%</td>
<td>5.2 ± 1.0%</td>
</tr>
<tr>
<td>Pellet</td>
<td>36.4 ± 0.6%</td>
<td>22.4 ± 2.4%</td>
</tr>
</tbody>
</table>

(1) Experiment 2 incubation condition 2: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.

(2) Experiment 3 incubation condition 3: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 88 μM [1-$^{14}$C]oleate in F12NCS5.
samples of experiment 3 than experiment 2. In experiment 2 (trace oleate), 45.6 ± 1.9% of the tritium was recovered in the floating lipid layer; in experiment 3 (88 nM oleate), 61.3 ± 2.6% of the tritium was recovered in the floating lipid layer (p=0.001). This increase in tritium recovered in the floating lipid layer is accompanied by a similar decrease (-14.0%, p< 0.001) in tritium recovered in the pellet fraction of experiment 3 compared to experiment 2.

In comparing the distribution of tritium within each of the centrifuged fractions of the samples from experiment 3 (see Table 32), it is noted that in the pellet fraction the percent tritium recovered in the region corresponding to the chromatographic mobility of the 15-ketosteryl esters decreased significantly (-14%, p= 0.0025) compared to experiment 2 while the percent of tritium recovered in the region of the free 15-ketosterol increased in the pellet (+14%, p= 0.0005). In the floating lipid layer, however, the relative distribution of tritium was not significantly different between the two experiments.

It is clear that the change in distribution is not due to any increase in incorporated 15-ketosterol as the quantities of free and esterified 15-ketosterol recovered in the samples from the different incubation conditions were very similar. In experiment 2, 30.8 ± 4.7 nmoles free 15-ketosterol per mg protein were recovered in the sum of all of the centrifuged fractions compared to 37.8 ± 2.1 nmoles free 15-ketosterol per mg protein in experiment 3. In experiment 2, 121.3 ± 14.6 nmoles 15-ketosteryl ester per mg protein was recovered in the sum of all of the centrifuged fractions compared to 115 ± 5.1 nmoles 15-ketosteryl ester per mg protein in experiment 3. Summing these values, in experiment 2, 152.1 ± 19.3 nmoles total 15-ketosterol (free + esterified) per mg protein was recovered in all of the centrifuged fractions compared to 153.2 ±7.2 nmoles total 15-ketosterol (free + esterified) per mg protein in experiment 3. The
Table 32. Comparison of chromatographic distribution of $^3$H from the centrifuged fractions derived from 15-ketosterol-adapted cells from experiment 2(1) and experiment 3(2).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 2</td>
<td>Experiment 3</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>1.9 ± 0.1%</td>
<td>2.5 ± 0.1%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>2.2 ± 0.5%</td>
<td>3.3 ± 0.7%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>10.5 ± 1.4%</td>
<td>22.4 ± 6.5%</td>
</tr>
<tr>
<td>Pellet</td>
<td>57.0 ± 0.9%</td>
<td>70.9 ± 2.2%</td>
</tr>
</tbody>
</table>

Mean ± S.D. (N=3)

(1) Experiment 2 incubation condition 2: 40 μM [2,4-$^3$H$\alpha$-cholest-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.

(2) Experiment 3 incubation condition 3: 40 μM [2,4-$^3$H$\alpha$-cholest-8(15)-en-3β-ol-15-one, 88 μM [1-$^{14}$C]oleate in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f = 0.06 - 0.11$ and had the same chromatographic mobility as an authentic sample of the free 15-ketosterol.

(b) Region B corresponds to a scraped region of $R_f = 0.31 - 0.40$ and had the same chromatographic mobility as an authentic sample of 15-ketosteryl palmitate.
amount of incorporated 15-ketosterol was essentially identical in both cases. Any explanation of the distribution change must not include any change in overall 15-ketosterol incorporation.

**Distribution of $^{14}$C**

In comparing the distribution of $^{14}$C in the samples from experiments 2 and 3, a similar shift in the overall distribution of $^{14}$C was observed as in the distribution of $^{3}$H (see Table 33). The floating lipid layer of experiment 3 contained significantly more $^{14}$C (~19%, $p = 0.00001$) than the corresponding fraction of experiment 2. There was 53% of the total $^{14}$C recovered in the floating lipid layer of experiment 3 while 34% of the total $^{14}$C was recovered in the floating lipid layer of experiment 2. This increase was accompanied by a similar decrease in the $^{14}$C recovered in the pellet fraction. The pellet fraction of experiment 3 contained significantly less $^{14}$C (~19%, $p = 0.0004$) than the corresponding fraction of experiment 2.

The most notable observations in comparing the $[^{14}$C]oleate-derived lipids in the samples of experiments 2 and 3 concern the $^{14}$C recovered in the regions having the same chromatographic mobilities as the 15-ketoseryl esters ($R_f = 0.37-0.46$) and triacylglycerols ($R_f = 0.49-0.57$) (see Table 34). When the K(15) cells were incubated in medium containing 0.33 μM $[^{14}$C]oleate (experiment 2), the majority of the $^{14}$C of the floating lipid layer and the intermediate zones was recovered in the regions corresponding to the 15-ketoseryl esters. Less than 4% of the $^{14}$C in the fractions was recovered in the region corresponding to the triacylglycerols. Under incubation conditions that included 88 μM $[^{14}$C]oleate (experiment 3), the $^{14}$C recovered in the region corresponding to the triacylglycerols increased significantly ($p = 0.00002$); 35.1% of
Table 33. Comparison of the distribution of $^{14}$C from the centrifuged fractions derived from 15-ketosterol-adapted cells from experiment 2(1) and experiment 3(2).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^{14}$C in fraction (of total $^{14}$C contained in all fractions)</th>
<th>Mean ± S. D. (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 2</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>34.2 ± 0.4%</td>
<td>53.0 ± 1.2%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>4.5 ± 0.8%</td>
<td>9.1 ± 3.2%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>9.1 ± 2.0%</td>
<td>4.9 ± 1.0%</td>
</tr>
<tr>
<td>Pellet</td>
<td>52.3 ± 1.8%</td>
<td>33.0 ± 2.4%</td>
</tr>
</tbody>
</table>

(1) Experiment 2 incubation condition 2: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.

(2) Experiment 3 incubation condition 3: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 88 μM [1-$^{14}$C]oleate in F12NCS5.
Table 34. Comparison of chromatographic distribution of $^{14}$C from the centrifuged fractions derived from 15-ketosterol-adapted cells from experiment $2^{(1)}$ and experiment $3^{(2)}$.

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 2</td>
<td>Experiment 3</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>87.7 ± 0.9%</td>
<td>54.7 ± 1.6%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>86.3 ± 1.9%</td>
<td>51.7 ± 4.6%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>76.9 ± 3.3%</td>
<td>36.6 ± 4.0%</td>
</tr>
<tr>
<td>Pellet</td>
<td>11.9 ± 0.6%</td>
<td>4.9 ± 0.7%</td>
</tr>
</tbody>
</table>

Mean ± S.D. (N=3)

(1) Experiment 2 incubation condition 2: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.33 μM [1-$^{14}$C]oleate in F12NCS5.

(2) Experiment 3 incubation condition 3: 40 μM [2,4-$^3$H]5α-cholest-8(15)-en-3β-ol-15-one, 88 μM [1-$^{14}$C]oleate in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f = 0.37-0.46$ and had the same chromatographic mobility as an authentic sample of 15-ketosteryl palmitate.

(b) Region B corresponds to a scraped region of $R_f = 0.49-0.57$ and had the same chromatographic mobility as an authentic sample of triolein.
the $^{14}$C recovered in the floating lipid layer was recovered in the triacylglycerols region.

At the same time, the percent $^{14}$C co-migrating with the 15-ketosteryl palmitate standard decreased from 87.7% in experiment 2 to 54.7% in experiment 3. The oleate supplement was incorporated into the 15-ketosteryl esters in a significantly less amount in experiment 3 than in experiment 2. The overall amount of [$^{3}$H]15-ketosteryl esters, however, was not significantly different between the two experiments. Fatty acids from within the cells must have been available for the esterification of the 15-ketosterol.

In both experiments, little $^{14}$C was recovered in the pellet fractions in the regions co-migrating with either the 15-ketosteryl esters or the triacylglycerol standards. About 78% of the $^{14}$C of the pellet fractions were recovered at the origin (data not shown in Table 34, see Tables 14 and 19). These results indicated that under an incubation condition primarily supplemented with 40 $\mu$M [$^{3}$H]5α-cholest-8(14)-en-3β-ol-15-one, the floating lipid layer derived from the K(15) cells consisted predominantly of 15-ketosteryl esters. When the experiment further included a $^{14}$C-oleate supplement (88 $\mu$M), the floating lipid layer was enriched in [$^{3}$H]5α-cholest-8(14)-en-3β-ol-15-one $^{14}$C-oleate and also contained a significant proportion of triacylglycerols containing $^{14}$C-oleate-derived fatty acids.
Comparison of sterol esters recovered in the floating lipid layer of
K(15) cells incubated in experiment 1 and experiment 4

In this section, experiment 1 (40 μM [2,4-\(^3\)H]5α-cholest-8(14)-en-3β-ol-15-one
+ 0.6 μM [4-\(^{14}\)C]cholesterol in F12NCS5 medium) is compared to experiment 4 (15
μM 5α-cholest-8(14)-en-3β-ol-15-one + 20.7 μM [4-\(^{14}\)C]cholesterol in Ham's
F12NCS5). The purpose of this comparison is to show that supplementation of the
growth medium of K(15) cells with additional cholesterol does not produce a floating
lipid layer enriched with cholesteryl esters in the same way that additional 15-ketosterol
produces a floating lipid layer enriched with 15-ketosteryl esters. The newborn calf
serum that is added in the proportion of 5% to Ham's F12 growth medium results in a
concentration of 18.8 μM free cholesterol. The total concentration of free cholesterol
in the growth medium of experiment 4 was 39.5 μM (20.7 μM cholesterol added via
ethanol solution). This concentration of cholesterol was chosen for comparison of the
results from samples of experiment 1 which contained 40 μM 15-ketosterol. It should
be noted for clarification, however, that the incubation conditions of experiment 1 (and
all other experiments) contained 5% NCS and therefore 18.8 μM free cholesterol as
well. The incubation conditions of experiments 1 and 4 were not designed to be rigorously analogous to one another. Rather, the intention was to investigate the effects of
the addition of a greater than routine concentration of free cholesterol to the growth
medium. It seemed logical to choose the same final concentration of cholesterol as the
concentration of the 15-ketosterone that produced a 15-ketosteryl ester-rich floating lipid
layer that was being studied in other experiments.

In the samples from experiment 4, it was calculated that there were only 4.9 ± 1.0
nmoles cholesteryl esters per mg protein in the floating lipid layer. In the case of the
samples from experiment 1, it was calculated that 61.6 ± 1.8 nmoles [\(^3\)H]15-ketosteryl...
esters per mg protein were recovered in the floating lipid layer. It was also calculated that 1.5 ± 0.02 nmoles [14C]cholesteryl esters per mg protein was present in the floating lipid layer of experiment 1.

A two-fold increase in cholesterol concentration in the growth medium did result in an increase of cholesteryl esters from 1.5 ± 0.02 nmoles per mg protein (experiment 1, 20.4 µM [14C]cholesterol) to 4.9 ± 1.0 nmoles cholesteryl esters per mg protein (experiment 4, 39.5 µM [14C]cholesterol). Although there was a three-fold increase in the amount of cholesteryl esters recovered in the floating lipid layer, this quantity of cholesteryl esters recovered in the floating lipid layer following incubation in 39.5 µM [14C]cholesterol, however, is only 8% of the quantity of 15-ketosteryl esters recovered in the floating lipid layer following incubation with 40 µM [3H]-15-ketosterol. It was concluded, therefore, that sterol esterification with subsequent formation of a floating lipid-rich layer containing the sterol esters is not an effect that can be observed by incubation of the K(15) cells in an increased concentration (39.5 µM free cholesterol from the serum supplement and added free cholesterol via the ethanol solution) of cholesterol.

In comparing the analysis of the CHO cells incubated in growth medium with and without the addition of the 15-ketosterol (experiment 5: 2.7 μM [4-14C]cholesterol in F12NCS5 medium; experiment 6: 5.8 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one + 0.7 μM [4-14C]cholesterol in F12NCS5 medium), the most important observation was that the presence of the 15-ketosterol had little affect on the distribution of [14C]cholesterol-derived lipids. Since the cholesterol contributed by the 5% NCS supplement to the growth medium was equivalent to 18.8 μM free cholesterol, the final concentration for experiment 5 was 21.5 μM cholesterol. The final concentration for experiment 6 was 20.7 μM cholesterol.

The overall distribution of 14C in all of the centrifuged fractions was similar between experiment 6 (+ 5 μM 15-ketosterol) and experiment 5 (0 μM 15-ketosterol). Table 35 presents a comparison between the distribution of 14C in the samples derived from the CHO cells incubated in experiments 5 and 6. Only a slight change was observed in the overall distribution of 14C in the centrifuged fractions (see Table 35). There was a small but significant decrease in the 14C recovered in the floating lipid layer in the samples from experiment 6 compared to experiment 5 (-5%, p=0.009). The 14C recovered in the pellet fractions, however, was not significantly different (p=0.37) between the two incubation conditions. Further, significant differences were not observed between the intermediate zones of experiment 5 and 6. It was concluded, therefore, that little difference was observed with respect to the overall distribution of 14C in the centrifuged fractions derived from the CHO cells.
Table 35. Comparison of the distribution of $^{14}$C from the centrifuged fractions derived from CHO K-1 cells from experiment 5$^{(1)}$ and experiment 6$^{(2)}$.

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Percent $^{14}$C in fraction (of total $^{14}$C contained in all fractions)</th>
<th>Mean ± S. D. (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 5</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>17.2 ± 1.3%</td>
<td>12.2 ± 1.3%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>1.2 ± 0.3%</td>
<td>0.9 ± 0.2%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>8.1 ± 1.9%</td>
<td>6.0 ± 2.7%</td>
</tr>
<tr>
<td>Pellet</td>
<td>73.4 ± 9.7%</td>
<td>80.9 ± 8.3%</td>
</tr>
</tbody>
</table>

(1) Experiment 5 incubation condition 5: 2.7 μM [4-$^{14}$C]cholesterol in F12NCS5.

(2) Experiment 6 incubation condition 6: 5 μM [2,4-$^{3}$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.7 μM [4-$^{14}$C]cholesterol in F12NCS5.
The distribution of $^{14}$C in the regions corresponding to the chromatographic mobilities of cholesterol and cholesteryl esters was not significantly different between the 15-ketosterol-supplemented and non-supplemented incubation conditions (see Table 36). The percentage values were not statistically significantly different in any of the corresponding fractions of the two experiments. It was concluded, therefore, that the addition of 5α-cholest-8(14)-en-3β-ol-15-one does not affect the distribution of $[^{14}$C]cholesterol-derived lipids in the CHO cells.
Table 36. Comparison of chromatographic distribution of $^{14}$C from the centrifuged fractions derived from 15-ketosterol-adapted cells from experiment 5(1) and experiment 6(2).

<table>
<thead>
<tr>
<th>Centrifuged Fraction</th>
<th>Region A (a)</th>
<th>Region B (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 5</td>
<td>Experiment 6</td>
</tr>
<tr>
<td>Floating Lipid Layer</td>
<td>2.1 ± 0.1%</td>
<td>1.8 ± 0.1%</td>
</tr>
<tr>
<td>Upper Intermediate Zone</td>
<td>6.7 ± 3.2%</td>
<td>7.1 ± 1.4%</td>
</tr>
<tr>
<td>Lower Intermediate Zone</td>
<td>32.0 ± 5.0%</td>
<td>26.9 ± 5.1%</td>
</tr>
<tr>
<td>Pellet</td>
<td>91.1 ± 1.2%</td>
<td>89.0 ± 0.2%</td>
</tr>
</tbody>
</table>

Mean ± S.D. (N=3)

(1) Experiment 5 incubation condition 5: 2.7 μM [4-$^{14}$C]cholesterol in F12NCS5.

(2) Experiment 6 incubation condition 6: 5 μM [2,4-$^{3}$H]5α-cholest-8(15)-en-3β-ol-15-one, 0.7 μM [4-$^{14}$C]cholesterol in F12NCS5.

(a) Region A corresponds to a scraped region of $R_f = 0.18-0.24$ and had the same chromatographic mobility as an authentic sample of free cholesterol.

(b) Region B corresponds to a scraped region of $R_f = 0.68-0.79$ and had the same chromatographic mobility as an authentic sample of cholesteryl oleate.
**CHO wild type cells versus 15-ketosterol-adapted cells: Comparison of experiment 6 (CHO) and experiment 1 (K(15))**

A comparison between the distribution of $[{}^3\text{H}]15$-ketosterol-derived lipids in the CHO cells and the K(15) cells is presented in this section. The CHO cells were incubated under the conditions of experiment 6 (5.8 $\mu$M $[2,4-{}^3\text{H}]5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one + 0.7 $\mu$M $[4-{}^{14}\text{C}]$cholesterol in F12NCS5 medium); the K(15) cells were incubated under the conditions of experiment 1 (40 $\mu$M $[2,4-{}^3\text{H}]5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one + 0.6 $\mu$M $[4-{}^{14}\text{C}]$cholesterol in F12NCS5 medium).

The CHO cells were grown in the presence of only 5.8 $\mu$M $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one as the growth of CHO cells is adversely affected by high concentrations of the 15-ketosterol. It has been demonstrated (Pinkerton et al., 1988) that the growth of CHO cells (as measured by total cell protein) significantly decreased when incubated in lipid-rich medium containing concentrations exceeding 10 $\mu$M $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one. In that experiment, CHO cells were plated into lipid-rich medium and incubated for 48 hours. At that time, the routine growth medium was replaced by F12FCS8 containing 0, 10, 12.5, 15, 20, 25, or 50 $\mu$M $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one. The incubation medium containing 0 $\mu$M 15-ketosterol served as a control. After an additional 48 hours, the cultures were assayed for protein following the procedure of Lowry et al. It was observed that a concentration of 10 $\mu$M $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one resulted in a 50% decrease in cell protein from the control plates. Because of these results, in the experiment discussed here, the distribution of $[{}^3\text{H}]15$-ketosterol-derived lipids was investigated in the CHO cells upon incubation in a concentration of 5.8 $\mu$M $5\alpha$-cholest-8(14)-en-3$\beta$-ol-15-one, a level well below the concentration shown to cause a major inhibition of cell growth.
The results of the CHO K-1 distribution were compared to the distribution of
[^3H]15-ketosterol-derived lipids in the 15-ketosterol-adapted cells when incubated at a
concentration of 40 μM, a concentration that does not affect growth of the adapted cell
line. The most striking difference between the two cell types is observed when
comparing the distribution of free 15-ketosterol and 15-ketosteryl esters. Figure 53
presents the distribution in the CHO cells and the K(15) cells. The obvious difference
is that while very little 15-ketosteryl esters (a total of 1.1 ± 0.06 nmols 15-ketosteryl
esters per mg protein) were recovered in the floating lipid layer of the wild type cells, a
total of 61.1 ± 1.8 nmols 15-ketosteryl esters per mg protein were recovered in the
floating lipid layer from the 15-ketosterol-adapted cells. That is a 60-fold increase in
15-ketosteryl esters recovered in the floating lipid layer while the concentration of free
15-ketosterol in the medium was increased only eight-fold!

The pellet fractions of both cell types contained a significant amount of free
15-ketosterol: 7.7 ± 0.9 nmols 15-ketosterol per mg protein in the CHO cells versus
33.4 ± 3.6 nmole 15-ketosterol per mg protein in the K(15) cells. The K(15) cells
contained four times as much free 15-ketosterol in the pellet as the CHO cells.
Recovery of more free 15-ketosterol was anticipated in the K(15) cells as there was an
eight-fold increase in the concentration of the free-15-ketosterol present in the growth
medium of the K(15) cells.

There was a 15-fold increase in the overall incorporation of the total 15-ketosterol
in the K(15) cells over the CHO cells. The total 15-ketosterol incorporated (free and
esterified) in the CHO cells was 10.0 ± 1.2 nmoles 15-ketosterol per mg protein; the
total 15-ketosterol (free and esterified) incorporated in the K(15) cells was 150.9 ± 9.5
nmoles 15-ketosterol per mg protein.
Figure 53. Overall distribution of free 15-ketosterol (striped bars) and 15-ketosteryl esters (solid bars) in the floating lipid layer, upper and lower intermediate zones, and pellet fraction derived from (A) K(15) cells incubated in F12NCS5 containing 40 μM [2,4,3-H]5α-cholesta-8(14)-ene-3β-ol-15-one and 0.6 μM [4-14C]cholesterol and (B) CHO cells incubated in F12NCS5 containing 5 μM [2,4,3-H]5α-cholesta-8(14)-ene-3β-ol-15-one and 0.7 μM [4-14C]cholesterol for 48 hours. The bars represent the mean value (N=3); the white bars represent the standard deviation.
The floating lipid layer of the K(15) cells incubated in 40 μM 5α-cholest-8(14)-en-3β-ol-15-one was composed predominantly of 15-ketosteryl esters. The association of a large concentration of 15-ketosteryl esters with the floating lipid layer may be correlated with the appearance of lipid-rich inclusions in the cytoplasm of these cells (see Figures 6 and 7 in the General Characterization chapter). This formation of 15-ketosteryl ester-rich inclusions may be the mechanism by which these 15-ketosterol-adapted cells remain unaffected at concentrations as high as 40 μM 5α-cholest-8(14)-en-3β-ol-15-one while a concentration of 10 μM 5α-cholest-8(14)-en-3β-ol-15-one causes a 50% decrease in cell protein in the CHO cells. The formation of large quantities of 15-ketosteryl esters by the K(15) cells may indicate that since the 15-ketosterol is in the esterified form and sequestered in these cytoplasmic inclusions, the cells have an increased ability to grow in a concentration of the 15-ketosterol that is extremely inhibitory in the CHO K-1 wild type cell.

The idea that incorporated 15-ketosterol in the form of 15-ketosteryl esters does not negatively affect cell growth in the same manner as the free 15-ketosterol is consistent with the results of other experiments described earlier. Cell growth is significantly less affected by incubation of CHO K-1 cells in high concentrations of the 15-ketosterol when the growth medium is supplemented with 88 μM oleate. With the addition of oleate, a 50% decrease in cell protein is not observed until 22.5 μM 5α-cholest-8(14)-en-3β-ol-15-one (compared to 10 μM 5α-cholest-8(14)-en-3β-ol-15-one without oleate supplementation). The availability of oleate may allow greater esterification of the 15-ketosterol; an increased synthesis of 15-ketosteryl esters may lead to formation of lipid rich inclusions, thereby sequestering the 15-ketosterol such that it has little or no effect on cell growth.

In previous studies in L cells, Schroepfer et al. (1979) found that incubation of the cells in medium containing palmitate and sulfate esters of the 15-ketosterol did not in-
hibit sterol synthesis. In this study, however, it was not determined whether the ester of the 15-ketosterol actually was incorporated by the cells. Taylor et al. (1984) investigated oxygenated sterols with respect to their relative binding to the oxysterol-binding protein. It was demonstrated that oxygenated sterols that have a 3-keto group substituted for the 3-hydroxy group or the addition of a 4,4-dimethyl group bind more poorly to the oxysterol-binding protein compared to their 3-hydroxy counterparts. Although the role of the oxysterol-binding protein has not yet been clearly defined, it is believed to play a role in the regulation of the HMG-CoA reductase enzyme (Kandutsch et al., 1984; Taylor et al., 1984). The long-chain fatty acid esters of the 15-ketosterol found in the K(15) cells may also bind poorly to the oxysterol-binding protein. The 15-oxygenated sterol that is very potent as an inhibitor of HMG-CoA reductase in the free form may not interfere with the regulation of the HMG-CoA reductase in the esterified form.
C. Excretion of Incorporated $5\alpha$-Cholest-8(14)-en-3β-ol-15-one
in the 15-Ketosterol-Adapted Cells and the CHO K-1 Cells
Introduction

It has been shown that incubation of the K(15) cells in 5α-cholest-8(14)-en-3β-ol-15-one-containing medium resulted in the formation of cytoplasmic lipid-rich regions that were composed predominantly of 15-ketosteryl esters. The purpose of this series of experiments was to determine the effect of removing the 5α-cholest-8(14)-en-3β-ol-15-one from the growth medium of K(15) cells that had already accumulated these lipid-rich regions. It was of particular interest to determine whether the 15-ketosterol would be excreted from the cells when exogenous 15-ketosterol was no longer present in the growth medium.

The general procedure included first loading the cells with radiolabeled 15-ketosteryl esters by incubation in growth medium containing [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one. The cells were subsequently transferred to routine growth medium, Ham's F12 + 5% NCS. The possible excretion of incorporated 15-ketosterol was monitored. CHO K-1 wild type cells were also assayed under similar conditions. In some cases, a trace of [4-14C]cholesterol was added to monitor the movement of cholesterol as well. At specified time intervals, aliquots of the medium were collected and the cells were harvested. Radioactivity was assayed for all samples.

As with the incorporation experiments, the samples of medium were extracted for lipids and analyzed by radio-TLC to determine the relative amount of free and esterified sterols present in the medium.

Methods

15-Ketosterol-adapted cells (2.2 x 10^6 cells/plate) were plated in 150 mm plates containing 25 ml Ham's F12 growth medium + 5% NCS containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one. After a 24 hour growth period, the culture plates
were changed to the experimental medium (designated as the incorporation medium) described below:

Experiment 1: Ham's F12 growth medium + 5% NCS containing 15 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one (3.7 μCi/plate) + 1.5 μM [4-14C]cholesterol (2.1 μCi/plate) or

Experiment 2: Ham's F12 growth medium + 5% NCS containing 40 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one (4.4 μCi/plate).

In the case of the CHO K-1 cells, the incorporation medium was:

Experiment 3: Ham's F12 + 5% NCS containing 5 μM [2,4-3H]5α-cholest-8(14)-en-3β-ol-15-one (2.9 μCi/plate) or

Experiment 4: Ham's F12 + 5% NCS containing 1.7 μM [4-14C]cholesterol (2.4 μCi/plate).

After the 24 hour incorporation or loading period in which the cells incorporated the [2,4-3H] 15-ketosterol and [4-14C]cholesterol and accumulated lipid-rich regions, the cells were rinsed two times with PBS (10 ml) and F12 growth medium + 5% NCS (25 ml) was added. This growth medium is sometimes referred to as the excretion medium because it is the medium into which the cells excreted the incorporated 15-ketosterol.

At designated time intervals from 0 to 48 hours after the radiolabeled medium was replaced with medium containing no 15-ketosterol supplement, aliquots (10 ml) of the medium were collected and the remaining medium was aspirated. This period of 48 hours is sometimes referred to as the excretion period. After rinsing twice with PBS (10 ml), the cells were harvested by trypsinization. Trypsin (5.0 ml; 0.05%) was
added and the plates incubated at 37 °C for 7.5 minutes. Soybean trypsin inhibitor (5.0 ml; 1.5 mg/ml), was added to each plate. The cells were collected in a 15 ml conical centrifuge tube and the plates were rinsed twice with growth medium (2.5 ml). The cells were collected by centrifugation for 5 minutes (1500 rpm). The supernatant was decanted and the cells were resuspended in water (2.0 ml) to promote cell swelling. The suspension was homogenized by aspirating four times through a 23 gauge needle fitted to a 3 ml syringe. Aliquots (100 µl) of the media and cell homogenates were transferred to scintillation vials; Scintisol (5 ml) was added and the samples were counted in the Packard 1500 scintillation counter. This procedure was repeated at 6,12, 24, and 48 hours after the removal of the 15-ketosterol supplement.

For the medium samples, aliquots (1.0 ml) were extracted by the method of Folch et al. (1957). The extracts were analyzed as described in the incorporation experiment.
Results

*K(15) cells: Experiment 1*

**Excretion of [2,4-\textsuperscript{3}H]5α-cholest-8(14)-en-3β-ol-15-one**

Figure 54 illustrates the excretion of incorporated 5α-cholest-8(14)-en-3β-ol-15-one as a function of time in the K(15) cells incubated in a loading medium consisting of Ham's F12 medium + 5% NCS containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one + 1.5 μM cholesterol. The top panel shows the quantity of the 15-ketosterol recovered in the medium expressed in nmoles. Clearly, the rate of excretion was maximal during the first 12 hours after removal of the 15-ketosterol supplement from the medium and by 12 hours, 72.7 ± 4.3 nmoles 15-ketosterol had been excreted into the medium.

The bottom panel of Figure 54 shows the amount of excreted 15-ketosterol expressed as a percent of the total 15-ketosterol (79.1 ± 6.2 nmoles) that had been incorporated during the previous 24 hour loading period. By 12 hours, 87.5 ± 0.6% of the total incorporated 15-ketosterol had been excreted into the growth medium.

Figure 55 illustrates the distribution of free and esterified 15-ketosterol recovered in the growth medium during the excretion period. The top panel show the nmoles of free 15-ketosterol and 15-ketosteryl esters recovered in the medium. The amount of free 15-ketosterol recovered in the medium was maximal after 12 hours; 53.5 ± 3.2 nmoles free 15-ketosterol was recovered. The amount of 15-ketosteryl esters recovered in the medium increased during the 48 hours of the experiment, reaching 31.7 ± 0.7 nmoles 15-ketosteryl esters in the medium.

The bottom panel presents the percent 15-ketosteryl esters of the total 15-ketosterol recovered in the medium. After 3 hours, 21.0 ± 0.4 % of the recovered 15-ketosterol
Figure 55. Distribution of 15-ketosterol (free and esterified) in the total 15-ketosterol excreted into F12NCS5 medium as a function of time following a 48-hour incubation in F12NCS5 + 15 μM [3H]15-ketosterol + 1.5 μM [3H]cholesterol, K(15) cells. Top: nmoles free 15-ketosterol [o-o] and nmoles 15-ketosteryl esters [●-●] recovered in the medium; Bottom: percent of 15-ketosteryl esters [■-■] of the total 15-ketosterol recovered in the medium.
was in the esterified form. By 48 hours, 37.1±1.2% of the total recovered 15-ketosterol was in the esterified form.

**Appearance of [4-^{14}C]cholesterol**

Figure 56 illustrates the appearance of incorporated radiolabeled cholesterol as a function of time in the K(15) cells incubated in a loading medium consisting of Ham's F12 medium + 5% NCS containing 15 μM [2,4-^{3}H]5α-cholest-8(14)-en-3β-ol-15-one + 1.5 μM [4-^{14}C]cholesterol. The top panel shows the quantity of cholesterol recovered in the medium expressed in nmoles. The amount of [^{14}C]cholesterol recovered in the medium increased during the 48 hours of the experiment, reaching 42.2 ± 0.4 nmoles [^{14}C]cholesterol in the medium after 48 hours.

The bottom panel of Figure 56 shows the amount of [^{14}C]cholesterol that appeared in the medium during the excretion period expressed as a percent of the total [^{14}C]cholesterol (96.7 ± 11.8 nmoles) that had been incorporated during the 24 hour loading period. After 3 hours, 12.2 ± 0.2% of the total incorporated [^{14}C]cholesterol was recovered in the growth medium. After 48 hours, 39.7 ± 0.2% of the total incorporated [^{14}C]cholesterol was recovered in the growth medium. It should be noted that in the case of cholesterol, the movement of cholesterol cannot be described solely as incorporation or excretion. As cholesterol is found in the plasma membrane, exchange of cholesterol between the growth medium and the plasma membrane also occurs. In the experiments described here, no effort was made to distinguish between exchange and incorporation or excretion. For the purposes of this experiment, all movements will be described as incorporation or excretion.
Figure 57 illustrates the distribution of free and esterified \(^{14}\text{C}\)cholesterol recovered in the growth medium during the excretion period. The figure shows the nmoles of free \(^{14}\text{C}\)cholesterol and \(^{14}\text{C}\)cholesteryl esters recovered in the medium. By 48 hours, only \(2.7 \pm 0.02\) nmoles \(^{14}\text{C}\)cholesteryl esters were recovered in the medium. This represented only 6\% of the total \(^{14}\text{C}\)cholesterol recovered in the medium.
Figure 57. Distribution of free and esterified $^{14}C$cholesterol in the total $^{14}C$-cholesterol recovered in the F12NCS5 medium as a function of time following a 48 hour incubation in F12NCS5 + 15 μM $^3$H15-ketosterol + 1.5 μM $^{14}C$cholesterol; K(15) cells. Nmoles free $^{14}C$cholesterol [o---o] and $^{14}C$cholesteryl esters [•---•] recovered in the medium.
**K(15) cells: Experiment 2**

**Excretion of [2,4-\(^3\)H]5α-cholest-8(14)-en-3β-ol-15-one**

Figure 58 illustrates the excretion of incorporated 5α-cholest-8(14)-en-3β-ol-15-one as a function of time in the K(15) cells incubated in a loading medium consisting of Ham's F12 medium + 5% NCS containing 40 μM 5α-cholest-8(14)-en-3β-ol-15-one. The top panel shows the quantity of the 15-ketosterol recovered in the medium expressed in nmoles. The rate of excretion was maximal during the first 12 hours after removal of the 15-ketosterol supplement from the medium. By 12 hours, 178.8 ± 9.0 nmoles 15-ketosterol had been excreted into the medium. Excretion of the 15-ketosterol continued to increase until 24 hours at which time 209.4 ± 9.5 nmoles 15-ketosterol were recovered in the medium.

The bottom panel of Figure 58 shows the amount of excreted 15-ketosterol expressed as a percent of the total 15-ketosterol that had been incorporated (291.0 ± 15.0 nmoles) during the loading period. By 24 hours, 65.7 ± 3.3% of the total incorporated 15-ketosterol had been excreted into the growth medium.

Figure 59 illustrates the distribution of free and esterified 15-ketosterol recovered in the growth medium during the excretion period. The top panel shows the nmoles of free 15-ketosterol and 15-ketosteryl esters recovered in the medium. The amount of free 15-ketosterol recovered in the medium was maximal after 24 hours; 193.9 ± 8.8 nmoles free 15-ketosterol was recovered. The amount of 15-ketosteryl esters continued to increase during the entire 48 hours of the experiment; after 48 hours, 36.1 ± 2.3 nmoles of the total 15-ketosterol recovered in the medium was in the esterified form. The bottom panel presents the percent 15-ketosteryl esters of the total 15-ketosterol incorporated (291.0 ± 15.0 nmoles) during the 24 hour loading period recovered in the
Figure 58. Excretion of incorporated 15-ketosterol into F12NCS5 medium as a function of time following a 48 hour incubation in F12NCS5 + 40 μM \(^{3}H\)15-ketosterol, K(15) cells. Top: nmole total 15-ketosterol [●-●] excreted. Bottom: percent of total incorporated 15-ketosterol [◇-◇] excreted.
Figure 59. Distribution of free and esterified 15-ketosterol in the total 15-ketosterol excreted into F12NCS5 medium as a function of time following a 48 hour incubation in F12NCS5 + 40 μM [³H]15-ketosterol; K(15) cells. Top: nmoles free 15-ketosterol [o--o] and 15-ketosteryl esters [●--●] recovered in the medium; Bottom: percent of 15-ketosteryl esters [■--■] of the total 15-ketosterol recovered in the medium.
medium. After 24 hours, about 7% of the total 15-ketosterol was in the esterified form.

By 48 hours, about 20% of the recovered 15-ketosterol was in the esterified form.
**CHO K-1: Experiment 3**

**Excretion of \(12.4^{3}H15\alpha\text{-cholesterol-8(14)-en-3\beta\text{-ol-15-one}}\)**

Figure 60 illustrates the excretion of incorporated 5\(\alpha\text{-cholesterol-8(14)-en-3\beta\text{-ol-15-one}}\) as a function of time in the CHO K-1 cells incubated in a loading medium consisting of Ham's F12 medium + 5% NCS containing 5 \(\mu\text{M} [2,4^{3}H]5\alpha\text{-cholesterol-8(14)-en-3\beta\text{-ol-15-one}}\). The top panel shows the quantity of the 15-ketosterol recovered in the medium expressed in nmoles. Clearly, the rate of excretion was maximal during the first 6 hours after removal of the 15-ketosterol supplement from the medium. By 6 hours, 25.0 \(\pm\) 0.0 nmoles 15-ketosterol had been excreted into the medium.

The bottom panel of Figure 60 shows the amount of excreted 15-ketosterol expressed as a percent of the total 15-ketosterol that had been incorporated (28.7 \(\pm\) 2.8 nmoles) during the 24 hours loading period. By 6 hours, 92.9 \(\pm\) 0.0% of the total incorporated 15-ketosterol had been excreted into the growth medium.

Figure 61 illustrates the distribution of free and esterified 15-ketosterol recovered in the growth medium during the excretion period. The figure shows the nmoles of free 15-ketosterol and 15-ketosteryl esters recovered in the medium. The amount of free 15-ketosterol recovered in the medium was maximal after 6 hours; 24.3 \(\pm\) 0.0 nmoles free 15-ketosterol was recovered. Less than 10% of the total 15-ketosterol recovered in the medium were recovered in the esterified form during the 48 hours of the experiment, reaching only 2.9 \(\pm\) 0.4 nmoles 15-ketosteryl esters in the medium compared to 29.5 \(\pm\) 4.2 nmoles free 15-ketosterol after 48 hours.
Figure 60. Excretion of incorporated 15-ketosterol into F12NCS5 medium as a function of time following a 48-hour incubation in F12NCS5 + 5 µM [3H]15-ketosterol, CHO K-1 cells. Top: nmoles total 15-ketosterol [●--●] excreted. Bottom: percent of total incorporated 15-ketosterol [◇--◇] excreted.
Figure 61. Distribution of free and esterified 15-ketosterol in the total 15-ketosterol excreted into F12NCS5 medium as a function of time following a 48-hour incubation in F12NCS5 + 5μM [3H]15-ketosterol, CHO K-1 cells. Nmoles free 15-ketosterol [o--o] and 15-ketosteryl esters [●--●] recovered in the medium.
CHO K-1: Experiment 4

Appearance of [4-\(14\)C]cholesterol

Figure 62 illustrates the excretion of incorporated [\(14\)C]cholesterol as a function of time in the CHO K-1 cells incubated in a loading medium consisting of Ham's F12 medium + 5% NCS containing 1.7 \(\mu\)M [4-\(14\)C]cholesterol. The top panel shows the quantity of [\(14\)C]cholesterol recovered in the medium expressed in nmoles. Although the rate of appearance of [\(14\)C]cholesterol was clearly greatest during the first hours of the excretion period, the amount of [\(14\)C]cholesterol recovered in the medium increased during the 48 hours of the experiment, reaching 30.9 ± 1.2 nmoles [\(14\)C]cholesterol in the medium after 48 hours.

The bottom panel of Figure 62 shows the amount of [\(14\)C]cholesterol that appeared in the medium during the excretion period expressed as a percent of the total [\(14\)C]cholesterol (42.2 ± 5.3 nmoles) that had been incorporated during the 24 hour loading period. After 12 hours, 46.7 ± 1.6% of the total incorporated [\(14\)C]cholesterol had been excreted into the growth medium. After 48 hours, 30.9 ± 1.7 nmoles or 66.1 ± 3.7% of the total incorporated [\(14\)C]cholesterol had been excreted into the growth medium.

Figure 63 illustrates the distribution of free and esterified [\(14\)C]cholesterol recovered in the growth medium during the excretion period. The figure shows the nmoles of free [\(14\)C]cholesterol and [\(14\)C]cholesteryl esters recovered in the medium. After 48 hours, only 1.1 ± 0.3 nmoles [\(14\)C]cholesteryl esters were recovered in the medium. This represents only 3% of the total [\(14\)C]cholesterol recovered in the medium.
Figure 63. Distribution of free and esterified $[^1{}^{14}C]$cholesterol in the total $[^1{}^{14}C]$cholesterol recovered in the F12NCS5 medium as a function of time following a 48-hour incubation in F12NCS5 + 1.7 $\mu$M $[^1{}^{14}C]$cholesterol, CHO K-1 cells. Nmoles free cholesterol [o-o] and cholesteryl esters [●-●] recovered in the medium.
Discussion of Results

In the K(15) cells incubated in a loading medium containing 40 μM [2,4-\textsuperscript{3}H]5α-cholest-8(14)-en-3β-ol-15-one, 66% of the incorporated 15-ketosterol was excreted by 24 hours. In K(15) cells incubated in a loading medium containing 15 μM [2,4-\textsuperscript{3}H]5α-cholest-8(14)-en-3β-ol-15-one, 88% of the incorporated 15-ketosterol was excreted within 12 hours. The CHO K-1 cells that had been loaded from a medium supplemented with 5 μM [2,4-\textsuperscript{3}H]5α-cholest-8(14)-en-3β-ol-15-one excreted 93% of the incorporated 15-ketosterol by 6 hours after removing the 15-ketosterol-supplemented medium. It appeared that the greater the amount of 15-ketosterol that had been incorporated during the loading period, the longer the time required to reach maximum excretion.

The movement of cholesterol was very similar in both the K(15) cells and the CHO K-1 cells. Data concerning the movement of cholesterol is more complicated to analyze. The loading medium was supplemented with 2.1 μCi [4-\textsuperscript{14}C]cholesterol/plate (55 μCi/μmol). Both the loading medium and the excretion medium contained 18.8 μM non-radiolabeled free cholesterol. (NCS was determined to have 14.5 mg free cholesterol per dl.) The amount of free non-radiolabeled cholesterol was the same in both cases. In this experiment, the incorporation of the radiolabeled cholesterol by the cells and subsequent reappearance of the radiolabeled cholesterol in the medium was monitored.

In the K(15) cells, after having been incubated in a loading medium containing 15 μM [2,4-\textsuperscript{3}H]5α-cholest-8(14)-en-3β-ol-15-one + 1.5 μM [4-\textsuperscript{14}C]cholesterol, it was found that the amount of [\textsuperscript{14}C]cholesterol recovered in the medium continued to increase for the duration of the excretion period, see Figure 56. After 48 hours, 40% of the incorporated [\textsuperscript{14}C]cholesterol was recovered in the medium.
Analysis of the floating lipid layer from the K(15) cells, (discussed in a previous section) revealed that only 27% of the incorporated cholesterol was recovered as cholesteryl esters in the floating lipid layer. The majority, 67%, of the incorporated cholesterol was recovered in the free form in the pellet fraction. One might propose, then, that much of the $[^{14}\text{C}]$cholesterol was incorporated into the plasma membranes of these cells. The radiolabeled cholesterol that was recovered in the growth medium during the excretion period may have been the result of an exchange of radiolabeled cholesterol incorporated in the plasma membrane with unlabeled cholesterol present in the medium rather than the result of excretion. Very little $[^{14}\text{C}]$cholesterol recovered in the medium was identified as $[^{14}\text{C}]$cholesteryl esters. $[^{14}\text{C}]$Cholesteryl esters represented only 6% of the total excreted $[^{14}\text{C}]$cholesterol after 48 hours.

In the CHO K-1 cells, movement of the $[^{14}\text{C}]$cholesterol was very similar to that observed in the K(15) cells. The appearance of $[^{14}\text{C}]$cholesterol continued throughout the 48-hour duration of the experiment. Less than 4% of the total $[^{14}\text{C}]$cholesterol recovered in the medium was identified as $[^{14}\text{C}]$cholesteryl esters after 48 hours.

In the K(15) cells incubated in an incorporation medium containing 40 μM $[2,4-^3\text{H}]$5α-cholest-8(14)-en-3β-ol-15-one, significant amounts of the 15-ketosterol recovered in the medium were identified as 15-ketosteryl esters. It is unknown whether the 15-ketosterol was directly excreted in the esterified form, or if esterification may have taken place in the medium. As shown in Figure 59, at 24 hours after the loading medium was changed, 7% of the total 15-ketosterol was in the esterified form. By 48 hours, 20% of the total 15-ketosterol was shown to be 15-ketosteryl esters.

The important feature to note is that while maximum excretion of 15-ketosterol into the medium had occurred by 24 hours, only 7% of the total 15-ketosterol was found in
the esterified form at that time. While excretion of total 15-ketosterol did not increase over the time period between 24 and 48 hours, 15-ketosterol esterification increased from 7% to 20%. It may be that since the amount of 15-ketosteryl esters increased three-fold well after the excreted 15-ketosterol had reached a maximum in the medium, that it may not have been excreted in the esterified form. There may be some mechanism for esterification of the 15-ketosterol in the medium. The possibility of lecithin:cholesterol acyl transferase (LCAT) activity in the serum has been investigated several times previously. From those previous experiments, it was determined that upon addition of the 15-ketosterol in ethanol solution to growth medium supplemented with 5% NCS, <1% of the 15-ketosterol was recovered from the same medium as 15-ketosteryl esters. Perhaps, though, the mechanism by which the 15-ketosterol is carried in the growth medium + serum when added in an ethanol solution is different than the 15-ketosterol that has been excreted by the cell. Upon excretion, the 15-ketosterol may somehow become available as an acceptable substrate for LCAT. This does not, however, appear to be the case with cholesterol. The level of cholesterol esterification did not increase in the same manner as the 15-ketosterol esterification over the 48-hour period.

The same phenomenon occurred when the 15-ketosterol-adapted cells were incubated in 15 μM 5α-cholesta-8(14)-en-3β-ol-15-one as loading medium, see Figure 55. The relative amount of esterification in the total excreted 15-ketosterol was much greater under these conditions, but the increase in esterification was only from 25% to 38% over the period ranging from 12 to 48 hours. Again, the excretion of the total 15-ketosterol had reached a maximum by 12 hours, but the relative 15-ketosterol esterification continued to increase for the duration of the experiment.
The important results of these experiments are summarized as follows. By 12-24 hours after removing exogenous 15-ketosterol from the growth medium of the 15-ketosterol-adapted cells, an average of 75% of the incorporated 15-ketosterol was excreted into the growth medium. During this time period, 62-95% of the 15-ketosterol recovered in the medium was in the free form. This may indicate that the free 15-ketosterol present in the cell is preferentially excreted or that the esterified 15-ketosteryl esters are hydrolyzed and then excreted. Although maximum excretion of the total 15-ketosterol was reached by 24 hours, the relative amount of 15-ketosteryl esters continued to increase during the 24-48 hour period of the experiment.
Chapter V.

Discussion
The results from previous studies of mammalian cells cultured in lipid-deficient media have shown that 5α-cholest-8(14)-en-3β-ol-15-one is a potent inhibitor of sterol synthesis (Pajewski et al., 1988; Miller et al., 1980; Schroepfer, et al., 1977a) and is highly active in lowering the levels of activity of enzymes involved in the synthesis and reduction of HMG-CoA (Miller et al., 1980). Upon incubation in lipid-deficient medium, the growth of CHO K-1 cells is markedly inhibited at low concentrations of the 15-ketosterol (0.1 - 1.0 μM) presumably due to a reduction in the availability of cholesterol for new membrane synthesis, as suggested by Kandutsch et al. for other oxygenated sterols (1974). The possibility that the 15-ketosterol may affect growth by inhibiting the formation of products of mevalonic acid other than sterols must also be considered.

It has also been shown that 5α-cholest-8(14)-en-3β-ol-15-one is active at higher concentrations (>10.0 μM) in the suppression of the growth of CHO K-1 cells in lipid-rich medium (containing a significant concentration of cholesterol, 41.5 μM) (Pinkerton et al., 1988). While the mechanism(s) involved in the inhibition of growth by the 15-ketosterol under these conditions has not been determined, it has been proposed that despite the presence of cholesterol in the medium, the availability of cholesterol for cell growth may be limited by not only inhibition of cholesterol synthesis by the 15-ketosterol, but also by a limitation in the uptake of exogenous cholesterol (Pinkerton et al., 1988).

Results from experiments investigating the distribution of sterols in the CHO K-1 cells presented in this thesis demonstrate that the uptake of exogenous cholesterol is significantly decreased when CHO K-1 cells are incubated in the presence of 5 μM 5α-cholest-8(14)-en-3β-ol-15-one. Over a 48-hour incubation period in lipid-rich growth medium in the absence of the 15-ketosterol, CHO K-1 cells incorporated a total
of 55.6 ± 7.2 nmoles cholesterol per mg total cellular protein. In the presence of 5 μM 15-ketosterol, however, 27.1 ± 3.4 nmoles cholesterol per mg total cellular protein were incorporated. At the same time, 10.0 ± 1.2 nmoles 15-ketosterol per mg total cellular protein were incorporated by the cells. In the control cells (0 μM 15-ketosterol), 70.5% of the incorporated cholesterol was recovered unesterified; in the cells incubated in the presence of 5 μM 15-ketosterol, 74.2% and 77.0% of the cholesterol and 15-ketosterol, respectively, were recovered in the unesterified form. Not only was there over a 50% decrease in the uptake of cholesterol in the presence of the oxygenated sterol but there was also a 33% decrease in the overall sterol incorporation (cholesterol + 15-ketosterol) by the CHO K-1 cells.

The values for the uptake of cholesterol and the 15-ketosterol in the CHO K-1 cells determined from the sterol distribution studies were consistent with those determined from the incorporation experiments. In the incorporation experiments, CHO K-1 cells incubated in lipid-rich medium in the absence of the 15-ketosterol for 48 hours incorporated 54.0 ± 1.2 nmoles cholesterol per mg total cellular protein (Table 6). After incubation in lipid-rich medium containing 5 μM 15-ketosterol for 48 hours, the CHO K-1 cells incorporated 9.2 ± 0.3 nmoles 15-ketosterol per mg total cellular protein (Table 5). In this incorporation experiment, cholesterol was not radiolabeled, therefore cholesterol incorporation under these conditions was not determined.

A 15-ketosterol-adapted cell line derived from the CHO K-1 cell line was isolated by incubating the parent line in increasingly higher concentrations of 5α-cholesterol-8(14)-en-3β-ol-15-one. Studies of this cell line, designated K(15), have contributed additional data concerning the effects of the 15-ketosterol on cell growth and the metabolism of both cholesterol and the 15-ketosterol. This cell line is routinely maintained in lipid-rich medium containing 15 μM 5α-cholesterol-8(14)-en-3β-ol-15-one. The
growth rate of the K(15) cells is not significantly different than that of the CHO K-1 parent line. It was calculated that the 15-ketosterol-adapted cells grown in lipid-rich medium containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one had a generation time of 14.0 ± 0.6 hours compared to 11.6 ± 1.5 hours for the CHO K-1 cells grown in lipid-rich medium containing no 15-ketosterol.

Possible mechanisms by which the K(15) cell line may have adapted to growth in 15 μM 15-ketosterol include the possibility of either a spontaneous mutation or a gene amplification. In a related case, CHO cells were adapted to growth in the presence of higher and higher concentrations of compactin, a competitive inhibitor of HMG-CoA reductase, by amplification of the gene that codes for the reductase enzyme (Luskey et al., 1983). Removal of these cells, designated UT-1 cells, from incubation in the presence of compactin for several months demonstrated that the amplification of the reductase gene was stable; the number of copies of the reductase gene was shown to be constant after the prolonged absence of the selection pressure. Further, the amplified gene was found to be functional upon re-introduction of the cells to compactin-containing medium; the activity of the HMG-CoA reductase returned to the previously observed amplified level within 44 hours.

Likewise in the K(15) cells, following twenty generations in lipid-rich medium alone (0 μM 15-ketosterol), the subsequent presence of 15 μM 15-ketosterol did not inhibit cell growth in the K(15) cells as is observed in the CHO wild type cells. Albeit a much less rigorous measure of the stability of the adaptation than was undertaken for the UT-1 cells, these results indicate that the characteristics of this cell line are due to a stable adaptation. If the adaptation had been unstable, a loss of the ability to grow at a normal rate at elevated concentrations of the 15-ketosterol would have been expected following the release of the selection pressure.
Studies of sterol distribution in the K(15) cells demonstrated that there was no difference in the incorporation of exogenous cholesterol when comparing the K(15) cells incubated in medium containing 15 μM 15-ketosterol or 40 μM 15-ketosterol. K(15) cells incubated for 48 hours in 15 μM 15-ketosterol incorporated 18.3 ± 2.3 nmoles cholesterol per mg total cellular protein; cells incubated for 48 hours in 40 μM 15-ketosterol incorporated 18.2 ± 1.8 nmoles cholesterol per mg total cellular protein. Uptake of cholesterol in the K(15) cell line was well below that of either the CHO K-1 cells incubated without the 15-ketosterol supplement for 48 hours (55.6 ± 7.2 nmoles per mg total cellular protein) or the CHO K-1 cells incubated in medium containing 5 μM 15-ketosterol (27.1 ± 3.4 nmoles cholesterol per mg total cellular protein).

It should be noted that the only metabolism of the 15-ketosterol that has been demonstrated in CHO K-1 cells is the conversion to its fatty acid esters (Pajewski et al., 1982, 1988). The results of studies presented in this thesis support these results as well. Thus, 15-ketosterol does not provide a source of cholesterol in this cell type (in contrast to the results observed in liver preparations (Monger and Schroepfer, 1988; Monger et al., 1980), and in vivo experiments in rats (Brabson and Schroepfer, 1988; Schroepfer et al., 1988b; Schroepfer, 1988c) and baboons (Pajewski et al., 1989; Schroepfer et al., 1987b, 1988a). The lack of conversion of the 15-ketosterol to cholesterol in CHO K-1 cells may be important in the sensitivity of these cells to the effect of the 15-ketosterol on cell growth. Further, simplified interpretation of results are possible due to the limited metabolism of the 15-ketosterol.

The results presented herein indicate that a correlation exists between the concentration of exogenous 15-ketosterol and the incorporation and subsequent esterification of the 15-ketosterol in the K(15) cells. K(15) cells were incubated in lipid-rich
medium containing either 15 μM or 40 μM 5α-cholest-8(14)-en-3β-ol-15-one. After
12 hours in medium containing 15 μM 15-ketosterol, 37.4 ± 0.5 nmols total
15-ketosterol per mg total cellular protein were recovered in the cell. After 12 hours in
medium containing 40 μM 15-ketosterol, 253.0 ± 11.0 nmols total 15-ketosterol/ mg
protein was recovered in the cell. In both cases, the quantity of unesterified
15-ketosterol recovered in the cells after 2 hours, the earliest assay time, remained
constant throughout the 48 hours of the experiment. In the 15 μM condition, the level
remained at an average of 21.5 ± 1.2 nmols free 15-ketosterol per mg total cellular
protein from the 2 hour to the 48 hour assay; in the 40 μM condition, the level
remained at an average of 74.0 ± 4.7 nmols free 15-ketosterol per mg total cellular
protein from the 2 hour to the 48 hour assay. The amount of esterified 15-ketosterol,
however, increased until the time of the 12 hour assay. While there was a three to
four-fold increase in the free 15-ketosterol found in the cells that approximately
paralleled the increase in the concentration of the exogenous free 15-ketosterol in the
growth medium, there was about an eleven-fold increase in the 15-ketosteryl esters in
the 40 μM condition compared to the 15 μM condition.

Significant reduction in the inhibitory effect of the 15-ketosterol on the growth of
CHO K-1 cells by sodium oleate has been observed (Pinkerton, et al., 1988). It has
been suggested that the observed effect of oleate, and to a lesser extent, of linoleic acid
and linolenic acid, on the inhibitory action of the 15-ketosterol is probably related to the
ability of the cells to convert 15-ketosterol to its esters, via the action of ACAT. The
loss of the effect of sodium oleate on the inhibition of cell growth by the 15-ketosterol,
and the increased potency of the 15-ketosterol in the presence of N-(2,4-difluoro-
pheryl-N-[4-(2,2-dimethylpropyl]-phenyl]-methyl]-N-heptylurea (an ACAT inhibitor)
is consistent with this proposition. The formation of fatty acid esters of the
15-ketosterol in CHO K-1 cells has been demonstrated (Pajewski et al., 1982; 1988). CHO K-1 cells, incubated for 6 hours in lipid-deficient medium containing 6.0 μM 15-ketosterol, converted 20% of the incorporated 15-ketosterol into its esters (Pajewski et al., 1988).

Data have been presented in this thesis showing that the 15-ketosterol is readily esterified in the 15-ketosterol-adapted cell as well. Both incorporation studies (Chapter IV, section A) and sterol distribution studies (Chapter IV, section B) indicated that approximately 76% of the incorporated 15-ketosterol was esterified in the K(15) cells when incubated in lipid-rich medium containing 40 μM 5α-cholest-8(14)-en-3β-ol-15-one. Incorporation experiments showed that upon incubation of the cells in medium containing 15 μM 5α-cholest-8(14)-en-3β-ol-15-one, about 45% of the incorporated 15-ketosterol was esterified. These data strongly suggest that there is a correlation between the concentration of exogenous 15-ketosterol and the percent of esterification of the 15-ketosterol.

The K(15) cells display morphological differences compared to the CHO K-1 parent cell line. Not only are the K(15) cells significantly larger in size but there is also the appearance of abundant vacuole-like structures in these cells. Staining techniques have indicated that these structures are lipid-rich. Moreover, the size and number of these cytoplasmic structures appear to be directly related to the concentration of the 15-ketosterol contained in the growth medium. In an effort to identify the composition of these structures, a protocol was developed to fractionate the cell samples. This procedure resulted in the isolation of a distinct floating lipid layer, an intermediate zone, and a pellet fraction following centrifugation of homogenized cell samples.
Following incubation of the K(15) cells in lipid-rich medium containing 40 μM [3H]5α-cholest-8(14)-en-3β-ol-15-one, it was determined that the isolated floating lipid layer contained ~44% of the total 3H recovered in all the fractions. The intermediate zones contained a total of ~21% and the pellet contained ~36% of the total tritium recovered in all the fractions. Most of the radiolabeled material recovered in the floating lipid layer (~97%) was identified by radio-TLC as the 15-ketosteryl esters (accounting for ~42% of the total 3H recovered in all of the fractions); 21% of the total tritium was identified as the free 15-ketosterol in the pellet fraction.

The addition of sodium oleate (82 μM) to the growth medium resulted in a greater proportion (~61%) of the total 3H being recovered in the floating lipid layer. In the pellet fraction, ~22% of the total 3H was recovered and in the intermediate zones, ~16% of the total 3H was recovered. Most of the radiolabeled material recovered in the floating lipid layer (~96%) was again identified as the 15-ketosteryl esters (representing 59% of the total 3H recovered in all the fractions); ~16% of the total 3H was identified as the free 15-ketosterol in the pellet fraction.

The addition of sodium oleate resulted in a higher proportion (59%) of the total tritium which was associated with 15-ketosteryl esters in the floating lipid layer compared to 42% of the total 3H associated with 15-ketosteryl esters in the floating lipid layer from cells incubated in the absence of sodium oleate. The total incorporation of the 15-ketosterol, however, was unaffected by the addition of oleate. A total of 152.1 ± 19.3 nmoles 15-ketosterol per mg total cellular protein was recovered in the cells (70% in the esterified form) incubated with the addition of sodium oleate. An average of 150.2 ± 8.4 nmoles 15-ketosterol per mg total cellular protein was recovered in the cells (75% in the esterified form) incubated in lipid-rich medium containing 40 μM 5α-cholest-8(14)-en-3β-ol-15-one without oleate addition.
Upon addition of trace quantities (0.3 μM) of radiolabeled sodium oleate to the growth medium of the K(15) cells grown in lipid-rich medium containing 40 μM 5α-cholest-8(14)-en-3β-ol-15-one for 48 hours, ~34% of the total 14C was recovered in the floating lipid layer, ~14% of the 14C was recovered in the intermediate zones, and ~52% of the 14C was recovered in the pellet fraction. The results of radio-TLC analyses indicated that, of the total 14C recovered in all the fractions, ~29% of the radiolabeled oleate-derived lipids co-migrated with 15-ketosteryl esters in the floating lipid layer and ~40% was unidentified polar material present in the pellet fraction.

When radiolabeled sodium oleate was added at the level previously established to significantly reduce the inhibitory effects of the 15-ketosterol with respect to the cell growth in the CHO K-1 cells (82 μM), the distribution of the oleate-derived material changed slightly. Upon incubation in lipid-rich medium containing 40 μM [3H]15-ketosterol + 82 μM [14C]sodium oleate for 48 hours, ~53% of the total 14C was recovered in the floating lipid layer, ~14% of the 14C was recovered in the intermediate zones, and ~33% of the 14C was recovered in the pellet fraction. There were three regions identified as being rich in oleate-derived compounds. Similar to the previous distribution, 28% of the oleate co-migrated with the 15-ketosteryl esters of the floating lipid layer. Also, 25% of the recovered oleate remained at the origin (polar material) in the pellet fraction. The biggest difference was that 18% of the [14C]oleate co-migrated with triacylglycerols in the floating lipid layer. When oleate had been added in trace quantities, only ~1% of the oleate had co-migrated with the triacylglycerols of the floating lipid layer.

These results indicate that, in the K(15) cells, an increase in concentration of exogenous fatty acid (82 μM from 0.3 μM) does not result in increased incorporation
of the 15-ketosterol into the cell (152 nmoles per mg protein in the case of 82 μM oleate, 150 n mole per mg protein in the case of 0.3 μM oleate). Further, oleate addition did not lead to an increased percent of esterification of the incorporated 15-ketosterol. Presumably the incorporated 15-ketosterol is esterified and is subsequently stored in these lipid-rich regions in the cytoplasm. Interestingly, there was not much difference between the amount of total 15-ketosteryl esters that the K(15) cells synthesized with or without the addition of the oleate supplement. Further, upon the addition of the oleate, a significant proportion of the fatty acid was used to synthesize triacylglycerols.

The results of studies in the CHO K-1 cell lead to the proposal that the addition of exogenous sodium oleate, the acyl-CoA form of which is the favored substrate for ACAT (Spector et al., 1979), provides additional fatty acid substrate for the esterification of the 15-ketosterol, thus reducing the amount of free 15-ketosterol in the cells, and enhancing the ability of the cells to grow in otherwise inhibitory concentrations of this sterol (Pinkerton et al., 1988). While the results of studies with the 15-ketosterol-adapted cells presented here indicate that the addition of exogenous sodium oleate does not affect the overall percent esterification of the 15-ketosterol in this cell line, increasing the concentration of the exogenous 15-ketosterol in the growth medium does lead to a dramatic increase in the incorporation and subsequent esterification of the 15-ketosterol. While cell growth of the 15-ketosterol-adapted cell is not adversely affected by the presence of the same level of incorporated unesterified 15-ketosterol as the CHO K-1 cells, the K(15) cells do appear to esterify the incorporated 15-ketosterol into a storage form in an attempt to maintain a certain relatively low level of free 15-ketosterol in the cell.
In the cholesteryl ester cycle, described briefly in the Introduction, Jones and Glomset propose that incorporated cholesterol is esterified by the action of ACAT and stored in lipid droplets (1985). The resulting cholesteryl ester may then be hydrolyzed and the cholesterol may be used for either membrane synthesis or excreted from the cell if an appropriate carrier is present. Results of excretion studies in the K(15) cell demonstrated that incorporated 15-ketosterol is, in fact, excreted upon replacement of the 15-ketosterol-containing medium with lipid-rich medium containing no 15-ketosterol. It was found that by 24 hours after removal of the exogenous 15-ketosterol, 66-88% of the total incorporated 15-ketosterol was excreted into the medium. Although it was determined by radio-TLC that the majority of the excreted 15-ketosterol was in the unesterified form, after 48 hours, 20-37% was identified in the esterified form.

Based on the fact that up to 73% of the incorporated 15-ketosterol is in the esterified form and 66-88% of the total incorporated 15-ketosterol is excreted, it is logical to conclude that stored 15-ketosteryl esters must undergo hydrolysis, perhaps catalyzed by the neutral CEH, prior to excretion. The presence of some 15-ketosteryl esters in the medium suggests that some of the 15-ketosterol may be excreted in the esterified form. Further, the possibility of subsequent esterification of excreted free 15-ketosterol in the medium cannot be excluded.

The general pattern of incorporation, esterification, hydrolysis and excretion of the 15-ketosterol in the K(15) cell line is similar at a basic level to the cycle followed by cholesterol and its esters in other cell lines. The studies presented in this thesis provide evidence for proposing at least the skeleton of a 15-ketosteryl ester cycle that parallels to some extent the cholesteryl ester cycle described previously. The 15-ketosterol is incorporated by the K(15) cell and subsequently esterified, presumably by the ACAT
enzyme. The resulting 15-ketosteryl esters are stored in lipid-rich regions while the level of unesterified 15-ketosterol is maintained at a constant level. Upon removal of the exogenous source of the 15-ketosterol, 15-ketosteryl esters are presumably hydrolyzed by the neutral CEH, transported to the plasma membrane, and the unesterified 15-ketosterol is excreted into the medium.
References


