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Dependence of Sterol Ester Hydrolase Activity on the Position of Ethylenic Bond in Cholesteryl *cis*-Octadecenoates*

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ABSTRACT: The cholesterol esters of the 16 positional isomers of *cis*-octadecenoic acid were synthesized and their rate of hydrolysis with rat liver cholesterol hydrolase (EC 3.1.1.13) was studied. The results showed that the enzyme exhibited a distinct preference for the 9-octadecenoate. With the ethylenic bond moving to either end of the carbon chain the activity gradually decreased. The pattern of substrate preference did not change during purification of the enzyme. When the enzyme preparation was allowed to age, the activity was lost at uniform rates for each isomer and similar patterns of activities were observed. Comparison of the rates of cholesteryl 9,10-methyleneoctadecanoate with cholesteryl *cis*- and *trans*-9,10-octadecenoates showed that the presence of π electrons although perceived by the enzyme was not as important as the configuration of the acyl moiety.

Deykin and Goodman (1962) have presented an extensive study of liver cholesterol ester hydrolytic activity (cholesterol esterase, an enzyme belonging to EC 3.1.1.13 group). The subcellular distribution, properties, and relative activity of the enzyme against commonly occurring cholesterol esters were included in that report.

More recently the specificity of rat liver cholesterol ester hydrolase with regard to the hydrolysis of *cis*- and *trans*-fatty acid cholesterol esters was investigated (Sgoutas, 1968). It was found that *trans*-fatty acid cholesterol esters were hydrolyzed to a lesser degree than *cis* esters. Furthermore, a preference for the hydrolysis of the 9-*cis*-unsaturated octadecenoate was indicated, suggesting a selectivity of the enzyme in response not only to the configuration but also to the location of the *cis* functional group along the acyl chain.

This led us to investigate the cholesterol ester hydrolase activity further by using cholesterol esters of the complete series of *cis*-octadecenoic acids. The results showed that a remarkable selectivity of the enzyme with regard to the position of the *cis* double bond in the fatty acid chain exists.

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Materials and Methods

Synthesis of Octadecenoic Acids. The synthesis and characterization of all of the *cis*-octadecenoic acids have been described in detail elsewhere (Gunstone and Ismail, 1967). Their purity was determined by gas–liquid partition chromatography, by infrared spectroscopy, and by oxidative degradation. Traces of stearic acid, *trans* isomers, and positional isomers were removed by appropriate methods (Gunstone and Ismail, 1967). In brief, the 2-octadecenoic acid, prepared from palmitic acid *via* the C_{15}-bromide and C_{17}-alkyne, was 95% pure. The 3-octadecenoic acid prepared from 3-octadecynol, itself obtained by condensation of the lithium derivative of 1-hexadecyne with ethylene oxide, was 91–93% pure. The 4 to 9 isomers were synthesized by condensation of the dimethylamides of alkynoic acids with an alkyl bromide, and the 10 to 12 isomers were prepared from 1-alkynes and ω-halogeno acid amides. All these acids were almost 99% pure. The 13- to 17-octadecenoic acids were obtained from the corresponding C_{12} acids by using the enamine process for chain extension by six carbon atoms. These acids were contaminated mainly with their *trans* isomers and stearic acid: 13-octadecenoic acid (10%), 14-octadecenoic acid (4–5%), 15-octadecenoic acid (5%), 16-octadecenoic acid (2–4%), and 17-octadecenoic acid (6%). The methyl esters of these five acids were separated from the contaminants by chromatography on silver nitrate impregnated thin-layer plates.

Other Materials. Fatty acids (purity 99%) were obtained from Hormel Institute, Austin, Minn., and were rechecked for purity by gas–liquid partition chromatography. *cis*-9,10-Methyleneoctadecanoic acid was a generous gift from Mr. J. L. Williams. Cholesterol was purchased from Nutritional Biochemical Corp. and was found to be 98% pure by gas–liquid partition chromatography (Swell, 1966).

Cholesterol-7α-*t* from New England Nuclear Corporation was diluted with unlabeled cholesterol to a specific activity of 10 μCi/μmole and purified by argentation thin-layer chromatography (Morris, 1966). The final purity was better than 98% as determined by gas–liquid partition radiochromatography.

Synthesis of Cholesterol Esters. Radioactive cholesterol esters on a microscale (approximately 1 μmole) were synthesized according to the method of Pinter *et al.* (1964). The cholesterol esters were purified as previously described (Sgoutas, 1968) and their radiochemical purity was assessed by thin-layer chromatography in conjunction with liquid scintillation counting. In all cases, the radiochemical purity was better than 98%. Since the same stock of radioactive cholesterol was used for their synthesis, cholesterol-*t* esters had the same specific activity, namely, 10 μCi/μmole.

Preparation of Enzyme. Liver homogenates were prepared from fed, 180–200-g male rats of the Sprague–Dawley strain essentially by the method of Deykin and Goodman (1962). For most experiments reported here the 100,000*g* supernatant fraction free of floating fat was employed. When a purified enzyme preparation was used the steps of purification that were followed were those described by Deykin and Goodman (1962). To the defatted 100,000*g* supernatant fraction, solid ammonium sulfate was added to 30% saturation (1.8 mg/10 ml) at 0°. The mixture was stirred in ice for 30 min and centrifuged at 10,000*g* for 30 min. The precipitate was dissolved in a small volume of water and the protein was freed from ammonium sulfate by Sephadex B-25 chromatography (Porath and Flodin, 1959). The eluted protein was diluted (5 mg to 1 ml) with 0.01 M phosphate buffer (pH 7.4). Calcium phosphate gel[1] was added to the protein solution (28 mg of gel/100 mg of protein) at 0°. The suspension was agitated for 1 min, and allowed to stand for 30 min at 0°. After centrifugation for 15 min at 2800*g* the precipitate was discarded and the supernatant was adjusted to pH 6.5 with 0.1 N acetic acid. Additional calcium phosphate gel (100 mg of gel/100 mg of protein) was added, and after centrifugation (2800*g*, 30 min) the precipitated gel was retained. It was eluted with 0.1 M and 0.5 M phosphate buffer (pH 7.4; 1 ml of eluent/10 mg of gel). The 0.5 M phosphate eluent (eluent V) contained approximately 20% of the ammonium sulfate fraction activity and was diluted to 0.1 M with respect to phosphate before use. Protein was determined by standard biuret methods (Gornall *et al.*, 1949). The substrate specificity of the enzyme fractions during its purification were checked by comparing the hydrolytic activities against *p*-nitrophenyl acetate (Huggins and Lapides, 1947), *p*-nitrophenyl phosphate Na_2 salt (Bessey *et al.*, 1946), and cholesteryl-*t* oleate.

Enzymatic Assay. The incubation media were the same as those reported by Deykin and Goodman (1962) and they are described in the tables. Controls containing 0.1 mmole 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 single liquid phase was left overnight at room temperature. The chloroform–methanol extract was equilibrated with two-tenths volume of distilled water, the chloroform layer was collected by aspiration, and the solvent evaporated to dryness with a stream of dry N_2. Five milliliters of hexane and 1 mg each of 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, scintillation solution, 2 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis(2,5-phenyloxazolyl)benzene per l. of toluene, was added, and the radioactivity measured. All counts were corrected to disintegrations per minute by external standardization and channels ratio method.

Results

Initial experiments indicated that the rate of hydrolysis was found to be linear with time for 45-min reaction time with less than 20% deviation from linearity in 1 hr. Figure 1 shows the dependence of enzyme activity with enzyme protein concentration for the 100,000*g* supernatant fraction. There was a direct proportionality between activity and enzyme concentration up to 30 mg. With the calcium phosphate (see Methods) preparation, the activity was linear up to 4 mg of

[1] Obtained from Bio-Rad Laboratories, Richmond, Calif.

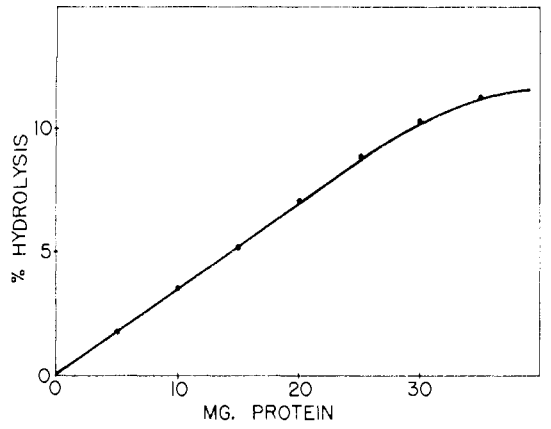

FIGURE 1: Relation of enzymatic concentration with activity. Increasing volumes of 100,000*g* supernatant were added to 30 mμmoles of cholesteryl-*t* oleate in 50 μl of acetone and in a final volume of 2 ml of 0.1 M potassium phosphate buffer. Incubations were carried out at 37° for 30 min.

enzyme protein in 20 μM cholesteryl-*t* oleate. Throughout these studies, the enzyme concentration was adjusted to obtain linear rate values. Also, groups of data to be directly compared were obtained on the same day and with the same enzyme preparation. Under these conditions the standard deviation in the assays was not larger than 2–4% of the mean.

Table I shows that increasing the concentration of each cholesterol ester from 40 to 100 μM did not significantly affect the rate of hydrolysis suggesting that the enzyme system was sufficiently saturated with substrate and that consumption of substrate during the early stages of the reaction would not change the rate. Table I also shows that cholesterol esters of fatty acids with the *cis* double bond located in the middle of the acyl chain were hydrolyzed at relatively higher rates. Among them cholesteryl *cis*-9-octadecenoate had the highest rate. As the double bond approached either end of the acyl chain the activity decreased.

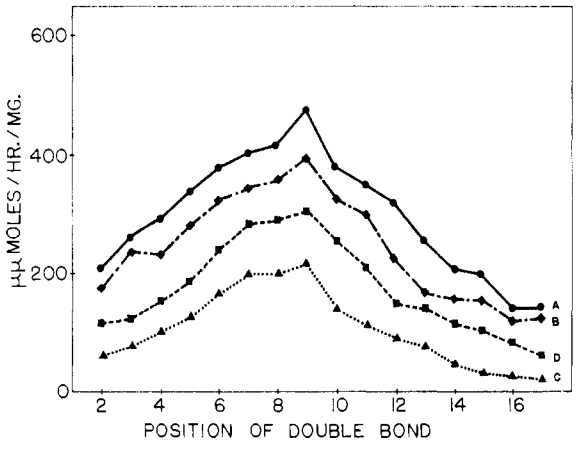

FIGURE 2: Cholesterol hydrolase specificities of fresh and aged enzymatic preparations from rat liver. The reaction mixture contained 100 mμmoles of cholesteryl-*t* ester in 50 μl of acetone, 200 μmoles of 0.1 M potassium phosphate buffer, and 27 mg of 100,000*g* supernatant protein in a final volume of 2 ml. The abscissa is numbered with reference to the carboxyl group. Curves obtained as described in text.

TABLE I: Cholesterol Ester Hydrolase Rates with Varying Concentrations of Cholesterol Esters.[a]

Cholesteryl Octadecenoate Positional Isomer[b]	μμmoles of Ester Hydrolyzed/hr mg of Protein		
	40 μM	75 μM	100 μM
2	190	210	205
3	270	265	265
4	280	290	280
5	330	340	360
6	390	380	400
7	410	405	390
8	420	420	410
9	460	475	480
10	370	375	390
11	350	350	360
12	300	320	310
13	255	255	260
14	220	210	200
15	205	200	205
16	140	140	160
17	150	140	120

[a] In 2 ml, the reaction mixture contained 200 μmoles of potassium phosphate buffer (pH 7.4), 40 mg of 100,000*g* supernatant protein, and the indicated amounts of cholesteryl-*t* ester in 50 μl of acetone. Incubation at 37°, for 1 hr. Each point represents the mean value of three experiments. [b] Number refers to the distance of the double bond from the carboxyl group.

Figure 2 presents results obtained with enzyme preparations of different activity. The reaction rates are displayed as a function of the position of the double bond in each isomer counting from the carboxyl group. Curve A, which had the fastest rates, was obtained with a freshly prepared enzyme. Curves B and C were obtained with the same enzyme preparation after it had been stored at 4° for 24 and 72 hr, respectively. Curve D gives the pattern for an enzyme preparation that was kept frozen for 48 hr prior to its use. From a comparison of the curves, it becomes apparent that there was no distinct difference among the preparations in the rates at which activities declined for each substrate. The same overall pattern of activity was maintained.

Table II gives the enzyme purification sequence as described in the Experimental Section. The data are in general agreement with those reported by Deykin and Goodman (1962). The approximately 70-fold purified enzyme (eluent V) exhibited no activity against *p*-nitrophenyl phosphate and a small activity against *p*-nitrophenyl acetate. For cholesteryl-*t* oleate the rate varied from 4.0 to 5.0 mμmoles per hr per mg of protein.

The relative hydrolysis rates of the complete series of cholesteryl *cis*-octadecenoates with the purified enzyme preparation (eluent V) are shown in Table III. Only the 6, 11, and 12 isomers were tested at different concentrations and their activities remained essentially constant over the range of tested concentrations. The other members were assumed to behave

TABLE II: Partial Purification of Rat Liver Cholesterol Ester Hydrolase.[a]

Fraction	Protein (mg)	Sp Act.	Rel Purifcn
Supernatant			
2,000*g*	4530	0.6	1.0
10,000*g*	3240	1.0	1.6
100,000*g*	1610	4.8	8.2
Ammonium sulfate precipitate	410	13.1	22.4
Calcium phosphate gel eluent V	24	42.5	72.5

[a] Millimicromoles of cholesteryl-*t* oleate hydrolyzed per milligram of enzyme protein per hour. Conditions of incubation: 200 μmoles of potassium phosphate buffer (pH 7.4), 100 mμmoles of cholesteryl-*t* oleate in 50 μl of oleate in 50 μl of acetone in a final volume of 2 ml, at 37° for 40 min.

TABLE III: Rates of Hydrolysis of Cholesterol Esters by the Purified Enzyme.[a]

Cholesteryl-*t* Octadecenoate Positional Isomer[b]	mμmoles of Ester Hydrolyzed/hr mg of Protein		
	30 μM	50 μM	75 μM
2		1.7	
3		1.8	
4		2.1	
5		3.2	
6	3.4	3.3	3.5
7		3.6	
8		4.0	
9		4.2	
10		3.6	
11	3.1	3.2	3.1
12	2.6	2.6	2.5
13		2.3	
14		1.8	
15		1.7	
16		1.4	
17		1.0	

[a] In 2 ml, each incubation mixture contained 200 μmoles of potassium phosphate buffer (pH 7.4), 0.3 mg of purified enzyme protein (eluent V, see Table II), and the indicated amount of substrate in 50 μl of acetone. Incubation at 37°, for 1 hr. Each value represents the mean value of two experiments. [b] Number refers to the distance of the double bond from the carboxyl group.

similarly in analogy to the constant activities that all members of the series exhibited with the 100,000*g* supernatant fraction. The pattern of the relative rates of hydrolysis was remarkably similar to that in Table I.

Experiments were also performed in which cholesteryl-*t* *cis*-9,10-methyleneoctadecanoate was included as substrate among others. It is well known that the configuration of cyclopropane derivatives is analogous to the *cis*-ethylenic derivatives but lacking π bonds. Data reported in Table IV show that the reaction rate for cholesteryl-*t* *cis*-9,10-methyleneoctadecanoate was closer to that of cholesteryl-*t* *cis*-9-octadecenoate and considerably higher than the rates for cholesteryl-*t* *trans*-9-octadecenoate or octadecanoate. These data serve to emphasize the finding that liver cholesterol ester hydrolytic enzyme is more sensitive to the configuration than to the presence of electrons at a given location in the acyl chain.

Discussion

The experiments described in this paper show that cholesterol esterase from rat liver is able to differentiate between isomers of cholesteryl octadecenoates which differ only in the position of the double bond of the acyl group. In agreement with earlier results (Deykin and Goodman, 1962; Sgoutas, 1968), it is concluded that the enzyme has the ability to perceive the presence of π electrons although it is distinctly more sensitive to the configuration of the acyl moiety.

An inspection of Tables I and IV would point to the fact that those members of the octadecene series with the double bond in the vicinity of the carboxyl or methyl group exhibit reactivities comparable to the reactivity of cholesteryl octadecanoate. This result corroborates the suggestion made previously (Reitz *et al.*, 1968, 1969) that classification of fatty acids as saturated and unsaturated seems to be an oversimplification and that each fatty acid should be regarded as having its own metabolic fate.

Deykin and Goodman (1962) studied the pattern of substrate preference of cholesterol ester hydrolase from rat liver during its purification. They found no difference with cholesteryl palmitate, stearate, oleate, and linoleate. In view of the fact that the cholesterol esters employed in the present study were highly unusual, we tested the selectivity of the enzyme at two different stages during its purification. It was shown that

TABLE IV: Cholesterol Ester Hydrolase Rates with Configurationally Isomeric Cholesterol Esters.[a]

Cholesterol-*t* Ester	$\mu\mu$moles of Ester Hydrolyzed/hr mg of Protein	
	40 μM	50 μM
cis-9,10-Methyleneoctadecanoate	400	400
cis-9-Octadecenoate	480	470
trans-9-Octadecenoate	200	200
Octadecanoate	150	160

[a] Each reaction mixture contained 200 μmoles of potassium phosphate buffer (pH 7.4), 28 mg of 100,000*g* supernatant protein, and the indicated amount of cholesterol-*t* ester in 50 μl of acetone in a total of 2-ml volume. Incubation at 37° for 40 min. Each value represents the average of two experiments.

the pattern of substrate preference did not change. This observation and the evidence that the activity was lost at uniform rates as the enzyme preparation was aged would suggest that we are dealing with a single enzyme and not with multiple forms or a mixture of cholesterol esterases each one acting on its own substrate. However, further purification and characterization of the enzymic activity will be necessary before we can conclusively state this.

In a previous study (Sgoutas, 1968) the melting points of substrate cholesterol esters and other similar measures of interaction of their hydrocarbon chain were considered and it was demonstrated that they did not correlate to the activities of the rat liver cholesterol ester hydrolase. It was postulated then that the enzymic properties that govern substrate specificity are a consequence of structural features of the enzymic surface. In that respect, full understanding of the enzymic reaction must await experimentation that would define both the structure of the enzyme protein and the electronic factors that reflect the enzymic properties.

The question naturally arises whether the observations described in the present paper have any biological significance. The importance of hepatic enzymes responsible for cholesterol ester hydrolysis becomes very obvious when one considers that hydrolysis within the liver is the immediate metabolic fate of almost all chylomicron cholesterol ester (Goodman, 1965) which is quite sizable due to the large volume of cholesterol circulating in the enterohepatic system. If similar cholesterol ester hydrolase selectivities occur *in vivo*, they would partly account for the enrichment of hepatic cholesterol ester in configurational and positional isomers of the naturally occurring fatty acids that was observed in some studies (Privett and Blank, 1964; Raulin, 1966). It must be noted, however, that the fatty acid composition of liver cholesterol esters is controlled by the interplay of several factors. One of them, the specificity of cholesterol ester hydrolase, was studied herein. Others include the selectivities of the enzyme systems synthesizing cholesterol esters, the nature of the fatty acid pool available to them, and competition among substrates for the enzymes involved either in the hydrolysis or the synthesis of cholesterol esters. All these factors must be taken into consideration in evaluating the mechanism by which changes in fatty acid composition of liver cholesterol esters occur *in vivo*.

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