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FURTHER STUDIES OF THE MECHANISM OF THE PLASMA CHOLESTEROL ESTERIFICATION REACTION

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SUMMARY

The role of the major plasma lipoprotein fractions in the plasma cholesterol esterification reaction has been studied. Human plasma was incubated at 37° for 24 h, and the lipoproteins were subsequently fractionated by ultracentrifugal flotation. Each fraction was compared to the corresponding non-incubated control with respect to content of total and unesterified cholesterol, lecithin, and lysolecithin. Although the total cholesterol content of the individual fractions did not change as a result of the incubation, the unesterified cholesterol of each decreased, the greatest decrement being associated with the low-density lipoproteins. In contrast, the greatest decrement in lecithin was in the high-density fraction, and the greatest increment in lysolecithin was in the "very high-density" fraction. When lipoprotein fractions obtained by precipitation with ethanol were incubated separately, it was found that the relative decrease in α-lipoprotein unesterified cholesterol was much greater than that in β-lipoprotein unesterified cholesterol, and comparable results were obtained with lipoproteins prepared by differential flotation. The results suggest that the high-density lipoprotein fraction is of particular importance *in vitro* as a lecithin donor, and that an important factor leading to the net formation of cholesterol esters at the expense of the plasma lecithin may be the dissociation of the resulting lysolecithin from the site of transesterification.

INTRODUCTION

In our previous studies of the mechanism of the plasma cholesterol esterification reaction[1,2], we obtained evidence indicating that free fatty acids are not involved in the reaction. Rather, the data suggested the direct transfer of fatty acids from other plasma esters to cholesterol, the chief source of fatty acids being the C-2 ester position of the plasma lecithin. The transfer reaction was found to be reversibly inhibited by *p*-hydroxymercuribenzoate, suggesting the existence of a sulfhydryl-dependent fatty acid transferase. Finally, the initial rate of the reaction in human plasma was found to be fast enough to account for the esterification of 5–10 % of the plasma-free cholesterol per h.

In the present communication additional studies of the plasma cholesterol esterification reaction are presented. These studies particularly concern the role of

the individual plasma lipoprotein fractions in the reaction, the previous studies having been carried out with whole plasma. The results are consistent with those obtained earlier and suggest that the respective roles played by the various plasma lipoproteins comprise an important feature of the mechanism of the reaction.

METHODS

The following techniques were employed in addition to those used previously[1,2].

Ultracentrifugal flotation

The method of BRAGDON *et al.*[3] was employed except that dry KBr was used for all density adjustments. The starting material was fasting human plasma containing EDTA (0.01 M, pH 7.4). The lipoproteins floating at densities of 1.019, 1.063, and 1.21 g/ml were designated the very low-density, low-density and high-density lipoproteins, respectively. The lipoproteins remaining in the final infranatant were designated the very high-density lipoproteins. All fractions were dialyzed for 48 h against 0.15 M NaCl–0.01 M EDTA (pH 7.4) before extracting the lipids unless otherwise mentioned in the text.

Amylopectin sulfate fractionation

Precipitation of the low-density lipoproteins of fresh serum was carried out by the method of BERNFELD[4].

Cohn fractionation

The method of LEVER *et al.*[5] was employed. The precipitates were dissolved in 0.1 M sodium phosphate–0.01 M EDTA (pH 7.4) and dialyzed against the same solution at 0° for 4–6 h before incubation.

RESULTS

Changes in lipoprotein composition resulting from the incubation of plasma

The following type of experiment was performed in order to determine the effect of the cholesterol esterification reaction on the lipid composition of the various plasma lipoproteins. Plasma was obtained from a normal fasting human, EDTA was added to a final concentration of 0.01 M, and two equal aliquots were taken. To the first (control) aliquot *N*-ethylmaleimide was added to a final concentration of 0.01 M in order to inhibit cholesterol esterification. After standing for 1 h at room temperature, excess *N*-ethylmaleimide was removed by thorough dialysis against 0.15 M NaCl–0.01 M EDTA (pH 7.4). The second aliquot was incubated in a Dubnoff-apparatus for 24 h at 37°. At the end of the incubation period, *N*-ethylmaleimide was added, the mixture allowed to stand, and excess blocking agent was removed by dialysis as in the case of the control. Both aliquots were then fractionated by ultracentrifugal flotation into very low-, low-, high- and very high-density lipoproteins. Subsequently, total and free cholesterol, lecithin and lysolecithin were measured in each fraction. The results of an experiment of this type are presented in Table I and in Fig. 1. A number of features of the experiment may be pointed out. First, although an overall decrease in free cholesterol of 44 % occurred, the relative distribution of

TABLE I

THE EFFECT OF INCUBATION FOR 24 h AT 37° ON THE COMPOSITION OF HUMAN PLASMA LIPOPROTEINS

Fasting human plasma was divided into two aliquots. *N*-Ethylmaleimide was added to the control aliquot in order to block the cholesterol esterification reaction. The second aliquot was incubated for 24 h at 37°. Subsequently, both aliquots were fractionated into very low-density (VLD), low-density (LD), high-density (HD), and very high-density (VHD) lipoproteins. After dialysis, the lipids were extracted and measured. "μmoles" means μmoles/ml original, plasma, "%" means percentage total component per ml original plasma. For further details, see the text.

Lipid	*VLD*		*LD*		*HD*		*VHD*		*Total*
	μmoles	%	*μmoles*	%	*μmoles*	%	*μmoles*	%	*μmoles*
Lecithin									
Control plasma	0.022	1.3	0.585	33.7	1.118	64.4	0.012	0.7	1.737
Incubated plasma	0.008	0.6	0.446	34.9	0.819	64.1	0.004	0.3	1.277
Δ	−0.014	3	−0.139	30.2	−0.299	65.0	−0.008	1.7	−0.460
Lysolecithin									
Control plasma	0.005	3.3	0.022	14.6	0.030	19.9	0.094	62.3	0.151
Incubated plasma	0.008	2.2	0.035	9.7	0.059	16.4	0.257	71.6	0.359
Δ	+0.003	1.4	+0.013	6.3	+0.029	13.9	+0.163	78.3	+0.208
Total cholesterol									
Control plasma	0.17	3.9	2.38	53.7	1.77	39.9	0.11	2.5	4.43
Incubated plasma	0.18	3.7	2.59	53.5	1.96	40.5	0.11	2.3	4.84
Δ	+0.01		+0.21		+0.19		0.00		+0.41
Free cholesterol									
Control plasma	0.05	5.4	0.64	63.7	0.30	29.9	0.010	1.2	1.00
Incubated plasma	0.02	3.8	0.36	63.8	0.17	31.1	0.006	1.0	0.56
Δ	−0.03	6.8	−0.28	63.6	−0.13	28.1	−0.004	1.5	−0.44

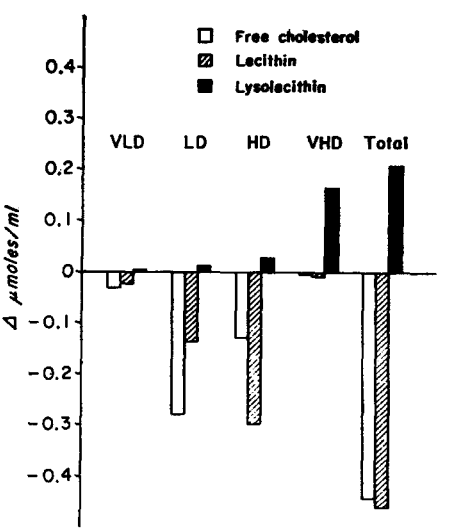

Fig. 1. The change in plasma lipoprotein-free cholesterol, lecithin, and lysolecithin on incubation of plasma. For details regarding the experiment, see text to Table I. The values for the non-incubated control plasma in Table I (1.00, 1.737 and 0.151 μmoles/ml plasma, respectively for free cholesterol, lecithin, and lysolecithin) are taken as 0.0. VLD, LD, HD, and VHD refer to very low-, low-, high- and very high-density lipoproteins.

total cholesterol among the various fractions was not appreciably altered. Secondly, the molar changes in free cholesterol, lecithin, and lysolecithin were approximately proportional to the original concentrations of these compounds in each lipoprotein fraction. Consequently, the relative distribution of these compounds between the several lipoprotein fractions did not change as a result of the incubation, even though the total amount of each compound did change. In Fig. 1 the changes in free cholesterol, lecithin, and lysolecithin in each lipoprotein fraction are compared graphically. Although the combined molar decrement in lecithin was very similar to that in free cholesterol, in agreement with earlier experiments[2], the decrement in high-density lecithin considerably exceeded that in high-density free cholesterol, while the opposite situation occurred in the case of the low-density fraction. Finally, although the great majority of newly formed cholesterol esters were associated with the lipoproteins of < 1.21 g/ml density, most of the change in lysolecithin was in the > 1.21 g/ml density proteins. This may be taken as evidence that the lysolecithin formed as a result of the transfer of a fatty acid from lecithin to cholesterol does not tend to remain at the original site of transesterification. Furthermore, this may be an important feature of the mechanism leading to the net formation of cholesterol esters during the incubation of plasma.

The separate incubation of individual lipoprotein fractions

Several experiments were performed in which the plasma lipoproteins were first fractionated and then incubated for 24 h at 37°. Initially fractionation was accomplished by precipitation with ethanol. The method of LEVER *et al.*[5] was employed except that only the two major fractions, I–III and IV–V, were prepared, the former consisting mainly of β-lipoproteins and γ-globulins and the latter mainly of α-lipoproteins and albumin. After dialysis against sodium phosphate buffer at 0° for 4–6 h, the fractions were incubated for 24 h at 37°, both separately and after recombination. The decrease in unesterified cholesterol of the recombined fractions corresponded to that usually obtained on the incubation of unfractionated plasma. However, when the two fractions were incubated separately, results were obtained which

TABLE II

THE EFFECT OF INCUBATION ON THE FREE CHOLESTEROL OF LIPOPROTEINS OBTAINED BY PRECIPITATION WITH ETHANOL

Fasting human plasma was fractionated by the method of LEVER *et al.*[5]. Fraction I–III contains largely β-lipoproteins and γ-globulins. Fraction IV–V contains largely α-lipoproteins and albumin. After dialysis against 0.1 M sodium phosphate–0.01 M EDTA (pH 7.4) for 4–6 h, the fractions were incubated for 24 h at 37°, the lipids were extracted, and the change in free cholesterol determined. "μmoles" refers to μmoles free cholesterol per ml incubation sample.

Fraction	*Preincubation (μmoles)*	*Postincubation (μmoles)*	*Δ (μmoles)*	*% decrease*
Expt. 1				
Fr. I–III	0.895	0.777	0.118	13.2
Fr. IV–V	0.259	0.041	0.218	84.2
Fr. I–V	1.154	0.636	0.518	44.9
Expt. 2				
Fr. I–III	0.917	0.746	0.171	18.6
Fr. IV–V	0.237	0.007	0.230	97.0
Fr. I–V	1.088	0.699	0.389	35.8

TABLE III

THE CHANGE IN FREE CHOLESTEROL ON INCUBATION OF LIPOPROTEINS OBTAINED BY ULTRACENTRIFUGAL FLOTATION

The fatty acid transferase activity of fasting human plasma was reversibly blocked by the addition of *p*-hydroxymercuribenzoate to a final concentration of 0.001 M. After dialysis against 0.05 M sodium phosphate–0.01 M EDTA (pH 7.4) for 24 h, the lipoproteins were fractionated by the method of BRAGDON *et al.*[3]. Subsequently, the fractions were dialyzed against phosphate–EDTA buffer for 48 h, mercaptoethanol was added to a final concentration of 0.01 M and incubation was performed for 24 h at 37°. After extraction of the lipids the change in free cholesterol was determined. Abbreviations are the same as in Table I. "μmoles" means μmoles free cholesterol per ml incubation sample.

Fraction	*Preincubation (μmoles)*	*Postincubation (μmoles)*	*Δ (μmoles)*	*% decrease*
Expt. 1				
LD	0.225	0.225	0.000	0.0
LD + VHD	0.213	0.180	0.033	15.5
HD	0.072	0.040	0.032	44.4
HD + VHD	0.072	0.017	0.055	76.4
LD + HD + VHD	0.275	0.193	0.082	29.8
Expt. 2				
LD	0.685	0.667	0.018	2.6
LD + VHD	0.655	0.517	0.138	21.1
HD	0.281	0.176	0.105	37.4
HD + VHD	0.311	0.049	0.262	84.2
LD + HD + VHD	0.910	0.536	0.374	41.1

differed from what might have been predicted on the basis of the experiments with whole plasma (compare Tables I and II). Thus, the relative decrease in the unesterified cholesterol of Fraction IV–V was not only much greater than that in Fraction I–III, but also very likely greater than would have been the case had the plasma been incubated first and then fractionated. This supposition is further supported by experiments in which other methods of fractionation were employed. Thus, in an experiment in which the β-lipoproteins of fasting human serum were removed by precipitation with amylopectin sulfate and the supernatant was subsequently incubated for 24 h at 37°, the α-lipoprotein unesterified cholesterol once again decreased by approx. 80 %. Moreover, the results of two experiments in which lipoprotein fractions obtained by ultracentrifugal flotation were incubated separately and in various combinations are shown in Table III. Here also the decrease in unesterified cholesterol of the high-density fraction was not only greater than that of the low-density fraction, but also greater than would have been predicted from Table I. These experiments also show an effect of the "very high-density" fraction. This may be due to the presence of fatty acid transferase activity, but may also be related to the fate of the lysolecithin produced by the cholesterol esterification reaction. This question is currently being investigated.

DISCUSSION

Evidence has been accumulating[1,2,6,7] concerning the role of lecithin as a fatty acid donor in the plasma cholesterol esterification reaction. The present findings suggest that *in vitro* this lecithin is to a large extent derived from the high-density lipoproteins,

even though most of the resulting cholesterol esters are associated with the low-density lipoproteins. Evidence of this may be found in the experiments in which the lipid composition of the various lipoproteins was determined before and after the incubation of whole plasma (see Table I). Also, the fact that the initial lecithin–free cholesterol ratio of the high-density fraction considerably exceeds that of the low-density fraction is compatible with this suggestion, since it seems likely that more of the high-density lecithin might be available for the cholesterol esterification reaction. If the high-density lipoproteins indeed are the major source of lecithin for the reaction, then the incubation of plasma containing relatively large amounts of high-density lecithin might be expected to result in a greater relative esterification of cholesterol. This might explain why the free cholesterol of rat plasma becomes almost completely esterified during incubation[1], while only about half of the free cholesterol of human plasma becomes esterified[8], since rat plasma contains relatively more high-density lipoprotein in comparison to low-density lipoprotein than does human plasma. That human plasma may indeed contain insufficient amounts of lecithin to permit a more complete esterification of the free cholesterol present is suggested by the finding of WAGNER[9], that the addition of dimyristoyl lecithin to human serum causes a considerable increase in the total amount of cholesterol esterified. Moreover, the present experiments in which human plasma lipoproteins were incubated separately are also in support of this. Thus a much greater proportion of the high-density free cholesterol was found to become esterified when the fraction was incubated separately then when incubated in the presence of the low-density lipoproteins (compare Tables II and III with Table I). Following the above line of reasoning, more of the high-density lecithin would be expected to be available for the esterification of high-density free cholesterol in the absence of competing low-density free cholesterol.

Aside from the relevance of the present results with respect to the source of lecithin for the cholesterol esterification reaction, they also appear relevant to the question of why fatty acid transferase activity should result in the net formation of cholesterol esters at the expense of the plasma lecithin. It was suggested previously that the subsequent breakdown of the lysolecithin formed by the reaction might be important. Thus, plasma contains lysolecithinase activity[10] and glycerylphosphorylcholine is formed during the incubation of plasma[11]. However, the formation of glycerylphosphorylcholine is relatively slow, since a considerable amount of lysolecithin accumulates during the first 24-h incubation of whole plasma (Table I). Furthermore, the addition of extraneous lysolecithin to fresh plasma does not decrease the extent of cholesterol ester formation (unpublished experiments). However, dissociation of the newly formed lysolecithin from the site of cholesterol esterification (presumably the < 1.21 g/ml density lipoproteins) as suggested by the data shown in Table I and Fig. 1 could also cause the fatty acid transfer reaction to proceed in the direction of cholesterol ester formation.

PHILLIPS[12] has shown previously that a large part of the total lysolecithin of plasma is associated with the > 1.21 g/ml density proteins. Moreover, he also reported that the lysolecithin of this fraction migrates with the albumin–α_1-globulin peak on zone electrophoresis. The finding that the lysolecithin of the > 1.21 g/ml density proteins greatly increases during the incubation of plasma gives rise to the possibility that the net formation of cholesterol esters might be dependent on the existence of a

lysolecithin-binding protein. It is also possible, however, that the lysolecithin might leave the surface of the parent lipoprotein by virtue of its own hydrophilic properties.

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REFERENCES

1 J. GLOMSET, F. PARKER, M. TJADEN AND R. H. WILLIAMS, *Biochim. Biophys. Acta*, 58 (1962) 398.
2 J. GLOMSET, *Biochim. Biophys. Acta*, 65 (1962) 128.
3 J. H. BRAGDON, R. J. HAVEL AND E. BOYLE, *J. Lab. Clin. Med.*, 48 (1956) 36.
4 P. BERNFELD, J. S. NISSELBAUM, B. J. BERKELEY AND R. W. HANSON, *J. Biol. Chem.*, 235 (1960) 2852.
5 W. F. LEVER, F. R. N. GURD, E. UROMA, R. K. BROWN, B. A. BARNES, K. SCHMID AND E. L. SCHULTZ, *J. Clin. Invest.*, 30 (1951) 99.
6 E. LEBRETON AND J. PANTALEON, *Compt. Rend. Soc. Biol.*, 138 (1944) 38.
7 J. ETIENNE AND J. POLONOVSKI, *Bull. Soc. Chim. Biol.*, 41 (1959) 813.
8 W. M. SPERRY, *J. Biol. Chem.*, 111 (1935) 467.
9 A. WAGNER, *Circulation Res.*, 3 (1955) 818.
10 R. M. C. DAWSON, *Biochem. J.*, 64 (1956) 192.
11 J. ETIENNE AND J. POLONOVSKI, *Bull. Soc. Chim. Biol.*, 41 (1959) 813.
12 G. B. PHILLIPS, *Proc. Soc. Exptl. Biol. Med.*, 100 (1959) 19.