Cordomier, C., and Bernardi, G. (1965), *Biochem. Biophys. Res. Commun. 20*, 555.
Dabrowska, W., Cooper, E. J., and Laskowski, M., Sr. (1949), *J. Biol. Chem. 177*, 991.
DeWitt, W., and Helinski, D. R. (1965), *J. Mol. Biol. 13*, 692.
Friedberg, E. C., and Goldthwait, D. A. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 934.
Goebel, W., and Helinski, D. R. (1969), *Bacteriol. Proc.* 46.
Hurwitz, J., Becker, A., Gefter, M. L., and Gold, M. (1967), *J. Cell. Physiol. 70, Suppl. 1*, 181.
Lehman, I. R. (1967), *Annu. Rev. Biochem. 36*, 645.
Lehman, I. R., Roussos, G. G., and Pratt, E. A. (1962), *J. Biol. Chem. 237*, 819.
Lesca, P., and Paoletti, C. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 913.
Melgar, E., and Goldthwait, D. A. (1968), *J. Biol. Chem. 243*, 4409.
Meselson, M., and Yuan, R. (1968), *Nature (London) 217*, 1110.
Nossal, N. G., and Heppel, L. A. (1966), *J. Biol. Chem. 241*, 3055.
Roulland-Doussoix, D., and Boyer, H. W. (1969), *Biochim. Biophys. Acta 195*, 219.
Siegel, J. E. D., and Hayashi, M. (1967), *J. Mol. Biol. 27*, 443.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Vinograd, J., and Lebowitz, J. (1966), *J. Gen. Physiol. 49*, 103.
Weissbach, A., and Korn, D. (1963), *J. Biol. Chem. 238*, 3383.

Further Studies on the Fatty Acid Specificity of Rat Liver Sterol–Ester Hydrolase*

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ABSTRACT: The specificity of rat liver cholesterol ester hydrolase activity (EC 3.1.1.13) was further investigated. Cholesterol esters of unsaturated fatty acids differing in the proximal or terminal position of the double bond were synthesized and their rate of hydrolysis indicated that a proximal portion of 9 carbon atoms for the fatty acid constituent is a requirement for an optimal hydrolysis of the corresponding cholesterol ester.

In addition, the enzymatic activity was shown to depend upon the chain length of the fatty acid moiety. When a series of saturated fatty acid cholesterol esters were studied, the activity increased from hexanoic to decanoic and then decreased gradually through the eicosanoic acid cholesterol ester. These observations are discussed in terms of a close matching of specially shaped acyl chains to a specially shaped complementary surface in the enzyme active site.

The most active hydrolytic enzyme in rat liver for cholesterol esters was found in the particle free cytoplasm of the cell (Deykin and Goodman, 1962; Swell *et al.*, 1964) and evidence indicated that it acted specifically on cholesterol esters of both common and uncommon fatty acids (Sgoutas, 1968).

Recently, the cholesterol esters of the 16 positional isomers of *cis*-octadecenoic acid were synthesized and their rate of hydrolysis with rat liver cholesterol ester hydrolase was studied (Goller *et al.*, 1970). It was clearly shown that cholesteryl *cis*-9-octadecenoate was hydrolyzed at the highest rate suggesting that this ester is the preferred substrate. Cholesteryl *cis*-9-octadecenoate, however, has the double bond in a symmetric position within the acyl chain and it does not by itself specify whether the proximal or the terminal portion governs the enzymatic selectivity.

Since it seemed desirable to us to emplore this parameter, we determined the enzymatic activity against cholesterol esters of unsaturated fatty acids with different chain lengths which had equal proximal but different terminal portions and different proximal but equal terminal portions. A dependence of the enzymatic activity upon the chain length of the acyl moiety was anticipated and since it was desirable to explore this parameter too, a homologous series of saturated fatty acid cholesterol esters were included as substrates. The results are reported in this communication.

It is hoped that relations between reactivity and structure of the cholesterol ester with regard to its acyl moiety might give useful information in predicting some features about the topography of the active site, the mode of binding, and the catalytic process.

Experimental Procedure

Microanalyses were performed by Clark Microanalytical Laboratories, Urbana, Ill. Instrumentation used in this study has previously been described (Sgoutas *et al.*, 1969). All solvents were reagent grade and were distilled before use.

Materials. Octadecanoic, hexadecanoic, tetradecanoic, 9-

* From The Burnsides Research Laboratory, University of Illinois, Urbana, Illinois 61801. *Received July 14, 1970.* This work was supported by Grant HE 10779-04 from the National Institutes of Health, U. S. Public Health Service.

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octadecenoic, 9-hexadecenoic, and 9-tetradecenoic acids (purity 99%) were purchased from the Hormel Institute, Austin, Minn. Eicosanoic, heptadecanoic, dodecanoic, decanoic, octanoic, and hexanoic acids (purity 95%) were products of Aldrich Co., Milwaukee, Wis.

10-Undecenoic acid was the product of Eastman Organic Chemicals, Rochester, N. Y., and it was fractionally distilled (Vogel, 1956) before use. Undecanoic acid was prepared from 10-undecenoic acid by hydrogenation over PtO_2 in methanol. 11-Dodecenoic acid was synthesized by the Arndt–Eistert homologation of 10-undecenoic acid according to Seher (1956). 9-Decenoic acid was prepared by stereoselective reduction with disiamylborane (Brown and Zweifel, 1961) of methyl 9-decynoate, itself synthesized during the course of another study (Sgoutas and Kummerow, 1964a). Purity of all acids was greater than 95% as judged by gas–liquid partition chromatography of their corresponding methyl esters.

Cholesterol from Nutritional Biochemical Corp. was purified through its dibromide (Fieser, 1955). Cholesterol-*4*-^{14}C and cholesterol-7α-^{3}t from New England Nuclear Corp., Boston, Mass., were diluted with nonradioactive cholesterol to a specific activity of 4 μCi/μmole and 10 μCi/μmole, respectively. Each radioactive compound was purified by argentation thin-layer chromatography (Morris, 1966) to a final purity better than 98% for both compounds as determined by thin-layer silicic acid chromatography and liquid scintillation counting.

Synthesis of 8-Heptadecenoic and 7-Hexadecenoic Acids. Commercial oleic acid was subjected to urea fractionation and purified to 96.3% purity as checked by gas–liquid partition chromatography. By bromination in carbon tetrachloride at $-10°$ it gave *threo*-9,10-dibromooctadecanoic acid by the method of Nevenzel and Howton (1957).

threo-1,8,9-Tribromoheptadecane. This compound was synthesized according to the Cristol–Firth (1961) and Davis *et al.* (1965) modification of the Hunsdiecker reaction (Hunsdiecker and Hunsdiecker, 1942). The reaction was conducted in the dark and precautions were taken to exclude moisture. A slurry of 0.1 mole of 9,10-dibromooctadecanoic acid with 0.1 mole of red mercuric oxide in 200 ml of dry CCl_4 were mixed and stirred with a magnetic stirrer. The mixture was heated to reflux and a small portion of 0.1 mole of bromine was added. Refluxing was continued, and after the reaction started (evolution of CO_2) the rest of the bromine was slowly added over a period of 30 min. The reaction mixture was stirred and refluxed for another 2 hr and then cooled and filtered. The filtrate was washed with 5% sodium hydroxide solution followed by water and then dried over anhydrous $MgSO_4$. The solvent evaporated and the crude product (49.6 g) was placed on a 4.0 (diam) $\times$ 90 cm column of silicic acid that was prewashed with petroleum ether (bp 40–45°). 1,8,9-Tribromoheptadecane was eluted with six column volumes of petroleum ether. The solvent evaporated and the residue was dried *in vacuo* over P_2O_5. The yield was 19.5 g, 42%. *Anal.* Calcd for $C_{17}M_{33}Br_3$: C, 42.78; H, 6.97; Br, 50.24. Found: C, 44.12; H, 7.32; Br, 49.76. The yellowish oil was found to lose bromine upon exposure to light.

threo-8,9-Dibromoheptadecanol. Bergstrom and his coworkers investigated the selective replacement of the primary bromine by hydroxyl group in 1,8,9,11,12-pentabromoheptadecane (Bergstrom *et al.*, 1953). A similar reaction was applied here for the synthesis of threo-8,9-dibromoheptadecanol. In a mixture of 10 ml of bromobenzene and 32 ml of 99% ethanol, 0.01 mole (4.8 g) of *threo*-1,8,9-tribromoheptadecane and 5.40 g of pure sodium trifluoroacetate were refluxed during a period of 12 days. Periodically aliquots were removed and the reaction was monitored by thin-layer chromatography on silica gel G plates. The plates were developed with petroleum ether (bp 38–46°)–diethyl ether (3:2, v/v). An aliquot from the reaction gave three spots: an upper spot (R_F 0.9) corresponding to the starting bromide, a middle spot (R_F 0.5) corresponding to 8,9-dibromooctadecanoyl acetate and a third spot (R_F 0.25) resulting from partial hydrolysis of the acetate. Identification was tentative with the aid of standards that were cochromatographed.[1] At the conclusion of the reaction 1 ml of 8 N methanolic HCl was added and the mixture boiled for 4 hr. After cooling, the solution was concentrated *in vacuo*, taken into ether, thoroughly washed with $KHCO_3$ and water, and dried over Na_2SO_4 and the solvents were distilled off. Attempted crystallizations from a variety of solvents failed. The greasy material (5.2 g) was placed on a silicic acid column 3.5 (diam) $\times$ 40 cm. Petroleum ether (1 l., bp 40–45°) eluted the unreacted 1,8,9-tribromoheptadecane and a compound tentatively identified as 8,9-dibromoheptadecanoyl trifluoroacetate. 8,9-Dibromoheptadecanol was eluted from the column with 300 ml of petroleum ether (bp 40–45°)–ethyl ether (1:1, v/v). The solvent was evaporated in vacuo and the residue (4.7 g, 72.1%) dried over KOH. *Anal.* Calcd for $C_{17}H_{34}OBr_2$: C, 49.29; H, 8.27; Br, 38.58. Found: C, 49.47; H, 8.26; Br, 37.49.

8-Heptadecenoic Acid. 8,9-Dibromoheptadecanol (4.3 g, 0.01 mole) was oxidized in glacial acetic acid (18 ml) with a solution of 2.2 g of CrO_3 in 7.5 ml of 66% acetic acid. The mixture was heated on a steam bath for 12 min. Working-up in the usual way 4.0 g of crude acid was obtained. Without purification a small portion was debrominated according to the method of Kaufmann and Mestern (1963). An aliquot from the isolated acid was methylated with diazomethane and tested by gas–liquid partition chromatography in both polar and nonpolar columns. From its behavior in comparison with standard methyl esters, it was identified tentatively as methyl heptadecenoate. The purity of the acid was 92% and the main impurities consisted of shorter chain fatty acids. Hydrogenation over PtO_2 in methanol gave methyl heptadecanoate. Oxidative cleavage (Von Rudloff, 1956) located the double bond at the 8–9 position and infrared analysis indicated 7–10% trans isomers.

7-Hexadecenoic Acid. By cycling the above reactions, 7-hexadecenoic acid was synthesized. It was indistinguishable from 9-hexadecenoic acid when their methyl esters were tested by gas–liquid chromatography. Hydrogenation of its methyl ester gave methyl hexadecanoate and oxidative cleavage located the double bond at the 7–8 position. Purity was 92–93% and again the main contaminants were shorter chain fatty acids.

Prior to their use for the synthesis of cholesterol esters, both cis isomers of 8-heptadecenoic and 7-hexadecenoic acids were

[1] Reference compounds that were used were prepared as follows: 9,10-dibromooctadecanol from 9,10-dibromooctadecanoic acid by the general method of reduction with $LiAlH_4$ (Nystrom and Brown, 1947); 9,10-dibromooctadecanoyl acetate by acetylation of 9,10-dibromooctadecanol with acetic anhydride in benzene and pyridine (Dakin and West, 1928).

separated from contaminating quantities of trans isomers by column chromatography of their methyl esters on silicic acid impregnated with silver nitrate (Sgoutas and Kummerow, 1964b). For the present study the use of acids free of trans contaminants was imperative.

Cholesterol Esters. Radioactive cholesterol esters on a microscale were synthesized according to Pinter *et al.* (1964). They were purified as previously described (Goller *et al.*, 1970) and their radiochemical purity was confirmed by thin-layer chromatography and liquid scintillation counting. In all cases, the radiochemical purity was better than 97–99%.

Preparation of Enzyme. Liver homogenates were prepared from fed, 180–200-g-mole rats of the Sprague–Dawley strain essentially by the method of Deykin and Goodman (1969). For the experiments reported here the 100,000*g* supernatant fraction free of floating fat layer was routinely employed. Protein was determined by standard biuret methods (Gornall *et al.*, 1949).

Enzymatic Assay. Unless otherwise stated the incubation media contained 200 μmoles of potassium phosphate buffer (pH 7.4), 28 mg of 100,000*g* supernatant protein, and the amounts of cholesterol esters indicated in tables and figures, in 50 μl of acetone to a total volume of 2 ml. Incubations were carried out at 37° for 1 hr. The kinetic constants were determined from data on the initial rates (6-min incubation). Controls containing 0.1 mM of *N*-ethylmaleimide which inhibited the enzymatic action were included.

The reaction was terminated by the injection of 50 ml of chloroform–methanol (2:1, v/v). The mixture which contained a simple liquid phase was left overnight at room temperature. The chloroform–methanol extract was equilibrated with 0.2 volume of distilled water, the chloroform layer was collected by aspiration and the solvent evaporated to dryness with a stream of dry N_2. Hexane (5 ml) and 1 mg of each free cholesterol and of cholesteryl oleate were added and aliquots were subjected to thin-layer chromatography. The plates were coated with silica gel G and cyclohexane–benzene–acetic acid (30:30:1, v/v) was used as the ascending solvent. The plates were dried in the air and exposed to iodine vapor. The spots corresponding to cholesterol ester and free cholesterol were scraped off and transferred to small chromatographic tubes packed with prewashed glass wool. The radioactivity was eluted with methanol (15 ml) into scintillation vials, the solvent evaporated, and scintillation solution, 2 g of 2,5-diphenyloxazole, and 50 mg of 1,4-di-(2,5-phenyloxazolyl)benzene per l. of toluene was added, and the radioactivity was measured (Sgoutas *et al.*, 1969).

Results

In agreement with previous studies (Goller *et al.*, 1970) the rate of hydrolysis was found to be linear with reaction time for 45 min and with enzyme concentrations up to 30 mg of protein, when the 100,000*g* defatted supernatant was used. Accordingly, in the experiments to be described the enzymatic concentration was adjusted so that linear reaction rates were obtained. Also, groups of data to be directly compared were obtained on the same day and with the same enzymatic preparation since it was observed that the specific activity of different preparations varied. Under these conditions the standard deviation in the assays was not larger than 4% of the mean. Noted that in view of the demonstration (Deykin and Goodman, 1962; Goller *et al.*, 1970) that any further purification of rat liver cholesterol ester hydrolase has no discernible effect on the fatty acid selectivity in the experiments reported herein, the enzyme was concentrated only to a relative purity of 20 times.

TABLE I: Dependence of Hydrolysis Rate upon the Proximal or Terminal Portion of the Acyl Chain in Cholesterol Esters.[a]

Expt	Cholesterol Ester Fatty Acid[b]	μμmoles of Ester Hydrolyzed/hr per mg of Protein		
		50 μM	80 μM	100 μM
A	9-Octadecenoic	480	495	485
	9-Hexadecenoic	595	590	590
	9-Tetradecenoic	635	635	640
	8-Heptadecenoic	340	335	345
	7-Hexadecenoic	370	385	380
B	9-Octadecenoic	340	340	350
	9-Hexadecenoic	390	370	390
	9-Tetradecenoic	510	520	540
	8-Heptadecenoic	190	210	200
	7-Hexadecenoic	260	250	260

[a] The conditions of incubation are described in the text. Values represent the mean from two experiments and are corrected for controls. In experiment A, a freshly prepared enzyme preparation was used. In experiment B, the same enzyme preparation after 48-hr storage at 4° was used. [b] All acids have the cis configuration.

On the other hand, analyses for cholesterol ester and free cholesterol content of rat liver homogenates showed that the whole homogenate contained 0.4 μmole of cholesterol ester and 1.8 μmoles of free cholesterol per ml. In contrast, the defatted 100,000*g* supernatant contained only free cholesterol at approximately 70 mμmoles per ml and no cholesterol ester. The limits of the determination (Sperry and Webb, 1950) were approximately 0.005 mg per ml ±0.01 and the data were in general agreement with those of Deykin and Goodman (1962). Since the defatted 100,000*g* supernatant was used as the enzymic source throughout this study, the contribution of endogenous substrates such as cholesteryl oleate and palmitate was negligible.

Table I shows the rate of hydrolysis for cholesterol esters of fatty acids having a 9 carbon atoms proximal or terminal portion. An increase in the concentration of each cholesterol ester from 50 to 100 μM did not affect the rate indicating that the enzyme acted at its maximal velocity. The relative order in hydrolysis rates was cholesteryl 9-tetradecenoate > 9-hexadecenoate > 9-octadecenoate > 7-hexadecenoate > 8-heptadecenoate suggesting that those acids with a 9 carbon atoms proximal portion formed cholesterol esters which were the preferred substrates. This order of hydrolysis remained unchanged when an enzyme that was aged at 0° for 48 hr was employed (Table I, Expt B).

The values recorded in Table I also pointed to a dependence of the activity upon the chain length of the fatty acid constituent of the cholesterol ester. Such evidence was further ob-

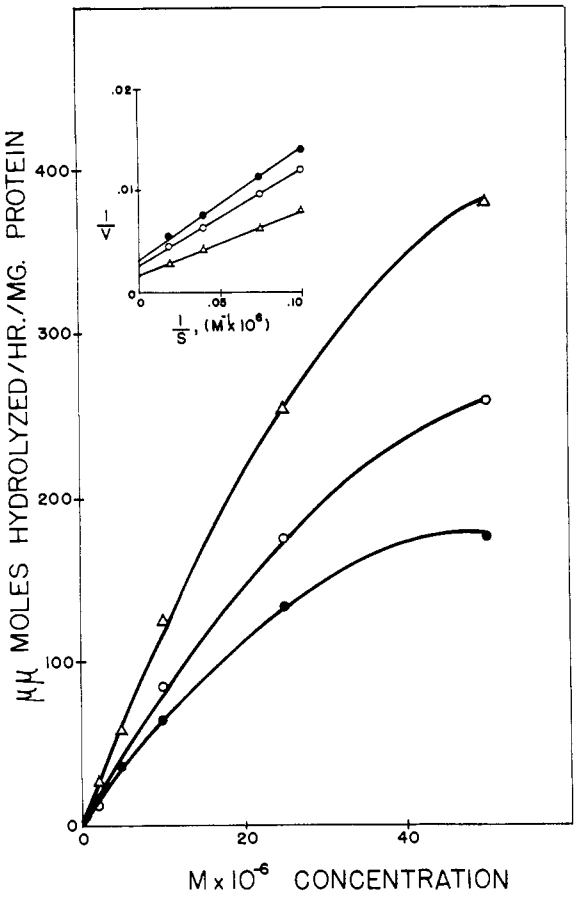

FIGURE 1: Concentration curves of hydrolysis of cholesteryl-*t* hexanoate (●—●), octanoate (○—○), and decanoate (△—△). Lineweaver–Burk plots are in the insert. Conditions for the reaction and the enzymatic assay are given in the text. Values represent the mean from two experiments and are corrected for controls.

tained when a homologous series of esters of cholesterol with *n*-fatty acids from 6 to 20 carbon atoms were used as substrates. Data in Table II show the relative activity and suggest that the series of esters with saturated acids could be roughly divided into two groups: with those acids above and with those below decanoic acid. The enzymatic activity increased from cholesteryl hexanoate to decanoate and then decreased sharply from decanoate to dodecanoate with a further gradual decrease to and through cholesteryl eicosanoate. In an ordinary chemical sense the lowest members of the series of cholesterol esters were expected to be much more reactive than the longer chain compounds. In the enzymatic hydrolysis, however, the reaction rate increased steadily with increasing chain length until a 10-carbon atoms length was reached.

In Figure 1, data from some preliminary kinetic experiments are presented. Cholesteryl-*t* hexanoate, octanoate, and decanoate were hydrolyzed individually and initial velocities of the reaction at different substrate concentration were determined. By extrapolation of the Lineweaver–Burk plots in the usual way substrate concentrations for half-maximal velocity were determined: 34.0 μM for cholesteryl-*t* hexanoate, and 35.0 and 32.0 μM for cholesteryl-*t* octanoate and for cholesteryl-*t* decanoate, respectively.

TABLE II: Chain-Length Specificity for Enzymatic Hydrolysis of Cholesterol Esters of Saturated Fatty Acids.

Cholesterol-*t* Ester Fatty Acid Component	μμmoles of Ester Hydrolyzed/hr per mg of Protein	
	50 μM	80 μM
Hexanoate	270	300
Octanoate	360	350
Decanoate	500	505
Dodecanoate	275	260
Tetradecanoate	210	205
Hexadecanoate	160	150
Heptadecanoate	90	100
Octadecanoate	125	120
Eicosanoate	100	105

[a] The conditions of incubation are described in the text. Values represent the mean from two experiments and are corrected for controls.

It should be pointed out, however, that there are major problems in applying enzyme kinetics derived for aqueous systems to the present studies. Due to the nonpolar nature of cholesteryl esters, the substrates were added in acetone and this mode of presentation increased the effectiveness of enzymic hydrolysis (Deykin and Goodman, 1962). Although, acetone increased the critical concentrations of micelle formation, the actual concentration of substrate in free solution remained an undetermined factor and in that case the values for K_m are calculated with no consideration to the particle size of the substrate.

In Figure 2, the hydrolysis of cholesteryl-*t* oleate was inhibited when a fixed amount of this substrate was incubated with increasing amounts of cholesteryl-^{14}C decanoate. The phenomenon was not linear and became asymptotic at higher concentrations of the inhibitor. Cholesteryl-^{14}C decanoate was hydrolyzed simultaneously with a rate that was almost zero at low substrate concentrations and only at higher substrate concentrations increased to substantial levels, after it overcame a lag period. Similar results were obtained with cholesteryl-^{14}C octanoate substituting for cholesteryl-^{14}C decanoate (Figure 2).

These mutual inhibitory effects between cholesteryl oleate and decanoate appeared to be caused by competitive reactions as shown in Figure 3. Reciprocal plots of initial velocities of hydrolysis of cholesteryl-*t* oleate showed convergent lines which intersected at the ordinate when increasing amounts of cholesteryl-*t* oleate were incubated in the absence and presence of varying amounts of cholesteryl decanoate.

The effect of a cis double bond in the ω position of medium chain fatty acids upon the reactivity of their cholesterol esters was also given some consideration. In particular, we wanted to find if the presence of a double bond at that distance from the esteratic site was important in promoting activity. To that purpose, cholesteryl-*t* 11-dodecenoate, 10-undecenoate, and 9-decenoate were synthesized and tested. The data in Table

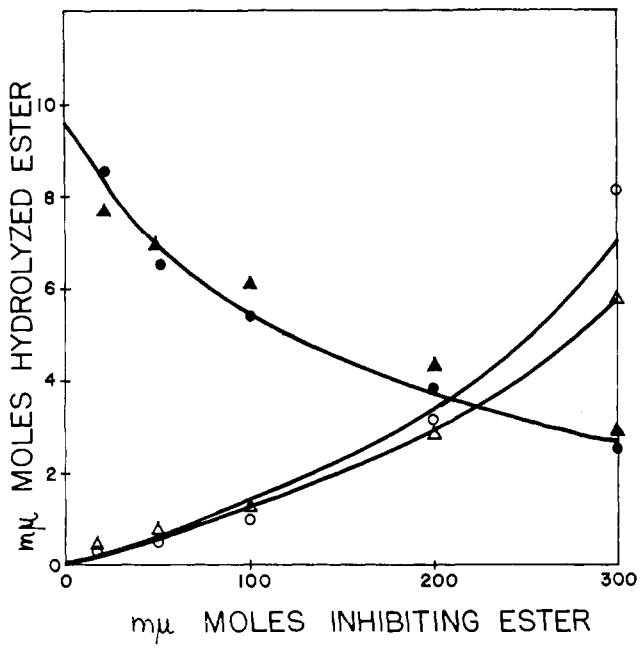

FIGURE 2: Mutual dependence in hydrolysis of 100 mμmoles of cholesteryl-*t* oleate by the indicated amounts of cholesteryl-^{14}C decanoate (●—●) and octanoate (▲—▲) with the simultaneous hydrolysis of cholesteryl-^{14}C decanoate (○—○) and octanoate (△—△). Conditions of reaction and assay are described in the text with the only difference that 20 mg of protein was incubated. Values representing the mean of two experiments and corrected for controls express mμmoles of hydrolyzed ester/hr per 20 mg of protein.

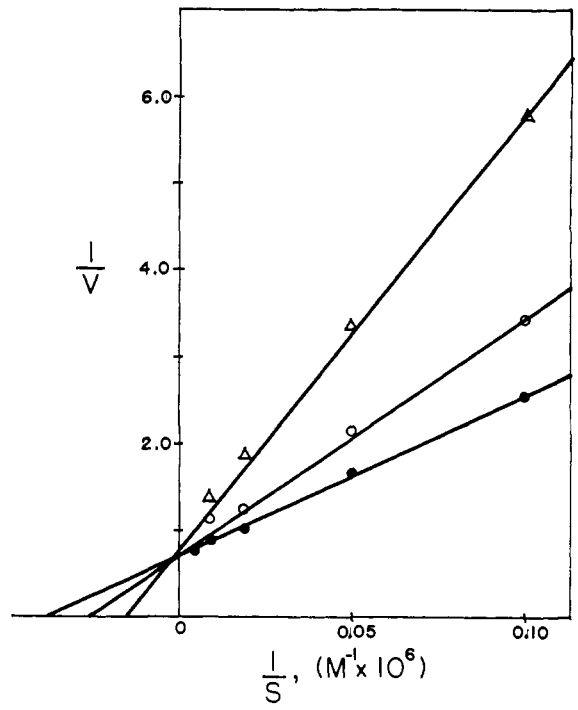

FIGURE 3: Reciprocal plots for cholesteryl-*t* oleate in the absence (●—●) and presence of 50 μM (○—○) and 100 μM (△—△) of cholesteryl decanoate. Conditions for reaction and assay are described in the text. Each point represents the mean of two experiments.

III clearly show that the hydrolysis rate of the indicated ω-unsaturated esters was of a higher order than that of their analogous saturated esters.

Discussion

It is evident that the preferred substrate for rat liver cholesterol ester hydrolase is either cholesteryl decanoate or cholesterol esters of monounsaturated fatty acids with a 9 carbon atoms proximal structure. In the latter case, only the cis configuration which results in a doubling back of the hydrocarbon chain is a determinant for a favorable enzymatic reaction (Sgoutas, 1968).

Generally, the association of substrate and enzyme which results in specificity, in higher reactivity of one compound than another and of one isomer than another is considered in terms of interactions of configurationally and conformationally oriented groups of substrates with complementarily located groups of sites of the enzymatic site (Hein and Niemann, 1961, 1962). It is usual that the structure of the substrates is well defined whereas the structure of the complementary groups of the enzyme is unknown. Studies of enzymatic reactions of substrates differing in structure may lead to inferences about the size, geometry, rigidity, and nature of the active site. In that respect, the data quoted in the present study strongly suggest that close to its hydrolytic site the enzyme possesses a patch which could accommodate nine methylene groups; thus, resembling a narrow strip one carbon wide and 16–18 Å long. Cholesteryl decanoate and 9-decenoate which are assumed to have exactly the correct length and "conformation" interact favorably with the active site and this interaction leads to a high reactivity.

Hydrophobic bonding between the alkyl chain of the substrate and the lipophilic patch of the enzyme protein is expected to play a critical role in binding. Within certain limits, our data indicated, that with increasing chain length of the acyl portion, reaction maximal velocities increased and that this occurred with relatively little change in the values of the Michaelis constants. Assuming that variations in alkyl size chains have a negligible effect upon the electronic character of

TABLE III: The Effect of Double Bond in the Binding of the Acyl Chain to Cholesterol–Ester Hydrolase.[a]

	μμmoles of Ester Hydrolyzed/hr per mg of Protein	
Cholesterol-*t* Ester	50 μM	80 μM
cis-9-Decenoate	680	660
Decanoate	500	505
cis-10-Undecenoate	310	325
Undecanoate	185	190
cis-11-Dodecenoate	420	425
Dodecanoate	280	275

[a] The incubation mixture is described in the text. Values represent the mean from two experiments and are corrected for controls.

the carboxyl group, the data suggested that a lengthening of the chain which would be expected to give only a tighter binding, resulted in an increase in the rate of attack at the acyl group. It should be noted, however, that K_m and V_{max} variations do not determine by themselves differences in binding or in the rate of breakdown of the enzyme–substrate complex. A detailed kinetic study would be required to determine whether the hydrophobic bonding energy of each additional methylene group is utilized directly to increase binding or to decrease the free energy of activation as the present data suggested. It is also indicated that a further increase in chain length decreased the enzymatic activity for both series of the homologous cholesterol esters that were tested (Tables I and II). Apparently, the additional methylene groups introduced some steric factors which produced a nuisance either to the binding or to the orientation of the substrate.

On the other hand, the requirement of the ω double bond for higher activity (Table III) could suggest that this group acted as a "handle" to bring about a better fit of the substrate to the enzyme. Whether a specific amino acid residue is implicated can not be stated at the present.

Finally, the present data coupled with those previously reported (Sgoutas, 1968; Goller *et al.*, 1970) leave a distinct impression about the high specificity of this enzyme considering the fact that it acts on relatively small substrates. Whether this is a unique feature of this hydrolytic enzyme or of other lytic enzymes, *i.e.*, glycerol ester hydrolase (EC 3.1.1.3) and phospholipases A_1 and A_2 (EC 3.1.1.4) that were found in rat liver cells (Olson and Alaupovic, 1966; Carter, 1967; Waite and Van Deenen, 1967) remains to be seen.

Acknowledgment

The authors thank Dr. F. A. Kummerow for his interest in this work. The technical assistance of Patricia Logsdon and Richard Archer is gratefully acknowledged.

References

Bergstrom, S., Paabo, K., and Rottenberg, M. (1953), *Acta Chem. Scan. 7*, 1006.

Brown, H. C., and Zweifel, G. (1961), *J. Amer. Chem. Soc. 83*, 3834.

Carter, Jr., J. R. (1967), *Biochim. Biophys. Acta 137*, 147.

Cristol, S. J., and Firth, W. C. (1961), *J. Org. Chem. 26*, 280.

Dakin, H. D., and West, R. (1928), *J. Biol. Chem. 78*, 91.

Davis, J. A., Herynk, J., Carroll, S., Bunds, J., and Johnson, D. (1965), *J. Org. Chem. 30*, 415.

Deykin, D., and Goodman, D. S. (1962), *J. Biol. Chem. 234*, 3649.

Fieser, L. F. (1955), *Org. Syn. 35*, 43.

Goller, H., Sgoutas, D. S., Gunstone, F. D., and Ismail, I. A. (1970), *Biochemistry 9*, 3072.

Gornall, A. G., Barnwill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Hein, G. E., and Niemann, C. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1341.

Hein, G. E., and Niemann, C. (1962), *J. Amer. Chem. Soc. 84*, 4495.

Hunsdiecker, H., and Hunsdiecker, C. (1942), *Ber. 75*, 291.

Kaufmann, H. P., and Mestern, H. Es. (1963), *Ber. 69B*, 2684.

Morris, L. J. (1966), *J. Lipid Res. 7*, 717.

Nevenzel, J. C., and Howton, D. R. (1957), *J. Org. Chem. 22*, 319.

Nystrom, R. F., and Brown, W. G. (1947), *J. Amer. Chem. Soc. 69*, 2548.

Olson, A. C., and Alaupovic, P. (1966), *Biochim. Biophys. Acta 125*, 185.

Pinter, K. G., Hamilton, J. G., and Muldrey, J. E. (1964), *J. Lipid Res. 5*, 273.

Seher, A. (1956), *Fette, Seifen, Anstrichm. 58*, 1077.

Sgoutas, D. S. (1968), *Biochim. Biophys. Acta 164*, 317.

Sgoutas, D. S., and Kummerow, F. A. (1964a), *Biochemistry 3*, 406.

Sgoutas, D. S., and Kummerow, F. A. (1964b), *J. Chromatog. 16*, 448.

Sgoutas, D. S., Sanders, H., and Yang, E. M. (1969), *J. Lipid Res. 10*, 642.

Sperry, W. M., and Webb, M. (1950), *J. Biol. Chem. 187*, 97.

Swell, L., Law, M. D., and Treadwell, Cr. R. (1964), *Arch. Biochem. Biophys. 104*, 1128.

Vogel, A. (1956), Practical Organic Chemistry, 3rd ed, New York, N. Y., Longmans, Green and Co., p 468.

Von Rudloff, E. (1956), *Can. J. Chem. 34*, 1413.

Waite, M., and Van Deenen, L. L. M. (1967), *Biochim. Biophys. Acta 137*, 498.