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Technical Information

USE OF SONICATED DISPERSIONS OF MIXTURES OF CHOLESTEROL WITH LECITHIN AS SUBSTRATES FOR LECITHIN:CHOLESTEROL ACYLTRANSFERASE*

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RUNNING TITLE: TRANSESTERIFICATION OF SONICATED SUBSTRATES.

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SUMMARY

1. Sonicated dispersions of mixtures of unesterified cholesterol with lecithin can serve as substrates for radioassay of lecithin:cholesterol acyltransferase activity in the ultracentrifugal $d > 1.21$ g/ml fraction.

2. Net esterification yields (24 h incubation) and initial reaction rates decrease with increasing molar proportion of unesterified cholesterol in the sonicated substrate.

3. Negligible lecithin:cholesterol acyltransferase activity is observed when lecithin in the sonicated substrates is replaced by phosphatidyl ethanolamine, phosphatidyl serine or sphingomyelin.

4. Ultracentrifugal fractionation of incubated assay mixtures shows product cholesteryl esters distributed primarily between the $d < 1.063$ and the d 1.063 - 1.21 g/ml fractions.

5. The present findings support the hypothesis that the significant differences in reactivity of the different classes of serum lipoproteins with lecithin:cholesterol acyltransferase may result from differences in the relative proportions of unesterified cholesterol and lecithin on the lipoprotein surface accessible to the enzyme.

INTRODUCTION

During incubation of human serum *in vitro*, the substrates of serum lecithin:cholesterol acyltransferase (LCAT) are the unesterified cholesterol (UCS) and lecithin (LEC) of the serum lipoproteins [1]. When each of the three major classes of serum lipoproteins (very low density lipoproteins, VLDL, d < 1.006 g/ml; low density lipoproteins, LDL, d 1.006 - 1.063 g/ml; and, high density lipoproteins, HDL, d 1.063 - 1.21 g/ml) is incubated separately with preparations of LCAT, a higher rate of transesterification has been observed with HDL than with LDL or VLDL [2,3]. The physical-chemical factor (or factors) responsible for these apparent differences in reactivity has not as yet been determined. The potential importance of the content and relative abundance of UCS and LEC to the enzyme's reactivity was suggested earlier by the work of Wagner and Rogalski [4] as well as more recently by Glomset [5] and Raz et al. [6]. Since the HDL class normally shows a substantially lower content of UCS, together with a markedly lower mole ratio of UCS to LEC than LDL or VLDL, the differences in reactivity of LCAT with the various lipoprotein classes may depend, in part, on the relative content of these lipids in the lipoprotein molecules. In order to investigate the reactivity of LCAT with substrates composed of UCS and LEC, we have developed a radioassay method utilizing sonicated dispersions of specific mixtures of UCS and LEC. As a lipoprotein-free source of LCAT activity, we used the ultracentrifugal d > 1.21 g/ml protein fraction (UPF), which contains the bulk of the LCAT activity of serum.

MATERIALS AND METHODS

Preparation of Ultracentrifugal Protein Fraction (UPF)

Freshly prepared serum was raised to a salt background density of d 1.21 g/ml by addition of KBr. 4 ml aliquots of this adjusted serum were layered underneath 2 ml aliquots of a d 1.21 g/ml KBr-NaCl solution (containing EDTA 0.1 mg/ml) in preparative tubes just prior to ultracentrifugation (48 h, 114,000 g at 16°). After ultracentrifugation the top 3 ml, containing essentially all of the serum lipoproteins, was removed and the remaining bottom 3 ml fraction was stirred and pooled. The ultracentrifugal protein fraction (UPF) was exhaustively dialysed against a 0.01 M phosphate - 0.26 M NaCl buffer of pH 7.4. After dialysis, this preparation served as our source of LCAT activity.

Preparation of Sonicated Dispersions of UCS and LEC

Mixtures of egg lecithin (General Biochemicals, Chagrin Falls, Ohio) and unesterified cholesterol (Supelco, Inc., Bellefonte, Pa.) dissolved in ether, were introduced into sonication vials. After addition of (^{3}H) cholesterol (usually 3000 DPM, 15 mC/mM) (New England Nuclear, Boston, Mass.) the solvent was evaporated under N_2. 5 ml of a 0.01 M phosphate buffer (pH 7.4, no NaCl) was added and the contents were sonicated for ten minutes using a 60-watt ultrasonic disintegrator (#3000, M.S.E. Ltd., London). During sonication the vial was cooled in an ice water mixture. Shortly following sonication, these dispersions were used as substrates for determination of LCAT esterifying activity. In one series of experiments, phosphatidyl ethanolamine, phosphatidyl serine and sphingomyelin (Supelco, Inc., Bellefonte, Pa.) were separately substituted for lecithin in the sonicated substrates.

Incubation Procedures

Substrate and UPF solutions were generally pre-incubated separately to 37° prior to mixing. Aliquots of substrate and UPF solutions were mixed in a proportion such that the concentration of the UPF components in the final assay media corresponded to their concentrations in the original serum. Incubations were performed at 37° in a Dubnoff shaker. After appropriate intervals of incubation, duplicate 1 ml aliquots were removed, immediately frozen and stored for subsequent analysis.

Ultracentrifugal Fractionation of Incubation Mixtures

In one set of experiments, incubated mixtures were ultracentrifugally fractionated to determine the distribution of labeled unesterified cholesterol and product cholesteryl esters. Two preparative ultracentrifugations (24 h, 114,000 g at 16°) were performed in sequence, first at d 1.065 g/ml and second at d 1.21 g/ml. Lipid-containing fractions floating at the above densities as well as sedimenting material of $d > 1.21$ g/ml were removed and subsequently analyzed.

Lipid Analysis

Lipids were extracted according to the method described by Sperry and Brand [7]. Chromatography of extracted lipids on silicic acid columns was performed after addition of an appropriate amount of carrier cholesteryl esters, unesterified cholesterol and lecithin. The carrier lipids were added to assure proper chromatography of the small amounts of labeled lipids. Aliquots of the stock solutions of the sonicated substrates were also extracted, chromatographed (without carrier lipids), and analysed for content of UCS and LEC. Analyses of chromatographed

lipids were performed by infrared spectroscopic techniques previously described [8].

Radioassay

Lipid fractions (cholesteryl esters and UCS) obtained from chromatography were separately placed in counting vials, phosphor was added, and counting was performed in a liquid scintillation spectrometer. Counting efficiency was determined by addition of (^3H) toluene as an internal standard.

RESULTS

Net Esterification Yields with Sonicated Substrates

Assay of LCAT activity, utilizing sonicated dispersions of defined mixtures of UCS and LEC as substrates, was performed by the net esterification method (sampling after 24 h incubation) and by the initial rate method (samplings during 0 - 30 min incubation). Table I shows the effect of variation of the initial UCS content of the sonicated substrate on the net esterification yield. The initial concentration of LEC in each of the assay media was approximately one third the total LEC concentration in the serum of human adults. This concentration approximates the separate contribution to the total LEC concentration of serum normally made by either the LDL or the HDL class of lipoproteins. Table I shows that with increasing content of UCS in the substrate, there results a marked reduction in the net esterification yield.

Table II shows the effect of variation of the initial LEC content of the sonicated substrates on the net esterification yield. This experiment was performed at two initial concentrations of UCS in the

incubation mixture; one concentration approximated the UCS level contributed by LDL to human serum, while the other approximated the level contributed by HDL. With increasing initial amounts of LEC in the substrates there was a marked increase in net esterification yield at both initial levels of UCS. At the same level of LEC concentration in the incubation mixture, the net esterification was greater for the substrate with the lower initial content of UCS content (approximating UCS level of LDL). These observations further indicated that the net esterification yield was significantly influenced by the relative initial amounts of UCS and LEC in the sonicated dispersions.

Table III shows the net esterification yields in assay media in which the initial concentrations of two different sonicated substrates, each with a fixed molar fraction of UCS, were varied over a wide range. These data show that the net esterification yield is determined primarily by the relative proportions of UCS and LEC in the sonicated substrate and not by the bulk concentration of UCS in the assay medium. The relative proportions of UCS and LEC in these substrates approximated those encountered in HDL and LDL of human serum.

Initial Rates of Reaction with Sonicated Substrates

The effect of variation of the initial substrate composition on initial esterification rates was also investigated. Fig. 1 shows a representative curve describing the progress of esterification over the first two hours on incubation. The curve is essentially linear

during the first 20 - 30 minutes of incubation and subsequently falls off to a lesser rate of increase. Hence, for determination of initial rates of esterification, we used sampling periods of 30 minutes or less. Initial rate values for the same UPF sample incubated with different batches of sonicated substrates of identical composition were highly reproducible. For the substrate concentrations used to obtain the data in Fig. 1, the percentages of cholesterol esterified at the times 5.4, 10.8, 15.6, 20.4, 30.0 and 120.6 min were 5, 11, 16, 20, 27, 43 and 58%, respectively. Initial rates of esterification for substrates of different composition are presented in Table IV. In these experiments the initial LEC content of the substrate was essentially the same while the UCS content was varied over a wide range of values. These data show a steady decline in the initial rate of esterification with increasing initial content of UCS in the substrates. Hence, the composition of the substrates markedly affects the initial rate of esterification as well as the net esterification yield.

The Effects of Substrate and Enzyme Concentration

The effect of increasing the total substrate concentration (using a sonicated substrate initially containing a 0.15 mole fraction of UCS) on the initial rate of esterification is shown in Fig. 2. With increasing substrate levels, values of the initial rate reach a plateau. Application of this assay technique to evaluation of enzyme activity would require utilization of the higher substrate concentrations where a smaller fraction of the initial cholesterol is esterified. The effect

of variation of UPF concentration on the initial reaction rate was also evaluated at high concentrations of substrate. Values of initial reaction rates decreased linearly with decreasing concentrations of UPF in the incubation medium.

Reactivity of other Phospholipids in Sonicated Substrates

The susceptibility of sonicated dispersions containing other phospholipids, such as phosphatidyl ethanolamine, phosphatidyl serine and sphingomyelin, to transesterification by the LCAT preparation was also evaluated (Table V). There was negligible esterifying activity when these phospholipids were used as substrates with UCS. The physical appearance of sonicates containing these phospholipids was highly comparable to those containing lecithin. Under identical incubation conditions, marked esterification occurred with LEC.

Ultracentrifugal Distribution of Substrate UCS and Product Cholesteryl Esters

The ultracentrifugal distribution of the radioactive substrate UCS and the product cholesteryl esters in the assay media was also evaluated. Ultracentrifugal fractionation of a sonicated substrate, incubated for 24 h in the absence of UPF, showed the radioactive UCS to be present almost exclusively in the $d < 1.063$ g/ml fraction. Fractionation of an incubated assay mixture containing both the sonicated dispersion and UPF, showed the presence of unreacted substrate UCS in both the $d < 1.063$ and d 1.063 - 1.21 g/ml fractions. The level of unreacted UCS in the $d < 1.063$ g/ml fraction was approximately twice that in the d 1.063 - 1.21 g/ml.

The product cholesteryl esters were also found distributed between the $d < 1.063$ and d 1.063 - 1.21 g/ml fractions with the major part in the d 1.063 - 1.21 g/ml. Such accumulation of esterification products with the d 1.063 - 1.21 g/ml fraction suggests complex formation of substrate and product lipids with proteins of the UPF. The physical and chemical proterties of these complexes are under investigation to determine their role in the esterification reaction.

DISCUSSION

In most procedures for assay of LCAT activity, plasma or serum lipoproteins serve as substrates [9]. Lipoproteins are incorporated into the assay system either as common constituents of the test plasma itself, or are added as part of a heat-inactivated plasma to the test plasma or LCAT-containing preparation. Other assay procedures use isolated lipoprotein fractions as substrates free of other plasma components. Preparation of substrates for radioassay of LCAT activity is usually effected by incorporation of radioactive cholesterol, in various forms of dispersion or solution, into heat-inactivated plasma [10]. Assays are performed after exchange of the labeled cholesterol with the lipoprotein cholesterol in heat-inactivated plasma. While heat inactivation of a substrate plasma is necessary to destroy its LCAT activity, it does at the same time alter the physical and chemical properties of the substrate plasma lipoproteins (unpublished experiments). Hence, in such heat-inactivated systems, the lipoproteins, although capable of serving as substrates for LCAT, differ in their compositional properties from normal plasma lipoproteins. Notwithstanding these considerations,

these preparations provide a reproducible source of dispersed lecithin and unesterified cholesterol appropriate for quantitative assay of LCAT activity. Such preparations, however, do not provide a capability for varying substrate composition or for investigation of specific molecular aspects of substrate-enzyme interaction.

Our experiments demonstrate that sonicated dispersions of UCS and LEC can serve as substrates for LCAT preparations and apparently are accessible to enzyme action in a manner comparable to UCS and LEC in plasma lipoproteins. The initial rates of esterification observed in our studies utilizing sonicated substrates, compare favorably with initial rates determined by Glomset from incubation studies with heat-inactivated plasma or isolated lipoproteins as substrates. In plasma of normal human subjects he found the initial esterification rate of about 0.11 μmoles/ml plasma/h [11]. The initial reaction rates for cholesterol esterification in isolated HDL and LDL fractions were 0.08 μmole/ml/h (initial UCS concentration 0.40 μmoles/ml) and 0.015 μmole/ml (initial UCS concentrations 0.60 μmoles/ml), respectively [2]. In our studies, the initial reaction rates for sonicated substrates similar in lecithin and unesterified cholesterol composition to plasma HDL and LDL were 0.064 μmoles/ml/h (initial UCS concentration 0.18 μmoles/ml) and 0.011 μmoles/ml/h (initial UCS concentration 0.58 μmoles/ml), respectively.

Our observations on the influence of the relative content of substrate UCS and LEC on esterification rates and yields may explain in

large part the strong reactivity of LCAT with the phospholipid-rich HDL [12,13]. The presence of high proportions of UCS in the sonicated substrates may decrease esterification rates and yields by reducing the closeness of fit of the enzyme's active site with the component substrates. Specifically, the interaction of the enzyme's active site with the substrate may be sensitive to alterations in the spacing and packing of the substrate components which may result from some condensation of the substrate surface by additional UCS. On the other hand, the esterification of a particular substrate UCS molecule may be strongly influenced by the composition of its immediate molecular neighbors. The presence of neighboring UCS molecules may be inhibitory to the esterification of a particular UCS molecule. This is suggested by our observation of maximal esterification rates and yields when assays are performed with sonicated substrates in which most UCS molecules are probably completely surrounded by LEC molecules. Wagner and Rogalski [4] observed inhibition of LCAT activity when serum was incubated in a vial glazed on the inside with unesterified cholesterol. However, unesterified cholesterol was not inhibitory in their studies when it was added to serum in a dispersion of sodium oleate. An apparent strong reactivity of the enzyme with LEC is also indicated from our experiments where we found that, under identical conditions, other phospholipids were inactive as substrates for LCAT. However, as pointed out by Dawson [14], the physical state of phospholipids may significantly influence the rate of an enzymatic reaction involving phospholipid

substrates (e.g., reactions with phospholipases). Hence, from our present data, it is not possible to attribute the marked apparent specificity of LCAT for LEC entirely to a structural specificity of the enzyme for LEC. Further experiments of this apparent specificity of LCAT for LEC are in progress.

Our data do not rule out the possible participation of proteins of the UPF, other than LCAT, in the esterification reaction. The presence of HDL and LDL peptides in the d > 1.21 g/ml fraction has been reported by several workers [15,16,17]. These lipophillic peptides could be incorporated, during incubation, into the substrate particles and thereby influence LCAT activity. Our present data show that, following 24 h incubation of UPF with a sonicated substrate, labeled product cholesteryl esters are found in both the ultracentrifugal d < 1.063 and the d 1.063 ÷ 1.21 g/ml fractions. Recently, each of these fractions has been demonstrated to contain also LEC, UCS and some protein material (unpublished observations). Hence, complex formation between the reactants and products of the LCAT reaction together with some protein moieties of the UPF does occur. Current studies (unpublished data of W. Ho and A. V. Nichols of this laboratory) also show that LCAT itself can be complexed together with sonicated dispersions.

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Fig. 1. Rate of cholesterol esterification in assay medium containing UPF and sonicated substrate. Assay conditions were as in Table I, except that the assay medium was sampled at the time intervals specified in the text. Initial concentration of UCS in assay medium was 0.12 μmoles/ml. Initial molar fraction of UCS in substrate sonicate was 0.15.

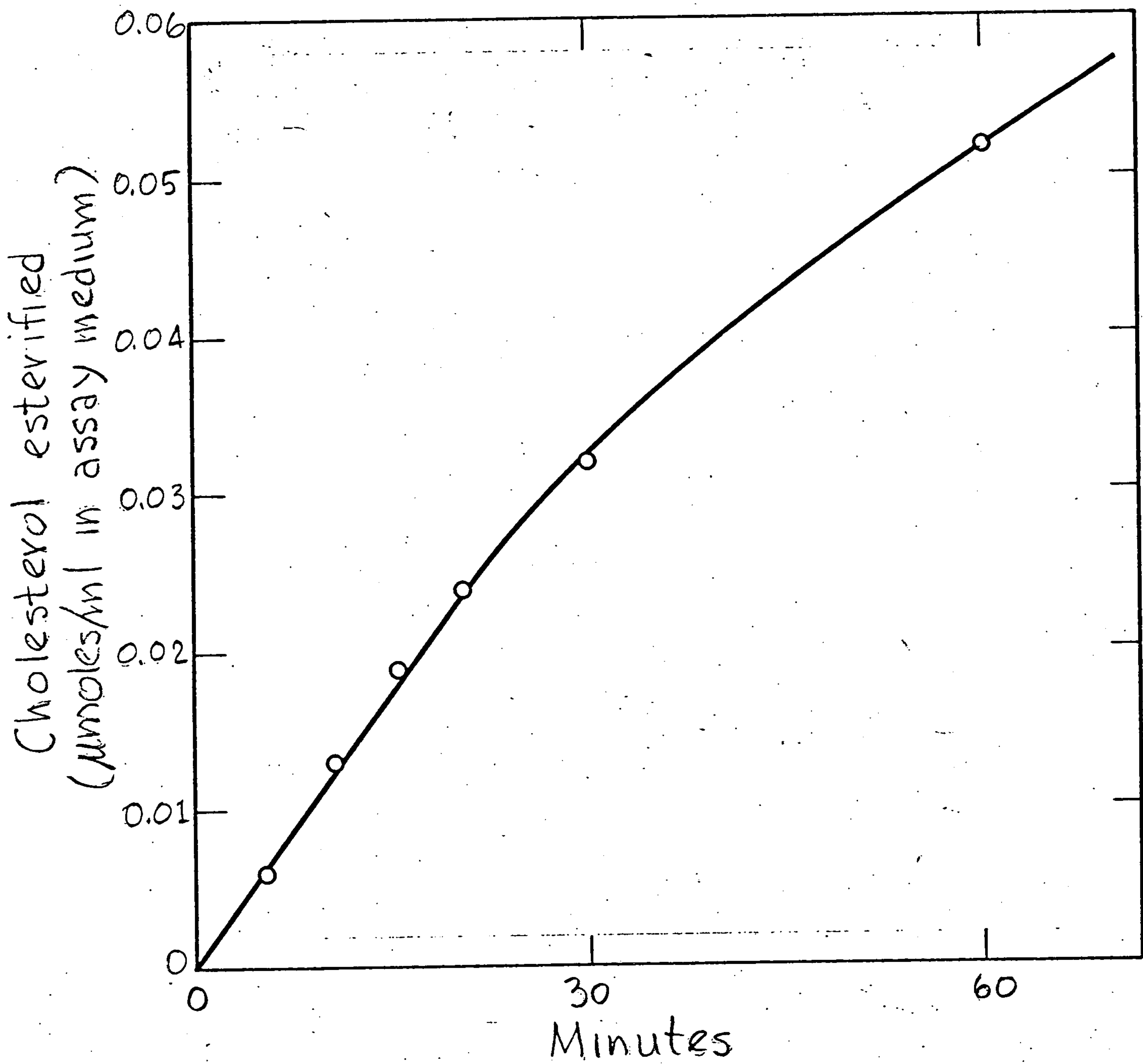
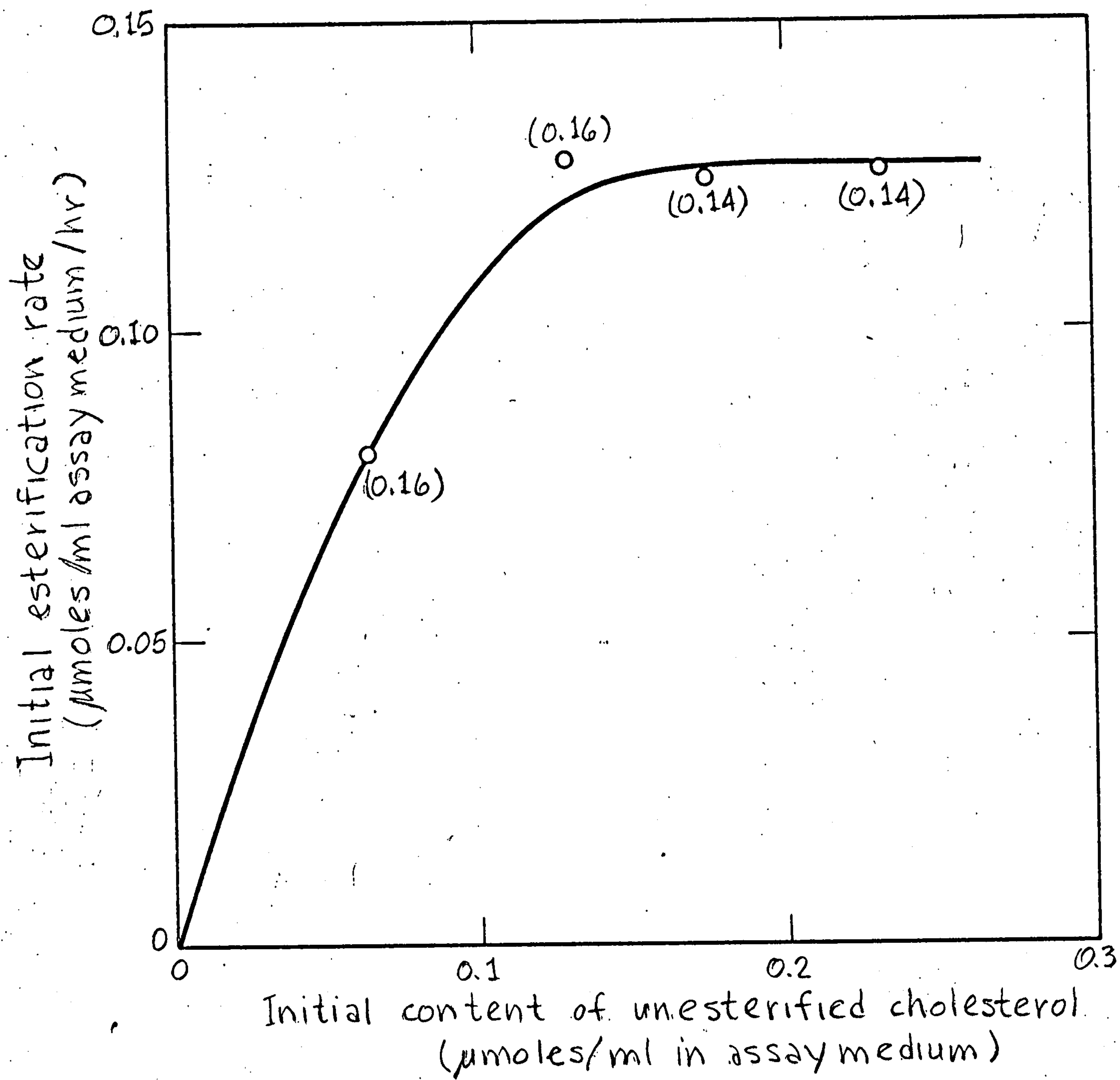

Cholesterol esterified
(µmoles/ml in assay medium)
0.06
0.05
0.04
0.03
0.02
0.01
0
0
30
60
Minutes

Fig. 2. Effect of total substrate concentration on initial esterification rate in assay medium. Assay conditions were as in Fig. 1. Values in parentheses are initial molar fractions (0.14-0.16) of UCS in sonicated dispersions.



0.15
0.10
0.05
0
Initial esterification rate
(µmoles/ml assay medium/hr)
(0.16)
(0.16)
(0.14)
(0.14)
0
0.1
0.2
0.3
Initial content of unesterified cholesterol
(µmoles/ml in assay medium)

TABLE I.

EFFECT OF INITIAL UCS CONTENT OF SUBSTRATE ON NET ESTERIFICATION YIELD (AT APPROXIMATELY SAME INITIAL LEC LEVEL IN ASSAY MEDIA). Assay media consisted of UPF plus sonicated UCS-LEC dispersion (3:1, v/v), final background solution consisted of 0.01 M phosphate - 0.19 M NaCl buffer, pH 7.4. Incubation for 24 h at 37°.

Substrate Composition (molar fraction of UCS + LEC)		Concentrations of Substrates UCS and LEC (μmoles/ml assay medium)		Net Esterification Yields (μmoles/ml assay medium)
UCS	LEC	UCS	LEC	
0.11	0.89	0.10	0.80	0.092 (92)*
0.21	0.79	0.21	0.79	0.086 (41)
0.34	0.66	0.39	0.77	0.059 (15)
0.42	0.58	0.54	0.75	0.043 (8)
0.52	0.48	0.83	0.76	0.033 (4)

* Values in parentheses are percentages of cholesterol esterified during incubation interval.

TABLE II

EFFECT OF INITIAL LEC CONTENT OF SUBSTRATE ON NET ESTERIFICATION YIELD (AT TWO LEVELS OF INITIAL UCS IN ASSAY MEDIA). Assay conditions as in Table I.

Substrate Composition (molar fraction of UCS + LEC)		Concentrations of Substrates UCS and LEC (μmoles/ml assay medium)		Net Esterification Yields (μmoles/ml assay medium)
UCS	LEC	UCS	LEC	
		Experiment 1		
0.41	0.59	0.21	0.30	0.027 (13)*
0.34	0.66	0.21	0.40	0.042 (20)
0.21	0.79	0.21	0.79	0.086 (41)
0.15	0.85	0.21	1.34	0.200 (95)
		Experiment 2		
0.52	0.48	0.57	0.52	0.034 (6)
0.40	0.60	0.65	0.97	0.072 (11)
0.25	0.75	0.62	1.83	0.223 (36)

* Values in parentheses are percentages of cholesterol esterified during incubation interval.

TABLE III

EFFECT OF INITIAL SUBSTRATE CONCENTRATION ON NET ESTERIFICATION YIELD (FOR TWO SUBSTRATES WITH DIFFERENT MOLAR FRACTIONS OF UCS). Assay conditions as in Table I.

Substrate Composition (molar fraction of UCS + LEC)		Concentrations of Substrates UCS and LEC (μmoles/ml assay medium)		Net Esterification Yields (μmoles/ml assay medium)
UCS	LEC	UCS	LEC	
		Experiment 1		
0.21	0.79	0.08	0.30	0.042 (53)*
0.21	0.79	0.21	0.79	0.086 (41)
0.23	0.77	0.41	1.41	0.168 (41)
0.25	0.75	0.62	1.83	0.223 (36)
		Experiment 2		
0.41	0.59	0.21	0.30	0.027 (13)
0.42	0.58	0.31	0.43	0.034 (11)
0.42	0.58	0.54	0.75	0.043 (8)
0.45	0.55	1.16	1.44	0.046 (4)

* Values in parentheses are percentages of cholesterol esterified during incubation interval.

TABLE IV

EFFECT OF INITIAL UCS CONTENT OF SUBSTRATE ON INITIAL ESTERIFICATION RATE. Assay conditions as in Fig. 1.

Substrate Composition (molar fraction of UCS + LEC)		Concentrations of Substrates UCS and LEC (μmoles/ml assay medium)		Initial Esterification Rate (μmoles UCS esterified/ml assay medium/h)
UCS	LEC	UCS	LEC	
0.16	0.84	0.13	0.70	0.127
0.21	0.79	0.18	0.68	0.064
0.30	0.70	0.30	0.70	0.038
0.46	0.54	0.58	0.69	0.011

TABLE V

SUBSTRATE PHOSPHOLIPID AND CHOLESTEROL ESTERIFICATION.

Assay conditions were as described in Table I. Initial molar fraction of UCS in each substrate sonicate was 0.14. Initial concentration of phospholipid in assay media was approximately 0.65 μmoles/ml.

Phospholipid in Substrate Sonicate	Cholesterol Esterified (nanomoles/ml assay medium) Incubation Interval		
	20 min	60 min	120 min
Phosphatidyl choline (lecithin)	30.6	46.6	58.5
Phosphatidyl ethanolamine	0.5	1.1	1.6
Phosphatidyl serine	0.6	0.8	0.8
Sphingomyelin	0.4	0.7	1.0

TABLE VI

ULTRACENTRIFUGAL FRACTIONATION OF ASSAY MEDIA: DISTRIBUTION OF RADIOACTIVE CHOLESTEROL AND CHOLESTERYL ESTERS.

Two mixtures were incubated for 24 h at 37° in 6 ml preparative ultracentrifuge tubes. One contained 3 ml UPF plus 1 ml sonicated substrate; final solution background consisted of 0.01 M phosphate - 0.19 M NaCl buffer (pH 7.4); the other contained 3 ml phosphate buffer plus 1 ml of the same sonicated substrate (same final background solution). Initial concentration of UCS in mixtures was 0.13 μmoles/ml. Initial molar fraction of UCS in substrate was 0.15. After incubation, mixtures were subjected to sequential preparative ultracentrifugation (24 h, 114,000 g, 16°), first at d 1.063 and then at d 1.21 g/ml. Lipid-containing fractions floating at the above densities as well as sedimenting material of $d > 1.21$ g/ml were removed and analysed for distribution of radioactivity in the UCS and the cholesteryl esters. (See Materials and Methods.)

Ultracentrifugal Fraction	Parent Incubation Mixture	Percent of Total Radioactivity In	
		UCS	CSE*
$d < 1.063$	Substrate & buffer	95	—
	Substrate & UPF	7	34
d 1.063 - 1.21	Substrate & buffer	1	—
	Substrate & UPF	3	50

* CSE, cholesteryl esters