The liver plays a central role in the metabolism of cholesterol absorbed from the gastrointestinal tract. Most of this cholesterol is transported as esterified cholesterol in chylomicrons (2, 3), by way of intestinal lymph. Recent experiments in this laboratory have demonstrated that soon after the intravenous injection into rats of chylomicrons containing labeled cholesterol esters, almost 90% of the labeled cholesterol was found in the liver, as esterified cholesterol (4). Slow hydrolysis of the esterified cholesterol then occurred within the liver, followed by gradual redistribution of the labeled cholesterol to other tissues.

These findings indicate that liver hydrolysis of chylomicron cholesterol ester is an important component of the metabolism of cholesterol. In addition, the liver participates in the metabolism of esterified cholesterol in other ways. Evidence exists that the liver may be the primary source of plasma cholesterol esters (5), and that the individual fatty acid esters of cholesterol are differently metabolized within the liver (6).

Very little information exists concerning the hydrolysis of long-chain fatty acid esters of cholesterol by liver enzymes. Niefi and Deuel (7) demonstrated net hydrolysis of cholesteryl palmitate, with the use of a saline extract of rat liver homogenates and a 21-hour incubation. Subsequent investigators, however, have employed short-chain esters, particularly cholesteryl acetate, to study liver cholesterol esterase activity (8, 9). It is shown below that this is not a satisfactory substrate for the study of the hydrolysis of long-chain fatty acid esters of cholesterol.

The present study reports the hydrolysis of long-chain fatty acid esters of cholesterol with enzymes present in the microsomal and soluble fractions of rat liver.

**EXPERIMENTAL PROCEDURE**

**Enzyme Preparations**—Fed male Sprague-Dawley rats, weighing 175 to 225 g, were killed by decapitation. The livers were excised and immediately rinsed in ice-cold 0.1 m potassium phosphate buffer, pH 7.4. The livers were passed through a tissue press, and the pulp was homogenized at ice temperature in a Potter-Elvehjem homogenizer with a loose fitting Teflon pestle, with 2.5 ml of the above buffer per g of liver pulp. After removal of nuclei and cell debris by centrifugation at 2,000 × g for 30 minutes, the supernatant fraction (S-2) was centrifuged at 10-

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transferring the 2 ml incubation mixtures to CHCl₃-MeOH (2:1, volume per volume) and washing with CHCl₃-MeOH to a final volume of 50 ml. Each mixture was separated into two phases by the addition of 10 ml of water, and an aliquot of 30 ml was withdrawn from the lower CHCl₃ phase, total volume 36 ml. Carrier cholesterol and cholesteryl palmitate, 1 mg of each, were added, and the samples evaporated to dryness with a stream of nitrogen.

The free and esterified cholesterol in each sample were separated by chromatography on alumina (Woelm, neutral, grade II activity). Small (10-mm internal diameter) columns containing 2 g of alumina were charged with the sample dissolved in 1 to 2 ml of light petroleum ether (Fisher reagent grade, b.p. 36-56°), and the cholesterol esters eluted with 15 ml of benzene-petroleum ether ether, 3:7 (volume per volume). Free sterols were then eluted with 10 ml of acetone-ethyl ether, 1:1 (volume per volume). In later experiments the sterol esters were eluted with 12 ml of benzene-petroleum ether, 1:1. Various tests showed that this simple procedure separated free and esterified cholesterol with a very high degree of efficiency. Analysis of an incubation mixture containing H⁺-cholesteryl palmitate but no enzyme resulted in the recovery of more than 98% of the H⁺ in the benzene-petroleum ether fraction. This radioactivity was not directly precipitable with digitonin, but became digitonin precipitable after saponification. Furthermore, the small amount of radioactivity (1 to 2%) found in the acetone-ether fraction was also not digitonin precipitable until after saponification. Similar results were obtained with heat-inactivated enzyme preparations. These experiments showed that only 1 to 2% of the esterified cholesterol appeared in the acetone-ether eluent, and that nonezymatic hydrolysis did not occur during incubation, extraction, and chromatography.

In another experiment, 25% of the H⁺-cholesteryl palmitate was hydrolyzed enzymatically, and the incubation mixture extracted and assayed as described. Only 0.4% of the H⁺ in the benzene-petroleum ether fraction was precipitable with digitonin in the presence of added unlabeled free cholesterol; the remaining 99.6% of this H⁺ was digitonin precipitable after saponification. All the H⁺ found in the acetone-ether eluent was directly precipitable with digitonin, except for the expected small chromatographic "leak" of sterol ester described above. This experiment demonstrated the almost quantitative separation of free and esterified cholesterol achieved during chromatography, and also served to identify the reaction product as free cholesterol, assayed as radioactivity recovered in the acetone-ether eluent. The results given below have all been corrected for the small chromatographic contamination of ester cholesterol in the acetone-ether fraction, measured in each experiment by inclusion of a control flask without enzymes.

When esters of labeled fatty acids were used, free fatty acids and fatty acid esters (triglycerides, ethyl palmitate) were separated by the solvent partition method of Börgström (12), after extraction of the reaction mixture with the solvent mixture of Dole (13). Control experiments indicated the efficiency of separation to be greater than 99%.

Assay of Radioactivity—All samples were dissolved in 15 ml of 0.5% diphenyloxazole in toluene, and assayed with a Packard liquid scintillation spectrometer. The efficiency for 0²⁴ was 50 to 55%, and for II²⁴ was 20 to 25%. No quenching was observed.

Other Esterase Activities—The hydrolysis of p-nitrophenyl acetate and of p-nitrophenyl phosphate was assayed spectrophotometrically at 400 μM (11). p-Nitrophenyl acetate (m.p. 75.5 to 80°) was synthesized by reacting p-nitrophenol and acetic anhydride in anhydrous pyridine, and crystallizing the product from ethanol; p-nitrophenyl phosphate was purchased from the Sigma Chemical Company.

Substrate Preparations—(a) Nonradioactive cholesterol esters. Cholesterol (Nutritional Biochemical Corporation) was purified through its dibromide as described by Fieser (15), followed by two crystallizations from 95% ethanol. Its melting point after purification was 147.7 to 148.9°. Linoleic, oleic, palmitic, and stearic acids were obtained from the Hormel Foundation; all acids were more than 99% pure by gas-liquid chromatography. Cholesteryl linolate, oleate, palmitate, and stearate were prepared by reacting the appropriate acyl chloride with cholesterol in pyridine solution, with a modification of the method of Swell and Treadwell (16). Purified linoleyl and oleyl chlorides were prepared from the corresponding fatty acids by reaction with oxalyl chloride, followed by distillation of the acyl chloride in a vacuum as described by Wood et al. (17). Palmitoyl and stearyl chlorides were prepared by reaction of the fatty acid with thiocyanate chloride, and were not purified before reaction with cholesterol (16). Cholesteryl palmitate and stearate were purified from the reaction mixtures by two crystallizations from acetone, followed by chromatography on a large column of alumina. Cholesteryl oleate was crystallized once from acetone, followed by chromatography on alumina. Cholesteryl linolate did not crystallize from acetone, but instead came out of solution as an impure oil, which was purified by chromatography on alumina. Approximately 5 g of each ester were synthesized. The column chromatographies used for purification employed approximately 100 g of alumina (Woelm, grade II). Elution of the product cholesterol ester was achieved in one step, with 76 ml of benzene-light petroleum ether 3:7 (volume per volume) per 10 g of alumina.

The melting points of the cholesterol esters were: linoleate, 41.4-43.4°; oleate, 49.8-51.2°; palmitate, 76-77.5°; stearate, 81-83°. Gas liquid chromatography of the fatty acid components indicated that the esters were each more than 97% pure. Infrared spectra obtained with a Perkin-Elmer Model 21 spectrophotometer showed no evidence for the presence of trans double bonds in either cholesteryl oleate or linolate. Elaidic acid was used as the standard compound for trans double bonds (18), and it was estimated that the presence of 2% trans bonds could have been detected in the range studied. There was complete loss of the absorption peak at 3800 cm⁻¹ characteristic of the —OH group, and present in the free sterol. Study of the ultraviolet absorption of cholesteryl linolate at 220 μM indicated the presence of approximately 2% of the double bonds as conjugated diene.

Cholesteryl acetate (m.p. 111.5-114°) and butyrate were purchased from Steraloids, Inc. and were chromatographed on alumina before use. The cholesteryl butyrate was found to be impure and suitable only for semi-quantitative work.

(b) Radioactive Cholesterol Esters—7-α-²⁴-Cholesteryl palmitate and stearate (specific activities 48 and 50 mc per mole, respectively) were purchased from New England Nuclear Corporation. Both esters were chromatographed on small alumina columns, to remove possible traces of contaminating free sterol. Gas-liquid chromatography of the fatty acid components of a few micrograms of each revealed the presence of only the expected fatty acid peak, at a sensitivity level where a 2% contamination by another component should have been detected.
HI-Cholesteryl oleate and linolate were prepared by a micro-
modification of the synthesis employed above on a macro scale.
To 1 mc of 7-a-HI-cholesterol (New England Nuclear Corpora-
tion, 1 mc per 0.48 mg) in a centrifuge tube were added 5.00 mg of
carrier cholesterol, 20 ml of the appropriate purified acyl
chloride, and 0.3 ml of dry pyridine (distilled from HzO). The
mixtures were flushed with nitrogen and heated at 50-60° for 3
hours. The reaction mixtures were transferred to cold water,
and the products extracted into petroleum ether. The petroleum
ether was washed with 0.1 N acetic anhydride and butyryl chloride,
respectively, in pyridine a micro scale by reacting 7-a-Ha-cholesterol with reagent grade
fatty acid peak in each case, with unidentified minor peaks com-
micro-syntheses with unlabeled cholesterol revealed the expected
fatty acid acid peak in each case, with unidentified minor peaks com-
non-chromatography on 5-g alumina columns. The yields of HI-
cholesteryl linolate and oleate were 77% and 82%, respectively.
Gas-liquid chromatography of the fatty acids from identical
micro-syntheses with unlabeled cholesterol revealed the expected
fatty acid acid peak in each case, with unidentified minor peaks com-

H2-Cholesteryl acetate and butyrate were also synthesized on a
micro scale by reacting 7-a-H2-cholesterol with reagent grade
acetate anhydride and butyryl chloride, respectively, in pyridine
solution. The reaction mixtures were transferred to small separ-
atory funnels containing cold water and extracted into petroleum
ether. The petroleum ether was washed with 0.1 N HCl, 0.1 N KHCO3, and water, and the products then purified by
chromatography on 5-g silicic acid columns, as described by
Hornig, Williams, and Hornig (19).

Other Lipid Substrates. Tripalmitin (m.p. 64.5-65.5°) was a
Fisher reagent chemical; it was further purified by silicic acid chromatography (19), with collection and use of the material eluted with a mixture of 30 ml of benzene and 20 ml of hexane.
C14-Carboxyl-labeled tripalmitin was purchased from Research
Specialties Company. It had a specific radioactivity of 7.5 mc
per mmole, and was also purified by silicic acid chromatography
before use.

Ethyl palmitate was prepared by heating palmitic acid, in ab-
solute ethanol containing 0.1% benzene and 2% concentrated
H2SO4, at 60° overnight. The product was extracted with light
petroleum ether after the addition of an equal volume of water,
and free fatty acids removed from the petroleum ether solution
by washing with 0.05 N KOH in 50% ethanol. Ethyl palmitate-
1-C14 was similarly synthesized from 1-C14-palmitic acid (Nu-
clear-Chicago, 24 mc per mmole). The yields of C14 and of unlabeled ethyl palmitate were both more than 95%.

RESULTS

A. Soluble Enzyme

Presentation of Substrate—Initial experiments were conducted
to compare several modes of presentation of the water-insoluble
lipid substrates to be tested for hydrolysis. Cholesteryl palmit-
ate was added to a whole homogenate in three ways: (a) in ace-
tone solution, (b) suspended in saline solutions of sodium glyco-
cholate or of Tween 20, or (c) suspended in a mixture containing
ethanol, acetone, and serum albumin. The data in Table I indi-
cate that the substrate was most effectively hydrolyzed when
added in acetone solution. Experiments in which identical quan-
tities of substrate were dissolved in varying amounts of acetone
indicated hydrolysis to be maximum with substrate added in 50
ml (final concentration of acetone, 2.5%). Addition of Tween
20, sodium glycocholate or taurocholate, human serum albumin,

<table>
<thead>
<tr>
<th>Flask</th>
<th>Substrate presentation</th>
<th>% Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetone</td>
<td>15.7</td>
</tr>
<tr>
<td></td>
<td>Tween 20</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>Sodium glycocholate</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Albumin-acetone</td>
<td>1.1</td>
</tr>
</tbody>
</table>

TABLE II

Subcellular distribution of cholesterol esterase activity

Each 2-ml incubation contained 200 ml of potassium phos-
phate buffer, pH 7.4, and amounts of enzyme fractions represent-
ing equivalent volumes of the whole homogenate. To each mix-
ture, 1.0 mpmole (0.046 pc) of H2-cholesteryl palmitate was added,
and the incubations conducted for 1 hour.

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Recovered radioactivity*</th>
<th>% Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ester</td>
<td>Free</td>
</tr>
<tr>
<td>S-2</td>
<td>17,110</td>
<td>2,620</td>
</tr>
<tr>
<td>S-10</td>
<td>18,193</td>
<td>2,682</td>
</tr>
<tr>
<td>S-104 (defatted)</td>
<td>14,120</td>
<td>7,140</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>18,898</td>
<td>971</td>
</tr>
<tr>
<td>Microsomes</td>
<td>20,317</td>
<td>1,155</td>
</tr>
</tbody>
</table>

* The c.p.m. listed are the actual values observed whereas the
% hydrolysis in the last column has been corrected for chroma-
tographic contamination.

human β-lipoprotein, or rat liver polyglycerolphosphatide to in-
cubations before the addition of substrate (in 50 µl acetone) all
inhibited hydrolysis. In the experiments that follow, the sub-
strate was always presented in 50 ml of acetone solution.

The subcellular distribution of hydrolytic activity is described in
Table II. The bulk of activity was associated with the soluble
supernatant fraction, although activity was detected in both
particulate fractions.

The data in Table III show the amounts of free and esterified
cholesterol in the subcellular fractions. Since the S-2, S-10, and
particulate fractions contained considerable endogenous cho-
lesterol ester, but the defatted S-104 contained none, the specific
activity of the substrate being hydrolyzed varied in each fraction
to the extent that the endogenous cholesterol ester in the enzyme
preparation itself was available for hydrolysis. It is probable,
therefore, that the lack of dilution of the substrate in the defatted
S-104 fraction was responsible for the greater per cent hydrolysis
of the added substrate by the S-104 fraction compared to the S-10 and S-2 fractions.

Purification of Soluble Enzyme—The hydrolytic activity of
the soluble enzyme was almost completely precipitable with am-
monium sulfate, at 30% saturation. Subsequent purification
fractions, rather than the S-104 fraction. The activity of the S-2 and S-10 fractions, compared to the S-104, was always very much greater when compared to the S-2 or S-10. The apparent purification of Fx. 5 was found to be constant for the initial 40 minutes of incubation, and decreased by less than 10% at 90 minutes, and less than 20% at 120 minutes. With progressively longer incubations the reaction velocity declined considerably.

Reesterification during the incubation was studied with the use of 10 mg of F₈₀, incubated for one hour with 10 µmoles of ATP, 10 µmoles of GSH, 2.5 µmoles of CoA, 50 µmoles of potassium oleate, and 25 µmoles of H⁺-cholesterol palmitate. Similar incubations were performed in which only potassium oleate, cholesterol, and F₈₀ were incubated, with no additional cofactors. No esterification was observed in either experiment.

The effects of inhibitors are shown in Table V. The enzyme was inhibited by p-mercuribenzoate and by N-ethyl maleimide, and slightly inhibited by iodoacetamide. These inhibitions were readily overcome by a 3- to 5-fold excess of GSH. DFP¹ was strongly inhibitory. Eserine was only slightly inhibitory at

¹ The abbreviation used is DFP, di-isopropyl fluorophosphate.

---

**Table III**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cholesterol (mg/ml)</th>
<th>Total Protein (mg/ml)</th>
<th>Specific Enzyme Activity (µmol/mg protein)</th>
<th>Total Enzyme Activity (µmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
<td>1.02</td>
<td>0.69</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>S-2</td>
<td>0.36</td>
<td>0.23</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>S-10</td>
<td>0.24</td>
<td>0.16</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>S-104</td>
<td>0.21</td>
<td>0.12</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>S-104 (defatted)</td>
<td>0.03</td>
<td>0.03</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>F₈₀</td>
<td>0.01</td>
<td>0.01</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fx. 5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Microsomes (conc. X 10)</td>
<td>1.15</td>
<td>1.02</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>Mitochondria (conc. X 10)</td>
<td>0.76</td>
<td>0.66</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

* Specific enzyme activity = % hydrolysis × 5000 c.p.m. per mg of protein, assuming linearity between amount of protein actually incubated and extent of hydrolysis.

† Total enzyme activity = specific enzyme activity times total protein.

was hindered by rapid loss of activity upon dialysis or lyophilization. Activity could not be recovered from DEAE-cellulose columns by elution with buffers ranging from 0.005 M, pH 7.9, to 1.25 M, pH 6.7. Further purification was effected with the use of Sephadex columns to remove the ammonium sulfate, and subsequent calcium phosphate gel adsorption of the Sephadex eluate.

The results of a typical purification experiment are summarized in Table IV. The most purified fraction (Fx. 5) after calcium phosphate gel elution usually displayed an 8- to 10-fold purification when compared to the S-104. The apparent purification was always very much greater when compared to the S-2 or S-10 fractions (see column headed "Specific enzyme activity" in Table IV). Because of the presence of endogenous esterified cholesterol, estimates of the specific enzyme activity of the initial fractions were in error to the extent that this endogenous material in the enzyme preparation was available as substrate for hydrolysis. This error is apparent in the paradoxically low total enzyme activity of the S-2 and S-10 fractions, compared to the S-104 (see Table IV), and undoubtedly accounts for the much larger apparent purification of Fx. 5 when compared to the S-2 or S-10 fractions, rather than the S-104 fraction.
10^{-4} \text{ m}, a concentration that completely inhibits cholinesterase activity.

**Effect of Enzyme Concentration**—The initial rate of hydrolysis of cholesteryl palmitate was linearly related to enzyme concentration at concentrations of F_{o30} below 4 mg of protein per ml. The rate of hydrolysis was, however, strikingly nonlinear at concentrations of F_{o30} in excess of 5 mg of protein per ml. Since the F_{o30} fraction contained negligible amounts of cholesterol ester, this could not result from the addition of endogenous substrate together with the enzyme fraction. In the various experiments presented here, the concentrations of protein employed were in the linear range of the enzyme concentration-activity curve.

The substrate specificity of the soluble enzyme was studied by comparing the hydrolytic activity of each fraction during the purification procedure with each of three different esters. p-Nitrophenyl acetate, an ester known to be hydrolyzed by a wide range of esterases, was employed as one model substrate. p-Nitrophenyl phosphate was used as a measure of phosphatase activity, and cholesteryl palmitate was used to measure cholesterol esterase activity. The results shown in Fig. 2 demonstrate that a selective purification of the cholesterol esterase activity was achieved with Fx. 5. The microsomal fraction showed striking hydrolytic activity for p-nitrophenyl acetate, whereas Fx. 3 displayed the most phosphatase activity. Fx. 3 also represented the greatest concentration of soluble activity toward p-nitrophenyl acetate. These results suggest that the soluble one-hour incubation contained the same buffer, Fx. 5, 0.75 mg of Fo30 protein, 200 pmole of potassium phosphate buffer, pH 7.4, and 20 mpmoles (0.020 pc) of substrate. In Experiment B, each one-hour incubation contained the same buffer, Fx. 5, 0.75 mg of protein and cholesteryl palmitate, 1.0 mpmole.

**TABLE V**

**Inhibition of cholesteryl palmitate hydrolysis with soluble enzyme**

The 1-hour incubations in Experiment A each contained 9 mg of F_{o30} protein, 200 pmole of potassium phosphate buffer, pH 7.4, and 20 mpmoles (0.020 \mu) of substrate. In Experiment B, each one-hour incubation contained the same buffer, Fx. 5, 0.75 mg of protein and cholesteryl palmitate, 1.0 mpmole.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>Recovered radioactivity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ester</td>
<td>Free</td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>6,897</td>
<td>2,096</td>
</tr>
<tr>
<td></td>
<td>N-ethyl maleimide, 1</td>
<td>7,688</td>
<td>763</td>
</tr>
<tr>
<td></td>
<td>N-ethyl maleimide, 6</td>
<td>7,966</td>
<td>247</td>
</tr>
<tr>
<td></td>
<td>N-ethyl maleimide 6 + GSH, 20</td>
<td>6,852</td>
<td>2,461</td>
</tr>
<tr>
<td></td>
<td>Iodoacetamide, 1</td>
<td>6,441</td>
<td>1,913</td>
</tr>
<tr>
<td></td>
<td>Iodoacetamide, 6</td>
<td>7,342</td>
<td>1,429</td>
</tr>
<tr>
<td></td>
<td>p-Mercuribenzoate, 0.8*</td>
<td>8,692</td>
<td>324</td>
</tr>
<tr>
<td></td>
<td>p-Mercuribenzoate, 2</td>
<td>8,672</td>
<td>281</td>
</tr>
<tr>
<td></td>
<td>p-Mercuribenzoate, 2 + GSH, 20</td>
<td>6,208</td>
<td>2,503</td>
</tr>
<tr>
<td></td>
<td>Eserine sulfate, 0.2</td>
<td>7,217</td>
<td>1,519</td>
</tr>
<tr>
<td></td>
<td>Eserine sulfate, 1</td>
<td>7,881</td>
<td>798</td>
</tr>
</tbody>
</table>

| B | None | 5,036 | 772 | 0 |
|   | DFP, 0.01 | 4,738 | 671 | 8 |
|   | DFP, 0.10 | 4,943 | 298 | 81 |
|   | DFP, 0.50 | 5,611 | 759 | 80 |

* This experiment was done on a different day and is referred to a different control.

**Fig. 2.** Relative esterase activities of liver homogenate fractions. Cholesteryl palmitate hydrolysis was studied with 2-hour incubations containing 1.25 mmoles of H\(^2\)-cholesterol palmitate. p-Nitrophenyl acetate and phosphate hydrolyses were both measured at pH 7.4. Five mmoles of p-nitrophenyl acetate were added in 0.25 ml of isopropanol to incubations of final volume 5 ml, and the hydrolysis rate measured and corrected for spontaneous hydrolysis. Two-milliliter incubations containing 2 mg of p-nitrophenyl phosphate were analyzed for hydrolysis after 30 minutes. The amounts of protein in the different fractions were approximately prorated as in Tables II and III. The S-104 was a de-fatted preparation. The data are plotted to indicate the relative activity of each fraction against each ester. The most active fraction for each ester was arbitrarily assigned an activity of 1.00 cholesterol esterase is a different enzyme from those involved in the hydrolysis of the other esters studied.

*The fatty acid specificity* of the soluble cholesterol esterase was tested in two ways. In one set of experiments, the different cholesterol esters were individually studied at several substrate levels, and substrate-concentration curves were determined. The results, shown in Fig. 3, demonstrate that considerably more hydrolysis was observed with cholesteryl olate and linoleate than with cholesteryl palmitate. There was very little hydrolysis of cholesteryl acetate. The hydrolysis of cholesteryl acetate was intermediate between that of oleate and palmitate; in a single
Hydrolysis of Cholesterol Esters

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FIG. 4. Hydrolysis of equimolar mixtures of cholesterol esters. Each 1-hour incubation contained 5 mmoles each of unlabeled cholesteryl palmitate, stearate, oleate, and linoleate. In each mixture, 0.3 to 0.5 m mole of one of the H*-cholesteryl esters was included as follows: H*-cholesteryl-palmitate = P; -stearate = S; -oleate = O; -linoleate = L. Incubations contained the following amounts of protein: S-104 (defatted), 10 mg; Fx. 5, 0.5 mg. *sample lost.

FIG. 5. The effect of pH on the hydrolysis of cholesteryl palmitate with microsomes. Each 1-hour incubation contained, in 2 ml, 15 mg of microsomal protein, 200 μmoles of Tris-malate buffer, and 0.9 m mole, H*-cholesteryl palmitate.

incubation cholesteryl butyrate was hydrolyzed to approximately the same extent as cholesteryl acetate.

A second study was conducted to measure the hydrolysis of each ester in an equimolar mixture of cholesteryl palmitate, stearate, oleate, and linoleate. To achieve this, four identical acetone solutions were prepared containing equal amounts of the four esters, but with radioactivity in only one of the esters. Each of the four solutions contained radioactivity in a different ester. The results of this study, shown in Fig. 4, also demonstrated a distinctly greater hydrolysis of cholesteryl oleate and linoleate. In contrast to the preceding results, in these experiments cholesteryl stearate was approximately as effective a substrate as cholesteryl palmitate. Fig. 4 also indicates that the pattern of substrate preference did not change during the purification of enzyme from S-104 to Fx. 5.

Other Lipid Substrates—The soluble enzyme was extremely active in effecting the hydrolysis of ethyl palmitate. A constant rate was observed, and more than 90% hydrolysis achieved in one hour, with substrate concentrations up to 100 m moles in 2 ml (the largest amount tested). Tripalmitin alone, or added with corn oil in an attempt to give a better dispersion, was not effectively hydrolyzed (5.3% hydrolysis of 10 or 20 m moles in one hour).

B. Microsomes

Studies were also conducted on the properties of the cholesterol esterase activity of washed microsomes.

Substrate presentation in the microsomal system was studied with the use of cholesteryl palmitate dissolved in acetone, and suspended in Tween 20, sodium taurocholate, and in ethanol-acetone-serum albumin. Optimal hydrolysis was again found with the substrate in acetone solution at a final concentration of acetone of 2.5%. Addition of Tween 20, sodium taurocholate, or human serum albumin to the incubations before the addition of substrate in acetone solution was all inhibitory. There was no requirement for GSH.

The effect of pH on the microsomal esterase was studied over the range 5.4 to 9.0. The results showed the optimal pH range to be 0.0 to 0.5, with an abrupt decline in activity below pH 5.3 and a more gradual decline above pH 6.5. As with the soluble enzyme, activity was greater in Tris-HCl buffer than in phosphate buffer.

TABLE VI

Inhibition of cholesteryl palmitate hydrolysis with microsomes

Each 1-hour incubation contained, in 2 ml, 15 mg of microsomal protein, 200 μmoles of potassium phosphate buffer, pH 6.1, 1 m mole (0.008 μc) of H*-cholesteryl palmitate, and additions as listed.

<table>
<thead>
<tr>
<th>Additions</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-ethyl maleimide, 1</td>
<td>24</td>
</tr>
<tr>
<td>N-ethyl maleimide, 6</td>
<td>52</td>
</tr>
<tr>
<td>N-ethyl maleimide, 6 + GSH, 20</td>
<td>3</td>
</tr>
<tr>
<td>Iodoacetamide, 1</td>
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<tr>
<td>Iodoacetamide, 6</td>
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<tr>
<td>p-Mercuribenzoate, 0.4</td>
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</tr>
<tr>
<td>p-Mercuribenzoate, 2</td>
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</tr>
<tr>
<td>p-Mercuribenzoate, 2 + GSH, 10</td>
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</tr>
<tr>
<td>Eserine sulfate, 0.2</td>
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</tr>
<tr>
<td>Eserine sulfate, 1</td>
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</tr>
<tr>
<td>DFP, 0.01</td>
<td>84</td>
</tr>
<tr>
<td>DFP, 0.1</td>
<td>95</td>
</tr>
<tr>
<td>DFP, 0.5</td>
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</table>

FIG. 6. Hydrolysis of 5 cholesterol esters at varying substrate concentrations, with microsomes. The incubations were similar to those of Table VI, with substrates similar to those of Fig. 3.
buffer at the same pH. Experiments with Tris-malate buffer to cover the entire pH range (Fig. 5) clearly demonstrated that the pH optimum was in the range 5.8 to 6.5.

The effects of enzyme and of substrate concentration were studied by measuring the hydrolysis of each of 4 different amounts of substrate (2, 6, 20, and 50 mmoles), with each of 5 different amounts of microsomal protein (3.5, 6.9, 21, 35, and 69 mg of protein). The results indicated that in the range of substrates tested, the relationship between enzyme concentration and rate of hydrolysis was never linear.

The effects of inhibitors are shown in Table VI. As with the soluble enzyme, N-ethyl maleimide, iodoacetamide, and p-methanobenzocate were all inhibitory, these inhibitions were readily overcome by addition of excess GSH. The microsomal enzyme was much more sensitive to DFP and to eserine than was the soluble enzyme.

The effects of divalent cations (Mg++, Mn++, Ca++, Co++, Ni++, Zn++, and Cu++) were studied at 0.5 mM concentration. Copper and zinc ions were markedly inhibitory, but the other cations tested did not affect hydrolysis.

Fatty acid specificity of the microsomal cholesterol esterase was investigated by the same techniques used to study the soluble enzyme. Substrate concentration curves for each of 5 different sterol esters are shown in Fig. 6. The rate of hydrolysis of cholesteryl acetate was much greater than that of any of the long-chain fatty acid esters, and was linear throughout the concentration range tested. This range extended to 50 mmoles of substrate, although Fig. 6 only shows the results for amounts up to 20 mmoles. The hydrolysis of cholesteryl butyrate (not shown) was less than that of acetate, but considerably more than that of linoleate. Of the long-chain esters, linoleate and oleate again served as better substrates than did cholesteryl palmitate and stearate. In addition, the hydrolysis of each component of equimolar mixtures of cholesteryl palmitate, stearate, oleate, and linoleate showed an order of preference similar to that of the soluble enzyme. These experiments were performed in the same manner as those of Fig. 4, and resulted in a 10.0% hydrolysis of cholesteryl linoleate, 9.8% of cholesteryl oleate, and 6.8% each of cholesteryl stearate and palmitate. These results, and the data shown in Figs. 3 and 6, indicate that the soluble and microsomal enzymes showed fairly similar patterns of specificity for the four long-chain esters studied, but strikingly different preferences for the short-chain esters.

Other Lipid Substrates—Ethyl palmitate was almost quantitatively (97 to 98%) hydrolyzed in 1 hour in concentrations up to 100 mmoles in 2 ml. Tripalmitin hydrolysis (15% of 20 mmoles in 1 hour) slightly exceeded that of cholesteryl oleate and linoleate at similar concentrations.

**DISCUSSION**

These experiments show that rat liver contains enzymes that hydrolyze the long-chain fatty acid esters of cholesterol, and that the bulk of hydrolytic activity is found in the soluble fraction of the liver homogenate. The data from Table II indicate that the S-10 fraction hydrolyzed 11.2% of the added substrate, but that an equivalent sample of the added substrate (of added substrate) hydrolyzed only 3.6% of the added substrate, or 32% of the amount of hydrolysis (of added substrate) effected by the S-10. Analytic measurements (Table III) showed that the endogenous cholesterol ester present in the microsomes was only 0.013/0.08, or less than ¼, the total cholesterol ester present in the S-10. It is thus clear that the lesser activity of the microsomes alone was not due to the presence of a relatively large pool of endogenous substrate in the microsomes, and that most of the hydrolytic activity of the S-10 was contributed by the soluble fraction. When compared to an equivalent sample of defatted S-104, the microsomes hydrolyzed only 11% as much of the added substrate. Therefore, the contribution of the microsomes lies between 11 and 32% of the observed total hydrolysis.

Studies of enzymic reactions involving lipid substrates routinely employ emulsified or finely suspended preparations of the water-insoluble substrates. With emulsions similar to those employed in this study (substrate added in acetone solution), it has been possible to study the characteristics of the enzymic cyclization of squalene (20) and of several reactions involving sterol precursors of cholesterol (21). In addition, other workers have applied kinetic analyses usually used for reactions occurring in true solution to enzymic reactions with steroid substrates added in propylene glycol (22) or in methanol (23) solution.

The effects of enzyme and of substrate concentration were studied by extrapolation of these plots in the usual way, approximate substrate concentrations for half-maximal velocity, for the soluble enzyme, were determined as follows: 14.3 X 10^-6 M for cholesteryl acetate, oleate, and linoleate, and 4.8 X 10^-4 M for cholesteryl palmitate. Extrapolated values for maximal velocities of hydrolysis (expressed as mmoles substrate hydrolyzed per 10 mgm of F-10 per 30 minutes) were: cholesteryl palmitate, 3.1; cholesteryl acetate, 9.1; and cholesteryl oleate and linoleate, 14.3.

The substrate concentration curves with individual cholesterol esters (Figs. 3 and 6) indicate that both the soluble and the microsomal enzymes hydrolyzed the unsaturated fatty acid esters (cholesteryl oleate and linoleate) more rapidly than the saturated ones. It is possible, however, that this observed difference resulted from different physical properties of the substrate suspensions of the individual cholesterol esters, rather than from true enzymic specificity. To investigate this possibility, experiments were performed with equimolar mixtures of all four long-chain fatty acid esters as substrate. It was felt that the mixed emulsions so formed would minimize any effects dependent, upon different properties of the individual ester emulsions.

This in fact occurred can be seen from the hydrolysis of cholesteryl stearate by the soluble enzyme. When presented alone, stearate was not effectively hydrolyzed, but when dispersed among the other three esters, it was hydrolyzed as effectively as palmitate. The preference for oleate and linoleate in the mixtures was similar to that observed with individual esters.

These results suggest that the more rapid hydrolysis of cholesteryl oleate and linoleate, compared to cholesteryl palmitate and stearate, reflected true enzymic specificity. It is recognized, however, that this conclusion cannot be established with certainty by these experiments.

The data shown in Fig. 4 indicate that the bulk of liver cholesterol esterase activity, present in the soluble fraction of the liver homogenate, was separable from the liver esterase activities involved in the hydrolysis of p-nitrophenyleacetate and -phosphate. The hydrolysis of p-nitrophenyleacetate has been found to reflect the activity of a variety of esterases, including pancreatic cholesterol esterase (27). This would seem not to be true for the liver soluble cholesterol esterase. The microsomes, in contrast, were associated with striking hydrolysis of p-nitrophenyleacetate.

The microsomal cholesterol esterase differed from the soluble esterase in several respects. The microsomal enzyme was inhibited by eserine in concentrations similar to those effective against cholinesterase; the soluble enzyme was not inhibited at these concentrations. The soluble enzyme was strongly inhibited by DFP at concentrations below $10^{-6}$ M. The soluble enzyme was also inhibited by DFP, but only when the concentration of inhibitor was an order of magnitude greater. The microsomal pH optimum near 6.1 was well below the optimum of the soluble enzyme. There was no enhancement of microsomal activity by divalent cations. Tripalmitin, not effectively hydrolyzed by the soluble enzyme, was hydrolyzed to a greater extent than either cholesteryl oleate or linoleate in the microsomal system. Most striking was the marked hydrolysis of cholesteryl acetate and butyrate with microsomes, far exceeding their relative hydrolysis by the soluble system. In addition, both enzymes displayed great activity toward ethyl palmitate.

The data shown in Figs. 3 and 6 indicate the extent of hydrolysis of cholesterol esters with soluble enzyme equivalent to 2 to 3 ml of liver whole homogenate, and with microsomes equivalent to 5 to 6 ml of whole homogenate, respectively. From these data, and from similar experiments (e.g. cf. Table II), it can be estimated that the soluble enzyme hydrolyzes 5 to 10 times more cholesteryl palmitate, stearate, oleate, and linoleate, than does the microsomal fraction. Figs. 3 and 6 also demonstrate that experiments based on the hydrolysis of cholesteryl acetate do not reflect the hydrolysis of physiologically significant esters.

Recent experiments have demonstrated that cholesterol esters in chylomicrons are taken up almost quantitatively from the blood by the liver. In the liver a slow net hydrolysis ensues, which is more than 80% complete in 3½ hours (4). Based on the rate of hydrolysis of cholesteryl oleate by the soluble enzyme observed here (Fig. 3), an estimated maximal rate of hydrolysis for a 7- to 8-g rat liver would be of the order of 600 mmoles of cholesteryl oleate hydrolyzed in one hour. This value is in remarkably close agreement with the net rate of hydrolysis observed in vivo (4).

SUMMARY

1. The hydrolysis of long-chain fatty acid esters of cholesterol with rat liver homogenates has been shown. The bulk of hydrolytic activity was associated with the soluble protein fraction, although the microsomes contributed between 11 and 39% of the observed total hydrolysis.

2. The soluble enzyme was partially purified by ammonium sulfate precipitation and calcium-phosphate gel absorption. The enzyme was unstable and lost activity after freezing, dialysis, or lyophilization. The soluble cholesterol esterase was separable from the major esterase activities involved in the hydrolysis of p-nitrophenyleacetate and p-nitrophenylphosphate.

3. The soluble enzyme displayed a broad pH optimum between pH 5.5 and 7.5; the microsomal enzyme displayed a sharper peak near pH 6.1. The soluble enzyme did not hydrolyze tripalmitin as effectively as did the microsomes. The soluble enzyme was not inhibited by $10^{-4}$ M eserine or by $10^{-4}$ M DFP; the microsomal enzyme was strikingly inhibited at these concentrations.

4. The order of hydrolysis of cholesterol esters by the soluble enzyme was found to be: Cholesteryl linoleate $>$ oleate $>$ palmitate $>$ stearate. The order of hydrolysis of cholesterol esters by the microsomes was found to be: Cholesteryl acetate $>$ oleate $>$ palmitate $>$ stearate.

5. The rate of hydrolysis of cholesterol oleate by the soluble enzyme is in close agreement with the net rate of hydrolysis observed in vivo.

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The Hydrolysis of Long-chain Fatty Acid Esters of Cholesterol with Rat Liver Enzymes
Daniel Deykin and DeWitt S. Goodman


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