Effect of Geometry and Position of Ethylenic Bond upon Acyl Coenzyme A–Cholesterol-*O*-acyltransferase*

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ABSTRACT: The specificity of acyl coenzyme A:cholesterol-*O*-acyltransferase (EC 2.3.1) from rat liver microsomes with regard to the configuration and position of the double bond in the acyl moiety was studied. The extent of cholesterol esterification, the composition of synthesized cholesterol esters, and the rate of cholesterol esterification with several isomers and homologs of the commonly found oleic and linoleic acids indicated that the enzyme is highly specific to a single *cis* double bond at a proximal distance of 9-carbon atoms from the carboxyl group.

trans-Fatty acids were not rapidly esterified and resembled stearic acid.

Rat liver microsomes contain an enzyme system which esterifies cholesterol in the presence of ATP and CoA. These cofactors could be completely replaced by the addition of preformed fatty acyl-CoA esters, suggesting that the enzyme involved is a fatty acyl-CoA–cholesterol-*O*-acyltransferase (Mukherjee *et al.*, 1958; Swell *et al.*, 1964).

The same studies have also shown that fatty acyl-CoA derivatives undergo esterification with free cholesterol *in vitro* in the following decreasing order: monounsaturated > saturated > polyunsaturated. No special attention, however, has been given to the specificity of the enzyme(s) for the configuration and location of the unsaturated bond(s) of the fatty acyl substrate.

In view of recent findings (Lands and Hart, 1965; Lands *et al.*, 1966; Reitz *et al.*, 1968) that acyl-CoA–phospholipid acyltransferase(s) from liver microsomes exhibit a high degree of specificity for the geometry and location of the ethylenic bond(s) in the acyl group, it became of interest to investigate whether similar properties apply to acyl-CoA–cholesterol-*O*-acyltransferase(s).

The present series of experiments was designed to determine whether the configuration and/or location of ethylenic bond influences the degree to which an unsaturated fatty acid is esterified to cholesterol by the microsomal fraction of the rat liver.

Experimental Procedure

Materials. The solvents and other chemicals were all reagent grade, of highest purity commercially available. Free fatty acid poor human serum albumin was prepared by the method of Goodman (1958); the preparation used had a mole ratio of free fatty acid to albumin of 0.2. CoA was obtained from Pabst Laboratories, and ATP was a Sigma Chemical product.

Unlabeled fatty acids were purchased from the Lipid Preparation Laboratory of the Hormel Institute. They were of known structure tested by ozonolysis and better than 95% pure as checked by gas–liquid partition chromatography. Stearolic acid synthesized by the method of Khan (1953) was a gift from Mr. J. L. Williams. Elaidic, linelaidic, *cis*-9,*trans*-12 and *trans*-9,*cis*-12 isomers of linoleic acid were synthesized as reported earlier (Sgoutas, 1968). The structures and purities were determined by ozonolysis, gas–liquid partition chromatography, argentation thin-layer chromatography, and infrared spectroscopy.

Cholesterol-7a-^{3}H and cholesterol-4-^{14}C were obtained from Nuclear-Chicago Corp. and each one was purified by argentation thin-layer chromatography (Morris, 1966) to a final radiopurity of 98%. They were diluted with unlabeled cholesterol, purified through its dibromide (Fieser, 1955) to a specific activity of 1 and 2 mCi per mmole, respectively.

Palmitic-1-^{14}C, oleic-1-^{14}C, and stearic-1-^{14}C acids were purchased from New England Nuclear Corp.; linoleic-1-^{14}C and palmitoleic-1-^{14}C acids from Applied Science Laboratories. They were purified by preparative gas–liquid partition chromatography of their respective methyl esters. Linoleic-1-^{14}C acid was freed from radioactive *trans* contaminants (8%) by thin-layer chromatography of its methyl ester on adsorbent of silver nitrate impregnated silica gel G. They were diluted with unlabeled (98% pure) acids to a specific activity of 2 mCi/mmole, radiopurity 94–98%.

Elaidic-1-^{14}C and linelaidic-1-^{14}C acids were synthesized from the corresponding *cis* acids by transmutation using thiophenyl radicals and characterized (Sgoutas and Kummerow, 1969). The methyl esters were purified by column chromatography on an adsorbent of silver nitrate impregnated silicic acid (Sgoutas and Kummerow, 1969). Each acid was diluted with pure (98%) unlabeled carrier to a specific activity of 2 mCi/mmole, radiopurity 98%.

Several monoenoic acids varying in chain length and the position of their double bond, and labeled with ^{3}H were used as substrates in this study. They were synthesized by a selective and stereospecific reduction of the corresponding alkynoic acid with tritiated disiamylborane (Brown and Zweifel, 1961; Nystrom *et al.*, 1964), followed by protonolysis with acetic-1-^{3}H acid. The alkynoic acids were prepared by bromination–dehydrobromination of commercially available monoenoic acids or by total synthesis.

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The following is given as a typical experiment of the bromination–dehydrobromination reaction. *cis*-6-Octadecenoic acid (280 mg, approximately 1 mmole) was brominated in dry ether at −5° and to the crude dibromo acid was added potassium *t*-butoxide (790 mg, 7 mmoles) in dry dimethyl sulfoxide (15 ml). The mixture was allowed to stay at 35° for 24 hr, then poured in 1 N sulfuric acid and ice, the product extracted with ether, and dried (Na_2SO_4). An aliquot was methylated with diazomethane and examined by gas–liquid partition chromatography with 20% ethylene glycol succinate and Apiezon L columns. The major component (78%) behaved similarly to methyl stearolate. It was freed from contaminants by column chromatography on silver nitrate impregnated silicic acid. The material (198 mg, 71.0%) which eluted from the column with 40–45% benzene in hexane (v/v), gave infrared absorptions identical with methyl stearolate and no allene band. No signal for olefinic protons in its nuclear magnetic resonance spectrum was present. Oxidative degradation (Von Rudloff, 1956) gave mainly hexanedioic (41%) and dodecanoic (38%) with varying amounts of C_7- and C_5-dibasic acids as well as of C_{11}- and C_{13}-monobasic acids, indicating that movement of the unsaturated bond occurred during bromination and dehydrobromination. Methyl 11-eicosynoate (83%) and methyl 13-docosynoate (71%) were synthesized and characterized in the above fashion.

Methyl 15-octadecynoate was synthesized from methyl 10-undecynoate which was converted into its silver salt (Isabelle and Leitch, 1958), reacted with diethyl iodide and then using the enamine synthesis for chain extension by six carbon atoms as described by Gunstone and Ismail (1967).

Reduction with concomitant introduction of ^3H was performed as in the case of methyl 6-octadecynoate which is presented as typical. Tritiated sodium borohydride (New England Nuclear Corp.) was diluted to specific activity of 16.4 mCi/mmole and was placed (37 mg, 1 mmole) in a dry 50-ml flask equipped with a magnetic bar. Freshly distilled under reduced pressure and over $LiAlH_4$ diglyme (3 ml) was added and the sodium borotritide was dissolved by stirring. In all stages of the reaction extreme care was taken to exclude ture. 2-Methyl-2-butene (175 mg, 2.5 mmoles) was added, the mixture was cooled to 0°, and freshly distilled boron trifluoride etherate (0.15 ml, 1.2 mmoles) was added drop by drop with constant stirring. At the end, the mixture was allowed to stay at 0° for 2 hr. To the disiamylborane-^3H thus prepared, was added methyl 6-octadecynoate (282 mg, 1.0 mmole) dropwise with external cooling and under constant stirring. After another 0.5 hr at 0° the solution was allowed to reach room temperature and stirring was continued for 2 hr. The mixture was cooled again, and ethylene glycol (0.1 ml) was added to decompose excess hydride. Tritiated acetic acid (0.5 ml, 0.8 mCi/mmole) was added and the protonolysis was completed at room temperature overnight. The crude product was recovered by pouring the mixture in cold water and extracting with hexane. It was purified by column chromatography on silicic acid with 10% anhydrous ether in hexane as the eluting solvent. The recovered material (270 mg, 92.4%) had a specific activity of 5 mCi/mmole (91.2%) and was ultimately purified by preparative gas–liquid partition chromatography. Radio gas–liquid partition chromatography (Dutton, 1961) indicated a radiopurity of 98% and a chemical purity of 97%, respectively. Infrared analysis showed no *trans* isomer. Oxidative degradation (Von Rudloff, 1956) indicated that the double bond was at C-6 position (82%). The cleavage products were free of ^3H suggesting that in the parent material ^3H atoms were located at the C atoms forming the double bond.

In a similar manner, the following methyl esters were synthesized: methyl *cis*-9-octadecenoate-9,10-^3H (88%; 4.9 mCi/mmole), methyl *cis*-15-octadecenoate-15,16-^3H (71%, 4.6 mCi/mmole), methyl *cis*-11-octadecenoate-11,12-^3H (72%; 4.9 mCi/mmole), methyl *cis*-11-eicosenoate-11,12-^3H (64%; 5.0 mCi/mmole), and methyl *cis*-13-dococenoate-13,14-^3H (68%; 4.3 mCi/mmole). All were better than 95% pure chemically and radiochemically as tested by radio gas–liquid partition chromatography. Saponification at 65° in 4 N KOH in 50% ethanol overnight gave the corresponding acids.

cis-9,*trans*-12-Octadecadienoic-9,10-^3H (85.2; 11.6 mCi/mmole) and *cis*-9,*trans*-12-nonadecadienoic-9,10-^3H (81.7; 11.4 mCi/mmole) acids were synthesized by tritioboration of the corresponding enynoic acids as reported (Sgoutas *et al.*, 1969). They were characterized, and analysis indicated a purity of at least 98% with the activity present solely in the acid.

Fatty acyl-CoA derivatives were synthesized from the acid chloride and free CoA by a modification (Lands *et al.*, 1966) of the method of Seubert (1960).

Radioactive fatty acyl-CoAs were prepared by the method of Galliard and Stumpf (1968). All of the acyl-CoAs migrated as single ultraviolet-absorbing spots on Whatman No. 1 paper with the solvent 1-butanol–acetic acid–water (5:2:3, v/v). In 3 mM Tris-HCl (pH 7.4), the A_{232}:A_{260} ratio was 0.55 or 0.57 for all preparations. Acyl-CoA concentrations were determined spectrophotometrically, assuming a molar absorptivity of 15,400 at 260 mμ (Bock *et al.*, 1956). The purity of the acyl-CoAs was checked by gas chromatography of the fatty acids liberated after hydrolysis with 0.1 N KOH for 1 hr at 25°.

Solutions of the potassium salts of labeled and unlabeled fatty acids (usually 0.04 M) were prepared by adding slight excess of aqueous KOH and warming. Solutions of saturated fatty acids were stored at 40–45°; those of unsaturated fatty acids were kept frozen under nitrogen.

Methods. White male rats (Holtzman Co.) were used in all experiments. They were maintained on a low fat diet containing 60% carbohydrate by weight and at the time that they were killed (by decapitation) they weighed 180–220 g. Their livers were perfused thoroughly with ice-cold 0.9% NaCl solution and homogenized in 2.5 volumes of 0.1 M potassium phosphate buffer (pH 7.4) at 0°. The homogenate was centrifuged at 2000*g* for 30 min to remove cellular debris which was discarded. The supernatant was then centrifuged at 10,000*g* for 30 min and the pellet was discarded. The supernatant was centrifuged at 104,000*g* for 60 min. The isolated microsomes were suspended in an amount of buffer medium equal to the volume of the original homogenate from which those subcellular fractions were obtained. The protein content of the suspensions was determined by the biuret reaction (Beisenherz *et al.*, 1953). Acyl-CoA derivatives and fatty acid salts were incubated with aliquots of these suspensions.

Unless otherwise stated, the basic incubation system contained in 3 ml: washed microsomes, 0.6 mg of protein; 300 μmoles of potassium phosphate buffer (pH 7.4); 3 mg of

TABLE I: Effect of Double-Bond Configuration upon Esterification of Cholesterol by Rat Liver Microsomes.[a]

	Cholesterol-^{14}C Converted into Cholesterol Ester	
Acyl-CoA Added[b]	75 mμmoles	150 mμmoles
cis-Δ^9-18:1	8.0	8.2
trans-Δ^9-18:1	2.6	2.4
cis-Δ^{11}-18:1	5.9	6.0
trans-Δ^{11}-18:1	2.8	2.5
18:2 cc	4.2	4.3
18:2 tt	2.6	2.8

[a] The incubation mixture was described in the text. Substrates added: cholesterol-^{14}C (60 mμmoles) and the indicated amount of fatty acyl-CoA ester. Values are mμmoles of ^{14}C-labeled cholesterol ester per 0.6 mg of enzyme protein per 40-min incubation time and they represent the average of duplicate observations of two experiments. [b] Fatty acids are designated by chain length; number of double bonds; position of double bond. The isomeric 9,12-octadecadienoates are designated cc for *cis*-9,*cis*-12; and tt for *trans*-9,*trans*-12.

TABLE II: Effect of Double-Bond Configuration upon Esterification of Cholesterol by Rat Liver Microsomes.[a]

		Labeled Substrate Converted into Cholesterol Ester	
Expt	Added Fatty Acid Salt[b]	75 mμmoles	150 mμmoles
A	*cis*-Δ^9-18:1	7.6	9.5
	trans-Δ^9-18:1	3.5	4.0
	cis-Δ^{11}-18:1	6.3	6.9
	trans-Δ^{11}-18:1	3.8	3.8
	18:2 cc	3.9	4.1
	18:2 tt	3.4	3.8
B	*cis*-Δ^9-18:1-^{14}C	8.3	
	trans-Δ^9-18:1-^{14}C	3.0	
	18:2 cc-^{14}C	3.9	
	18:2 tt-^{14}C	3.1	

[a] The conditions of incubation are described in the text. Substrates were added as follows. In expt A, cholesterol-^{14}C (60 mμmoles) and varying amounts of each fatty acid as indicated, and in expt B, 60 mμmoles of cholesterol plus the indicated amount of ^{14}C-labeled fatty acid salt. Values are mμmoles of ^{14}C-labeled cholesterol ester per 0.6 mg of enzyme protein per 40-min incubation time and they represent the average of duplicate observations of two experiments. [b] Fatty acids are designated as in Table I.

fatty acid deficient serum albumin; varying amounts of fatty acyl-CoAs; and cholesterol (added always in 0.1 ml of acetone). In some experiments acyl-CoA derivatives were replaced by 6 μmoles of ATP, 6 mmoles of $MgCl_2$, 0.3 μmole of CoA, varying amounts of potassium salts of fatty acids, and cholesterol (added in 0.1 ml of acetone). Incubations were conducted at 37° with shaking.

The reaction was stopped by pouring the contents of the tubes into 50 ml of chloroform–methanol (2:1, v/v) containing 0.5 ml of 1 N sulfuric acid. After shaking, 12 ml of 0.4% KCl was added and the aqueous layer was removed. The chloroform layer was dried (Na_2SO_4) and taken to dryness under a stream of nitrogen. The lipid residue was dissolved in hexane. The free and esterified cholesterol were fractionated on alumina columns as previously described (Sgoutas, 1968). Where radioactive fatty acids were employed, lipids were separated on silicic acid columns (Horning *et al.*, 1960) with the following solvents (v/v): benzene–hexane (15:85) (sterol esters), ether–hexane (7:93) (triglycerides and free fatty acids), chloroform (cholesterol and partial glycerides), and methanol (phospholipids). Free fatty acids were separated from triglycerides by extraction with 0.1 N NaOH in 50% ethanol (Borgstrom, 1952).

The distribution of radioactive cholesterol in different subclasses of cholesterol esters was determined by thin-layer chromatography on silica gel G impregnated with $AgNO_3$. The method which separated cholesterol esters according to the number, geometry, and sometimes position of the double bond (Sgoutas, 1968) in the fatty acid moiety is essentially that of Morris (1966) with different conditions. Standard cholesterol esters that were available from another study (Sgoutas, 1968) were used as markers. The separated compounds were visualized with 2,7-dichlorofluorescein spray under ultraviolet light. Each band was scraped into filter paper and eluted with chloroform into scintillation vials. Radioactivity was determined as reported earlier (Sgoutas *et al.*, 1965).

Alternatively, the incorporation of the substrate fatty acid into various lipids was determined by analysis of an aliquot of the lipid extract into lipid classes on thin-layer plates covered with silica gel G. The plates were developed in petroleum ether (bp 30–60°)–ether–acetic acid (90:10:1, v/v). The different bands identified by comparison with standards were scraped off and radioactivity was counted in the scintillation solution containing Cab-O-Sil according to Snyder and Stephen (1962).

Results

In preliminary experiments not reported here and in agreement with previously presented data (Goodman *et al.*, 1964) the rate of esterification was practically linear for enzyme concentration up to 0.4 mg of protein/ml and for 30-min reaction time with 20% deviation from linearity in 1 hr. Addition of 20–100 mμmoles of free cholesterol did not increase the velocity indicating that the microsomes contained initially an excess of endogenous free cholesterol sufficient to saturate the enzyme.

Table I shows the extent to which free cholesterol-^{14}C and geometric isomers of fatty acyl-CoA esters were converted into cholesterol esters by microsomal protein. Addition of oleate (*cis*-Δ^9-18:1) at both concentration levels increased the amount of cholesterol ester formed. Linoleate (18:2 cc), elaidate (*trans*-Δ^9-18:1), *trans*-vaccenate (*trans*-Δ^{11}-18:1), and linelaidate (18:2 tt) produced a minimal effect.

A similar order in cholesterol esterification was observed when the fatty acid salts were incubated (Table II). The data indicated, in accordance with previous experiments (Goodman *et al.*, 1964), that formation of acyl-CoA *in situ* could replace the preformed acyl-CoA. In fact, free fatty acids, Mg^{2+}, ATP, and CoA were as efficient if not better to acyl-CoA esters in acylating free cholesterol. There was no acylation of free cholesterol in the absence of added acyl-CoA or Mg^{2+}, ATP, and CoA, suggesting that the pool of acyl-CoA that exists in microsomes is negligible.

Table II also shows that the values calculated for the number of millimicromoles of cholesterol esters formed from the labeled cholesterol in the presence of a given unlabeled fatty acid were in reasonable agreement with the values calculated for unlabeled cholesterol in the presence of the same labeled fatty acid. This stoichiometric relationship already indicated that at the tested concentrations of exogenous substrate the contribution of endogenous fatty acid[1] was small.

A direct participation of each exogenous fatty acid in the esterification reaction was shown by analysis of the cholesterol-^{3}H esters synthesized after addition of exogenous fatty acid (Table III, expt A). The cholesterol-^{3}H ester composition was markedly influenced by the fatty acid added to the incubation mixture. Among the pairs of isomers more radioactivity was found in the cholesterol ester of *cis*-Δ^{11}-18:1 and *cis*-Δ^9-18:1 than of *trans*-Δ^{11}-18:1 and *trans*-Δ^9-18:1; more in the respective band with *cis*-9,*trans*-12 than with *trans*-9,*cis*-12 isomer of linoleate. Addition of 200 mμmoles of each exogenous fatty acid resulted in the predominant formation of the corresponding cholesterol ester (85–95%). On the other hand, addition of 40 mμmoles of acyl-CoA resulted in the almost exclusive synthesis of the corresponding cholesterol ester (Table III, expt B).

In another experiment, the relative ability of *cis*- and *trans*-fatty acyl-CoA isomers to undergo esterification with free cholesterol was investigated by incubating artificial mixtures which contained thiol esters in approximately equimolar concentrations and with only one labeled. The results are shown in Table IV. The relative order in incorporation into cholesterol esters was oleate > linoleate > elaidate = linelaidate. In phospholipids, oleate was the least incorporated among the other acyl-CoA esters, thus, suggesting that the biochemical processes involved in cholesterol ester and phospholipid synthesis were characterized by different relative specificities. Furthermore, the above experiments ruled out the possibility that differences in substrate solubility could be reflected in the extent of cholesterol esterification (Shah *et al.*, 1965).

In a repetition of this experiment the incubation time was increased to 90 min. It was found (data not shown) that the distribution of radioactivity among the lipid subclasses was practically identical with that shown in Table IV for the 30-min incubation.

Additional information on the effect of one configurational isomer upon the esterification of the other is presented in Figure 1. Incubation of a *cis* isomer labeled with ^{14}C with increasing amounts of its unlabeled *trans* isomer and *vice versa* showed a relation between a depressive effect and the concentration of the fatty acid added. The phenomenon was not linear and became asymptotic, indicating that when greater amounts of acids were added, other factors played an important role in the esterification in addition to a possible competition effect.

In another line of evidence, evaluation of the enzyme specificity based on substrate–concentration curves was made (Figure 2). Again, oleate was the most active substrate. Equally active was the *cis*-9,*trans*-12 isomer of linoleate whereas elaidate and the all *trans* isomer of linoleate exhibited rates that were comparable with stearyl-CoA ester. Palmitate and the all-*cis*-linoleate had activities somewhere in between, and they were included for the purpose of comparison. From the above data, double-reciprocal plots of initial velocity against substrate concentration yielded straight lines. By extrapolation of these plots, apparent K_m values were calculated: 4.1 μM for oleate, 2.8 μM for linoleate, and 2.2 μM for

TABLE III: Composition of Cholesterol Esters Formed with Added Fatty Acid Salts or Fatty Acyl-CoA.[a]

		Distribution of ^{3}H Act. in Cholesterol Esters[b] % Total of Esters					
		A	B	C	D	E	F
	Added Fatty Acid Salt[c]						
A	None	36		45		13	6
	cis-Δ^{11}-18:1	12	56	29		3	
	trans-Δ^{11}-18:1	24	26	38		10	2
	cis-Δ^9-18:1	16		77		5	2
	trans-Δ^9-18:1	18	31	40		8	3
	18:2 cc	23		28		49	
	18:2 ct	18		22	54	6	
	18:2 tc	23		34	42	5	
	Fatty Acyl-CoA[c]						
B	*cis*-Δ^9-18:1	2		95		3	
	trans-Δ^9-18:1	2	92	6			
	18:2 cc	1		5	1	93	
	18:2 ct	1		3	90	6	
	18:2 tc			4	88	8	

[a] Conditions for incubation as in the text. Substrates added: 60 mμmoles of cholesterol-^{3}H and 40 mμmoles of each fatty acid salt (expt A) or fatty acyl-CoA (expt B). Duration of incubation, 1 hr. [b] Cholesterol ester bands obtained by argentation thin-layer chromatography. Cholesterol esters used as standards migrated as follows: saturated, A; *trans*-monounsaturated and *cis*-vaccenate, B; oleate, C; ct and tc, D; cc, E; and polyunsaturated, F. [c] Fatty acids are designated by chain length; number of double bonds; position of double bond. The isomeric 9,12-octadecadienoates are designated cc for *cis*-9,*cis*-12; ct for *cis*-9,*trans*-12; tc for *trans*-9,*cis*-12.

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[1] The endogenous pool was analyzed for free cholesterol (Sperry and Webb, 1950) and free fatty acids (Dole, 1956) and the average values with four samples of unincubated microsomes were 24 and 16 mμmoles per mg of microsomal protein, respectively.

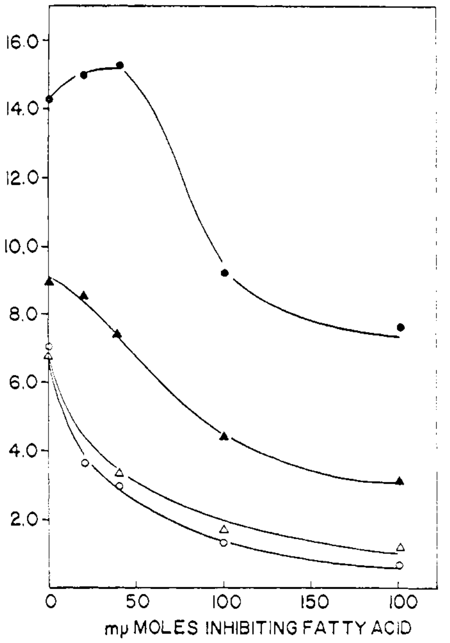

FIGURE 1: Effect of elaidic (●), linelaidic (▲), oleic (○), and linoleic (△) acid concentration upon the esterification of [^{14}C]oleic, [^{14}C]-linoleic, [^{14}C]elaidic, and [^{14}C]linelaidic acid, respectively. The incubation mixture was described in the text. Cholesterol (60 mμmoles) and 40 mμmoles of each [^{14}C]fatty acid were incubated for 1 hr. Values are the mean of duplicate observations from two experiments.

TABLE IV: Incorporation of Each Fatty Acyl-CoA, of an Equimolar Mixture of Oleate, Elaidate, Linoleate, and Linelaidate into Lipid Subclasses.[a]

		Distribution of Recovered Lipids-^{14}C (mμmoles)			
Expt	^{14}C-Labeled Fatty Acyl-CoA[b]	Cholesterol Esters	Triglycerides	Free Fatty Acids	Phospholipids
A	*cis*-Δ^9-18:1	8.4	1.4	20.4	3.6
	trans-Δ^9-18:1	1.3	1.1	20.8	13.2
	18:2 cc	2.2	1.3	15.4	18.4
	18:2 tt	1.1	1.2	21.0	12.8
B	*cis*-Δ^9-18:1	13.0	1.3	16.8	4.8
	trans-Δ^9-18:1	1.6	1.6	17.1	10.4
	18:2 cc	2.3	0.4	14.1	17.0
	18:2 tt	1.6	1.4	17.6	12.2

[a] Conditions of incubation as described in the text. In expt A, 60 mμmoles of cholesterol and 40 mμmoles of each of four acyl-CoA esters with only one of them labeled were added. In expt B, 150 mμmoles of cholesterol was added. Incubation lasted for 30 min and the values represent the average of duplicate observations. [b] Fatty acyl-CoA esters designated as in Table I.

elaidate, respectively. It should be noted, however, that the estimated kinetic parameters must be considered as tentative. The enzyme was a multienzyme reaction system, and the substrate had a limited solubility as the low apparent K_m values suggested and as the experiments of Zahler and Cleland (1969) indicated. In addition, kinetic analysis is further complicated by the detergent effects of acyl-CoA esters and by their binding to the enzyme protein.

In the second portion of this study, the effect of a number of acyl-CoA esters differing in configuration, position of double bond(s), and chain length upon the esterification of cholesterol was tested more intensively. A number of labeled acyl-CoA esters which differed by number, position, configuration of double bond, and chain length were tested and their reaction rates were collated in Table V, in decreasing order. An apparent correlation between all substrates with high reaction rates was that they had a single *cis* double bond at C-9 counting from the carboxyl group. These were good substrates regardless of chain length within the range of C-16 to C-20. The presence of a *trans* double bond in a methylene interrupted fashion did not produce any effect. In contrast, the presence of a second *cis* double bond in the above fashion decreased the rate considerably. One or two *trans* double bonds, regardless of position reduced the reaction rate to the level of saturated fatty acid. A difference of two positions in either direction of a single *cis*-ethylenic bond could be sensed by the enzyme and resulted in a reduced reaction rate. However, the effect was more appreciable when the bond was located further from position 9 as in *cis*-6-octadecenoate and even more in *cis*-15-octadecenoate. In these experiments the acyltransferase probably was proceeding at a maximal velocity since the rates of reaction were essentially independent of the thiol ester concentrations used.

In the reported experiments, groups of data to be directly compared were, as far as possible, obtained on the same day with the same preparation of microsomes, since it was observed that the specific activity of different microsomal preparations varied.

Nevertheless, to ensure that rate measurements were made under uniform conditions, a reference substrate was used as an "internal standard" for purposes of comparison. In several experiments the CoA ester of oleic-9,10-^{3}H acid was simultaneously and in equimolar concentrations incubated with fatty acyl-CoA substrates labeled with ^{14}C and the CoA derivative of oleic-1-^{14}C acid with substrates labeled with ^{3}H. It was assumed that variations in the activity between incubations could correspondingly effect the metabolism of both incubated thiol esters. Both isotopes were measured simultaneously in subsequent samples and the results were expressed as the ratio of ^{3}H:^{14}C. At equal concentrations between the substrate and the internal standard the ratio of the two isotopes recovered in cholesterol esters for a number of substrates were recorded in Table VI.

The data clearly show that the ratio of incorporated ^{3}H- and ^{14}C-labeled substrates reflected the ratio of their reaction rates as described in Table V. For example, an equal rate of esterification for oleate-^{3}H and palmitoleate-^{14}C (*cis*-Δ^9-16:1) was in harmony with an unchanged ^{3}H:^{14}C ratio between the substrate mixture added and the isolated cholesterol ester. The unchanged ^{3}H:^{14}C ratio of oleate-^{3}H and oleate-^{14}C pointed to the equivalence of their labels in agreement with a previous study (Sgoutas *et al.*, 1965).

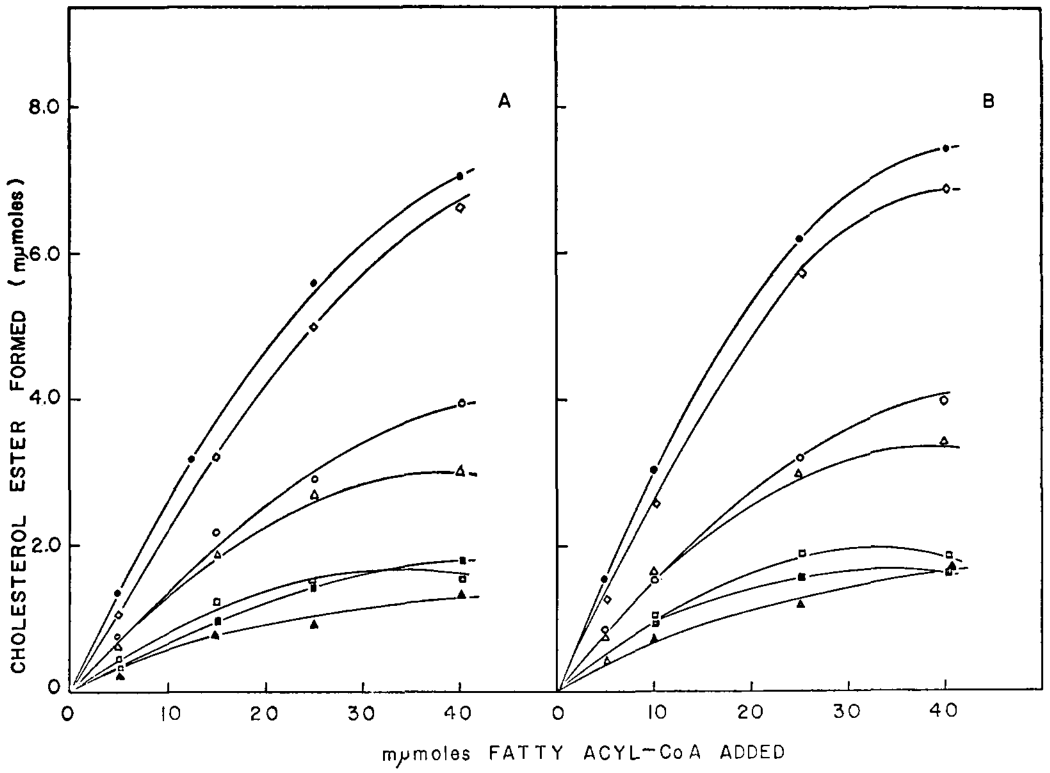

FIGURE 2: Esterification of cholesterol with varying amounts of [^{14}C]oleate (●), [^{3}H]*cis*-9,*trans*-12-octadecadienoate (◇), [^{14}C]palmitate (○), [^{14}C]linoleate (△), [^{14}C]elaidate (▲), [^{14}C]linelaidate (■), and [^{14}C]stearate (□). The incubation mixture was given in the text. Cholesterol, 30 mμmoles in expt A and 100 mμmoles in expt B. Fatty acyl-CoA esters at the indicated amount were incubated for 30 min. Values represent mμmoles of formed cholesterol esters per 30 min per 0.6 mg of protein and are the mean of two experiments.

Discussion

The availability of a number of positional and configurational isomers of unsaturated fatty acids permits a more searching evaluation of the specificity of rat liver microsomal acyl-CoA–cholesterol-*O*-acyltransferase(s) than has been possible previously. A marked difference in specificity between *cis* and *trans* isomers and a strong preference for a single *cis*-ethylenic bond at position 9 of the acyl moiety, were the significant properties of the enzyme.

Based on the evidence in the literature and on the data obtained here, acyl-CoAs apparently are involved in a number of competing reactions, namely, hydrolysis and incorporation into phospholipids. In order to rule out the possibility that the observed differences in cholesterol esterification were secondarily derived from the competing reactions, several experiments were conducted under different conditions. The amount of added cholesterol was varied and the duration of incubation was prolonged in several experiments. Despite these variations, the relative specificity of cholesterol esterification remained unchanged.

It is readily seen from an inspection of Tables I and II that the same rank order of relative specificity for cholesterol ester synthesis was observed when fatty acid salts, Mg^{2+}, ATP, and CoA were substituted for the corresponding thiol ester. This system, involving ATP, CoA, and fatty acid salt is actually an assay for a combination of two reactions, namely, the formation of the fatty acyl-CoA and its utilization in the acylation of cholesterol. The slowest reaction undoubtedly controls the overall rate of the reaction. In this respect, it is of interest to point out that no method has been found which will separate acyltransferase activity from the enzyme which activates fatty acids in the presence of ATP, CoA, and Mg^{2+}.

Preliminary studies (D. Sgoutas and P. Weston, 1969, unpublished data) indicated that rat liver microsomes were able to synthesize isomeric octadecenoyl coenzyme A thiol esters under conditions similar to those described by Pande and Mead (1968). Although differences in the reaction rates of activation were observed among the isomers, the observed values for the acyl-CoA synthetase(s) were at least 100-fold higher than the rates reported here for the acyl-CoA–cholesterol-*O*-acyltransferase. These findings suggest that in the esterification of free cholesterol with fatty acid salt, ATP, and CoA, the rate-limiting reaction should be attributed to the acylation step.

The data presented here are insufficient to define the molecular basis for the specificity of acyl-CoA–cholesterol-*O*-acyltransferase. It is quite possible that the enzyme responds to a physical property of the substrate, *i.e.*, melting point, solubility, etc., as well as specific structural features, *i.e.*, size, shape, etc., of the substrate molecule.

The current data and those previously reported (Goodman *et al.*, 1964; Swell *et al.*, 1964) clearly show that saturated and polyunsaturated fatty acids with marginal differences in their melting points and their solubilities have practically the

TABLE V: Rates of Esterification of Labeled Fatty Acyl-CoA Esters by Rat Liver Microsomes.[a]

	$\mu\mu$moles/min per mg of Protein	
Acyl-CoA[b]	75 mμmoles	150 mμmoles
cis-Δ^9-18:1	550	560
cis-Δ^9-16:1	533	520
18:2 ct	514	528
19:2 ct	453	490
cis-Δ^{11}-18:1	440	450
cis-Δ^{11}-20:1	416	434
cis-Δ^6-18:1	327	350
16:0	250	290
18:2 cc	240	230
cis-Δ^{15}-18:1	217	220
cis-Δ^{13}-22:1	180	180
18:2 tt	126	110
trans-Δ^9-18:1	118	120

[a] Conditions of incubation are given in the text. Cholesterol (60 mμmoles) and the indicated mμmoles of each fatty thiol ester labeled either with ^{14}C or ^{3}H (see Experimental Procedures) were incubated for 30 min. The values represent the average of duplicate observations of two experiments. [b] Fatty acyl-CoA esters are designated as in Tables I and III; 19:2 ct is *cis*-9,*trans*-12 nonadecadienoate.

same activities. Thus, if a physical property of the substrate exists to which the enzyme responds, then it must be expressed in a more complicated manner than melting point or solubility.

In past discussions (Malkin, 1931; Reitz *et al.*, 1969) the contribution of the tilt of the chain with respect to the plane of the terminal group of the acyl molecule as a parameter was considered. The argument can be advanced that since a correlation between tilt and melting point exists and since the melting point is rejected as a factor, so must be the tilt. Nevertheless, it is important to note that the enzymatic properties are studied with monomers in solution or micelles, whereas the melting point expresses the hydrocarbon chain interaction in the solid state.

A strong possibility exists, that we are dealing with a spectrum of several acyl-CoA–cholesterol-*O*-acyltransferases and that there is a correlation between the physical properties mentioned above, as well as others, and the specificity of individual acyltransferases. If this is the case, then, further separation and purification of enzymatic activities will be necessary before we can conclusively demonstrate it.

Finally, the enzymatic properties may well be a consequence of the spatial pattern and charge of the surface of the enzyme. A "correct" binding *i.e.*, that which results in esterification at high rates could depend on complementarity between substrate groups and specific enzyme side chains as well as substrate-induced changes of enzyme conformation, in accord with concepts of the formation of substrate–enzyme complexes in general. The definite structural identification of the mode of binding, however, will require the development of suitable methods.

TABLE VI: Ratio of Appearance in Cholesterol Esters of Incubated Substrates in Relation to a Reference Substrate.[a]

	Added Fatty Acyl-CoA		^{3}H:^{14}C Ratio	
Expt	Tested	Reference	Control	Cholesterol Ester
A	*cis*-Δ^9-18:1-^{14}C	*cis*-Δ^9-18:1-^{3}H	2.5	2.9
	trans-Δ^9-18:1-^{14}C	*cis*-Δ^9-18:1-^{3}H	2.1	15.0
	cis-Δ^9-16:1-^{14}C	*cis*-Δ^9-18:1-^{3}H	2.4	2.6
	18:2-^{14}C cc	*cis*-Δ^9-18:1-^{3}H	2.2	6.7
B	18:2-^{3}H ct	*cis*-Δ^9-18:1-^{14}C	5.8	5.2
	cis-Δ^6-18:1-^{3}H	*cis*-Δ^9-18:1-^{14}C	2.5	1.4
	cis-Δ^{11}-20:1-^{3}H	*cis*-Δ^9-18:1-^{14}C	2.4	1.3

[a] Incubation mixtures prepared as described in the text. Cholesterol (60 mμmoles) and 40 mμmoles of both tested and reference acyl-CoA were simultaneously incubated for 30 min. The values represent duplicate observations of one experiment. [b] Notations for fatty acyl-CoA esters as in Tables I and III; the label in parentheses.

Apart from these, competitive studies lead to the recognition that several enzymes that incorporate fatty acids into lipid classes could compete among themselves for the acyl-CoA when the substrate enzyme ratio is low in the medium. Then, in terms of biosynthesis, the channeling of fatty acids toward any lipid class becomes the result of interplay of tendencies that expresss enzyme specificity, concentration of metabolites, competitive inhibition, and other factors.

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Synthesis of Estriol 16α-(α-D-Glucoside) by Enzymic Transglucosylation*

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ABSTRACT: Formation of alkyl glucosides was demonstrated when lower alkanols and maltose were incubated together in the presence of the culture filtrate of *Aspergillus niger* (NRRL 330) or of commercial fungal amylase preparations. Of several steroidal alcohols tested, those with a 16α,17β- or a 16α,17α-diol grouping were also converted into their glucosides. The product from estriol has been isolated in crystalline form and identified as an α-glucoside of estriol. The isolation of estriol 3,17β-dimethyl ether from the acid hydrolysate of the fully methylated glucoside established the structure of the glucoside as 3,17β-dihydroxy-$\Delta^{1,3,5(10)}$-estratrien-16α-yl α-D-glucopyranoside.

Most studies of the oligosaccharide transglycosylases have dealt with the synthesis of polysaccharides or new oligosaccharides, with sugars (mono-, oligo- or polysaccharides) as the glycosyl acceptors (Hehre, 1951; Edelman, 1956; Hassid and Neufeld, 1962; Li and Shetlar, 1964; Manners *et al.*, 1968). Examples are known of compounds other than sugars acting as the glycosyl acceptors, with the resultant formation of heterosaccharides (Edelman, 1956). Syntheses of glycosides of lower alkanols (Whelan and Jones, 1953; Bealing, 1953), polyols (Whelan and Jones, 1953; Boos *et al.*, 1966), glucuronic acid (Barker *et al.*, 1959), riboflavin (Whitby, 1954; Tachibana *et al.*, 1957; Suzuki and Uchida, 1969), and aniline (Miwa *et al.*, 1960), by the use of the oligosaccharide transglycosylases have been reported. However, no steroidal alcohol has been considered capable of acting as the glycosyl acceptor in this type of reaction.

As a continuation of our earlier studies on the transglycosylase activity of the culture filtrate of *Aspergillus niger* (Pan *et al.*, 1951, 1953; Wolfrom *et al.*, 1951), we showed that the same enzyme preparation can transfer a glucosyl group from maltose not only to simple alkanols, but also to certain steroidal alcohols, *e.g.*, estriol. This paper presents a detailed account of these findings, especially on the isolation and identification of estriol 16α-(α-D-glucoside) thus synthesized.

Syntheses of steroid glucosides by conventional chemical methods have been amply documented (Miescher *et al.*, 1942; Miescher and Meystre, 1943; Huebner *et al.*, 1944; Elce *et al.*, 1967; Schneider and Bhacca, 1969). By an enzymic glycosyl transfer from uridine diphospho derivatives of monosaccharides, *e.g.*, glucose or glucuronic acid, steroid glucosides, and glucuronides have also been synthesized (Dutton, 1956; Eichenberger and Newman, 1968). The

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