Role of plasma lecithin:cholesterol acyltransferase in the metabolism of high density lipoproteins

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ABSTRACT The role of the plasma lecithin:cholesterol acyltransferase reaction in the esterification of the cholesterol of human and baboon plasma high density lipoproteins has been studied.

Human plasma was incubated in vitro, and the initial rate of cholesterol esterification in lipoprotein fractions obtained by chromatography on hydroxylapatite was determined. The rate of esterification was greater in the high density lipoprotein fraction than in the low density lipoprotein fraction.

High density lipoproteins from human and baboon plasma were filtered through columns of Sephadex G 200, and the relative concentrations in the effluent of key lipids involved in the acyltransferase reaction were determined. The ratio of esterified to unesterified cholesterol varied across the lipoprotein peak obtained from either type of plasma. The relative concentration of lecithin compared to sphingomyelin also varied across the peaks obtained with human high density lipoproteins.

When human or baboon plasma was incubated with cholesterol-^{14}C and the high density lipoproteins were filtered through Sephadex, the specific activity of the esterified cholesterol varied across the lipoprotein peak. Similar results were obtained when plasma esterified cholesterol was labeled in vivo by the injection of labeled mevalonate into baboons.

The data suggest that the acyltransferase reaction is the major source of the esterified cholesterol of the high density lipoproteins.

KEY WORDS acyltransferase · plasma · cholesterol · phospholipid · lipoproteins · high density · gel filtration · man · baboon

THE PHYSIOLOGICAL role of the plasma cholesterol esterification reaction (1, 2) has yet to be defined. A number of observations suggest that it may be the major source of the cholesterol esters in plasma from fasting human subjects. For example, both the rate and the fatty acid specificity of the reaction (3) appear compatible with this possibility. However, before the importance of the reaction can be evaluated, several aspects of the metabolism of plasma cholesterol esters require clarification.

One question is whether the relative rate of cholesterol esterification in high density lipoproteins (HDL) and low density lipoproteins (LDL) in vitro is consistent with the rate of incorporation of labeled mevalonate into HDL and LDL cholesterol esters in vivo (4). A second question concerns the possible heterogeneity of the HDL in vivo. If the transfer of acyl groups from lecithin to cholesterol in plasma is a physiologically important source of HDL cholesterol esters, the continuous operation of the enzyme on a given type of HDL molecule, continuously being secreted into the plasma, should result in the formation of a family of HDL "substrate" and "product" molecules of different cholesterol and phospholipid composition. There is, in fact, evidence that two or more HDL may be present in plasma. However, most of this evidence has been derived from ultracentrifugal flotation experiments (5–8), and this technique has been reported to cause the "artifactual" breakdown of HDL

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; NEM, *N*-ethyl maleimide.

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(9). The object of the present investigation was to obtain evidence concerning the role of the acyltransferase reaction as a source of HDL cholesterol esters, and particularly to seek evidence of HDL heterogeneity through the use of a technique other than ultracentrifugation, viz. gel filtration.

METHODS

Experimental Subjects

Plasma was prepared from the freshly drawn blood of fasting humans and baboons (*Papio doguera* and *P. cynocephalus*). Sodium EDTA (0.005 μmole/ml) was added to the whole blood as an anticoagulant. The baboons were anesthetized with Sernylan[1] (1–2 mg/kg) before the blood was drawn. When rapid withdrawal of large volumes of blood was required in the experiments with labeled mevalonate, the Sernylan was supplemented with Nembutal (10–15 mg/kg), the femoral vein was isolated under sterile conditions, and a polyethylene catheter was inserted through it into the abdominal vena cava. A physiological saline solution (ca. 20 ml/hr) was infused through the catheter to maintain its patency.

Lipoprotein Fractionation

In most experiments the method of Cramér and Brattsten (10) was used to separate HDL from LDL prior to gel filtration. The hydroxylapatite was prepared as described by Levin (11); and 30 g of cellulose powder (A.B. Munktell, Grycksbo, Sweden) was added to each preparation of the final product to facilitate rapid flow through the packed columns. For most preparations of hydroxylapatite, 4 volumes of hydroxylapatite–cellulose mixture per volume of plasma was sufficient to allow complete separation of HDL and LDL, judged by the distribution of cholesterol on rechromatography, ultracentrifugal flotation, and zone electrophoresis. Fraction 1 contained HDL as well as the plasma proteins of density > 1.21 g/ml. Fraction 2 contained the LDL and probably also some very low density lipoproteins (VLDL).[2]

Gel filtration was performed at 6–8°C by the principle of reverse flow (13) on Sephadex G 200 (Pharmacia Fine Chemicals Inc., Piscataway, New Market, N.J.). The sizes of the columns were 4.5 × 150 cm (size A) for samples of 30–40 ml, and 2.5 × 120 cm (size B) for 5.0 ml samples. The columns were equilibrated with a buffer, pH 7.4, containing 0.01 M Tris [tris(hydroxymethyl) amino methane]–HCl, 0.001 M EDTA, and 0.14 M NaCl. The effluent from the size A and B columns was collected in 20- and 5-ml fractions, respectively, except where specifically noted. A single filtration usually required 2–3 days.

Preparative ultracentrifugal flotation was performed serially in a No. 40 rotor of a Spinco Model L ultracentrifuge at successive densities of 1.063, 1.125, and 1.21 g/ml. Antecedent lower density material was collected before each adjustment of the density with solid KBr. The centrifugation times were 24, 24, and 48 hr, respectively.

Analytical Procedures

Protein was determined by measuring absorbance at 280 mμ. Lipids were extracted with chloroform–methanol (14). Phospholipids were fractionated by thin-layer chromatography on plates of washed (15) Silica Gel H (Brinkman Instruments Inc., Westbury, N.Y.) with chloroform–methanol–water 100:55:8. After exposure to I_2 vapor, appropriate areas of silicic acid (determined on the basis of the position of standards chromatographed on the same plate) were scraped into test tubes and digested with perchloric acid for 2 hr at 220°C, and microphosphorus determinations were performed by the method of Bartlett (16). The lower limit of sensitivity for this method is 0.015 μmole of phosphorus. However, for amounts this small, the absorbance was only five times that of the silica gel blank from the chromatoplate. Recovery of phosphorus from the plates was 90–95%.

Neutral lipids were chromatographed in hexane–ether–acetic acid 90:20:1. Areas of silicic acid corresponding to esterified and unesterified cholesterol were scraped from the plates and heated for 1 hr at 80°C in 1 N ethanolic KOH. The cholesterol was extracted with hexane,[3] and analyzed by the procedure described by Courchaine, Miller, and Stein (17). For samples containing less than 0.04 μmole of cholesterol, the volume of the reagents was reduced by a factor of four and 1 ml cuvettes were used. In this way samples containing as little as 0.013 μmole of cholesterol could be measured. Blank readings from the washed Silica Gel H were essentially zero. Recoveries varied from 85 to 95%.

Radioactivity Determinations

Labeled cholesterol (7α-^{3}H or 4-^{14}C) and DL-mevalonate (5-^{3}H or 2-^{14}C; dibenzylethylenediamine salt) were obtained from New England Nuclear Corp. (Boston, Mass.). The purity of the labeled cholesterol was checked, and when necessary the cholesterol was further purified by thin-layer chromatography.

[1] Sernylan was the gift of Dr. Graham Chen (Parke, Davis & Co., Detroit, Mich.).

[2] Borgström, Naito, and Wlodawer have shown (12) that chylomicrons remain adsorbed to hydroxylapatite under the conditions of LDL elution employed here, and their experiments suggest that VLDL may be only partially eluted. Therefore, the "LDL" fraction probably contains small amounts of VLDL.

[3] The authors are indebted to Dr. Frank Parker for making this method available to them.

In the experiments with labeled mevalonate the specific activity of the plasma cholesterol was determined as follows. After saponification of the sterol-containing fractions from the thin-layer plates (see above), the sterols were extracted with hexane and finally precipitated with digitonin[4] (18). The precipitates were washed with acetone–ether 1:1 and with ether and subsequently dissolved by being heated in methanol for 1 hr at 65°C. The solution was cooled to room temperature and aliquots were taken for determination of total cholesterol and radioactivity. In the latter case the aliquots were pipetted directly into counting vials and the methanol was evaporated at 90°C. Toluene, 1 ml, was added, the cap secured, and the vial heated at 90–100°C for 1 hr. The vials were then cooled, 10 ml of Liquifluor (Pilot Chemicals, Inc., Watertown, Mass.) was added, and the radioactivity was determined in a Packard Tri-Carb spectrometer. External standardization was employed to detect and correct for quenching. Sufficient counts were obtained to limit the counting error to a level of 5% or less. Similar counting conditions were employed in the other radioactivity experiments except where specifically noted.

Initial Rate Determinations

Initial rates of cholesterol esterification in vitro were determined as follows. A sterile suspension of cholesterol-^{3}H in a solution of human albumin (19) was added to human plasma (10^5 cpm/ml of plasma). The mixture was incubated at 37°C in sterile, glass-stoppered flasks. The first aliquot was withdrawn after 15 min. Successive aliquots were withdrawn at 30-min intervals for 2 hr and then at gradually lengthening intervals for 24 hr. Upon withdrawal of each aliquot, crystalline *N*-ethylmaleimide (NEM) was added to a final concentration of 0.05 M; this inhibits plasma acyltransferase activity (3). As soon as the NEM was dissolved, the plasma was chromatographed on hydroxylapatite columns. The flow rate through the hydroxylapatite was regulated with N_2 pressure in such a way that the lipoprotein fractions in 3 ml of plasma were separated within 10 min. Control experiments indicated[5] that fraction 1 contained not only HDL but also the emulsified cholesterol, while no emulsified cholesterol was present in fraction 2. Consequently, the rate of cholesterol esterification in the LDL fraction could be calculated from the total LDL cholesterol ester radioactivity and the specific activity of the LDL unesterified cholesterol. Since equilibration of the added, unesterified ^{3}H-emulsion with the LDL required 2–4 hr, the number of micromoles of LDL cholesterol esterified per interval of incubation was calculated by dividing the increment in cholesterol ester radioactivity during that interval by the mean unesterified cholesterol specific activity within the same interval. This type of calculation was not possible for the HDL, because it was contaminated with the labeled cholesterol emulsion. To obtain the amount of HDL cholesterol esterified during an interval, we subtracted the increment in LDL cholesterol ester, calculated as described above, from the total amount of cholesterol esterified in the combined lipoprotein fractions during that interval. The latter value was obtained most accurately by measurement of the total decrement in unesterified cholesterol rather than the increment in cholesterol ester; it has been repeatedly shown that Δ μmoles of cholesterol ester $= -\Delta$ μmoles of unesterified cholesterol for whole plasma (1).

RESULTS

Initial Rates of Cholesterol Esterification in Human Plasma Lipoproteins In Vitro

Fresh human plasma was incubated with an emulsion of cholesterol-^{3}H, HDL and LDL were fractionated by chromatography on hydroxylapatite, and the incorporation of the labeled material into lipoprotein cholesterol esters was studied. The results of one such experiment are shown in Figs. 1 and 2. Fig. 1 demonstrates that the specific activity of the esterified cholesterol in the HDL fraction was consistently higher than that in the LDL fraction. These results are compatible with those obtained by Goodman (4) after he had injected mevalo-

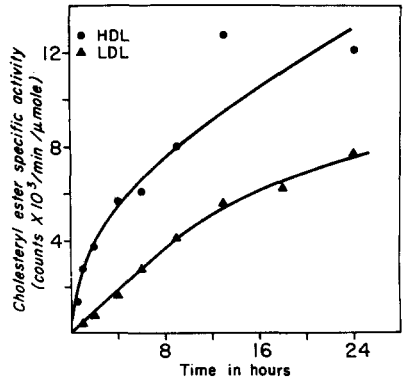

FIG. 1. Specific activity of esterified cholesterol in lipoprotein fractions from the plasma of a fasting human female. 10^5 cpm of cholesterol-^{3}H added per ml of fresh plasma; mixture incubated at 37°C. At intervals shown, aliquots were removed, NEM was added, and plasma lipoproteins were fractionated by chromatography on hydroxylapatite. Lipids were extracted with chloroform–methanol, and esterified cholesterol was isolated by thin-layer chromatography.

[4] None of these steps is completely specific for cholesterol. It is possible, therefore, that some labeled contaminants may have been present in the "cholesterol" fractions.

[5] Cholesterol emulsions prepared in albumin solution are absorbed onto and eluted from columns of hydroxylapatite under conditions closely similar to those required for the absorption and elution of albumin. This suggests that the emulsified cholesterol may be coated with albumin.

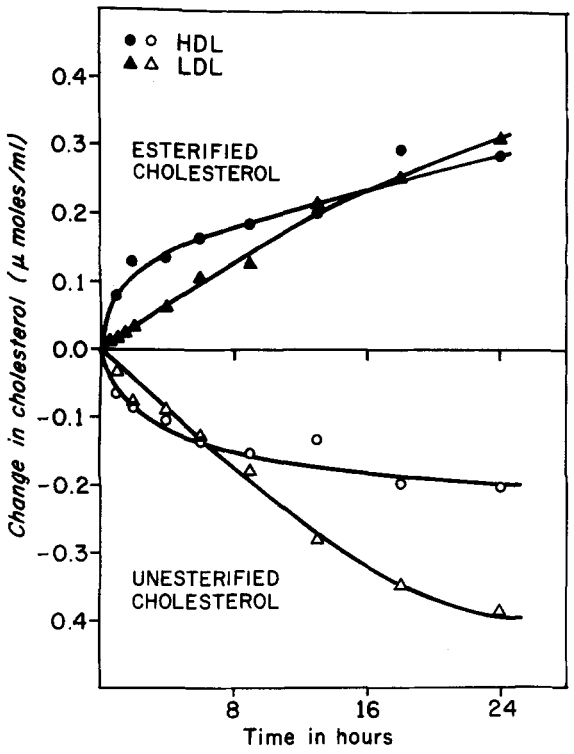

FIG. 2. Calculated rates of cholesterol esterification and measured changes in unesterified cholesterol in the experiment shown in Fig. 1. Initial concentrations of esterified and unesterified cholesterol in the HDL fraction were 1.109 and 0.400 μmoles/ml of plasma, respectively. The corresponding values for the LDL fraction were 1.992 and 0.697 μmoles/ml of plasma.

nate-^{14}C into human subjects. Fig. 2 shows the calculated initial rates of cholesterol esterification and indicates that the number of micromoles of cholesterol esterified per hour was initially greater in the HDL than in the LDL fraction. The directly measured changes in unesterified cholesterol are shown in the lower part of the figure. Note that the changes in HDL esterified and unesterified cholesterol are not commensurate and that the same is true for the LDL. Similar results were obtained in each of the three experiments in this series (see Discussion).

The calculated turnover times[6] of the HDL and LDL cholesterol esters in these three experiments, as well as the initial rate data, are presented in Table 1. These are the turnover times that theoretically would apply in vivo if the plasma acyltransferase reaction were the sole source of the cholesterol esters of fasting plasma, and if the rate of esterification in vivo is equal to that determined in vitro. It should be noted that neither the quantitative importance of other possible sources of plasma cholesterol esters nor the over-all rate of plasma cholesterol ester formation is known in man.

[6] The "turnover time" of a given cholesterol ester pool is taken as the time required for biosynthesis of the total amount of cholesterol esters in that pool (amount of cholesterol ester/rate of cholesterol esterification).

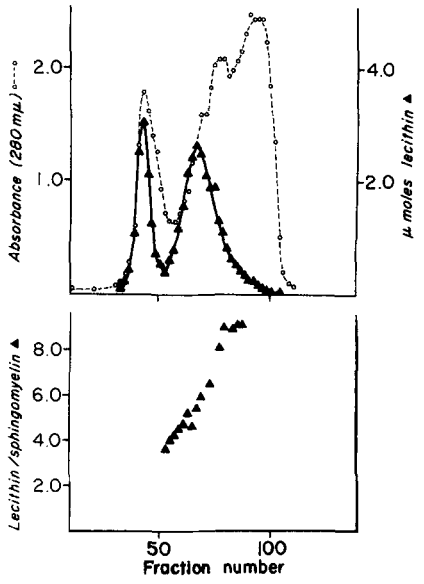

FIG. 3. Gel filtration of human whole plasma: 30 ml of plasma from a fasting human female fractionated on a size A (4.5 × 150 cm) column of Sephadex G 200. Total protein concentration in the effluent was determined by measurement of absorbance, and the lipoprotein concentration was determined by measurement of lecithin phosphorus (upper part of figure). Ratios of lecithin/sphingomyelin across the second (HDL) peak are shown in the lower part of the figure. Ratios were determined only for fractions in which at least 0.04 μmole of sphingomyelin or lecithin was actually measured.

Heterogeneity of HDL Demonstrated by Gel Filtration

One effect of the plasma acyltransferase on HDL in vivo could be to cause heterogeneity with respect to cholesterol and phospholipid composition. In an attempt to obtain evidence of this type of heterogeneity, gel filtration on columns of Sephadex G 200 was employed. Initially, human whole plasma was applied to the columns (Fig. 3). In this and all subsequent experiments,

TABLE 1 CHOLESTEROL ESTER CONTENTS, ESTERIFICATION RATES, AND CALCULATED "TURNOVER TIMES" OF HUMAN PLASMA LIPOPROTEIN FRACTIONS

	HDL Cholesterol Ester			LDL Cholesterol Ester		
	Total*	Initial Rate	"Turnover Time"†	Total*	Initial Rate	"Turnover Time"†
	μmoles/ml	μmole/ml/hr	hr	μmoles/ml	μmole/ml/hr	hr
Expt. 1	1.023	0.11	9.3	2.750	0.020	138
Expt. 2	0.627	0.090	7.0	1.294	0.035	66
Expt. 3‡	1.109	0.080	13.9	1.992	0.015	133

Three experiments with the same design (see legend to Fig. 1) were conducted.

* Measured at the beginning of incubation.

† Quotient of two preceding columns.

‡ Same experiment as that shown in Figs. 1 and 2.

the total plasma protein concentration in the effluent was determined by measuring the absorbance at 280 mμ. This measurement gave the approximate positions of the LDL and HDL peaks, for we found, like others (20, 21), that LDL always emerged with the first protein (macroglobulin) peak and that HDL emerged slightly ahead of the second protein peak. In this particular experiment the concentration of lipoproteins in the effluent was determined by the analysis of phospholipid. Although only the values for lecithin are plotted in the upper part of the figure, those for sphingomyelin also could be represented graphically as two smooth peaks. The ratios of lecithin to sphingomyelin across the second (HDL) peak are plotted in the lower part of the figure. The increase in the ratios as a function of effluent volume can be considered evidence of HDL heterogeneity. Similar results were obtained in a second whole plasma experiment, as shown in Fig. 4. In this experiment, the ratio of esterified to unesterified cholesterol was also determined across the HDL peak. This ratio increased with increasing effluent volume until most of the HDL had emerged from the column, and then appeared to decrease rapidly.

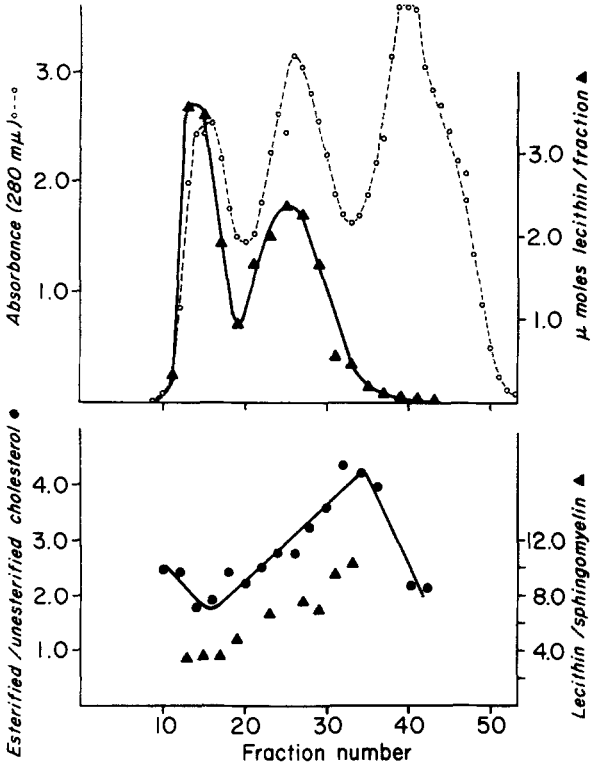

FIG. 4. Gel filtration of whole plasma from a second fasting woman. Plasma, 33 ml, was fractionated on a size A Sephadex column. Total plasma protein and lipoprotein concentrations were determined as in the experiment shown in Fig. 3 (upper part of figure). Ratios of lecithin/sphingomyelin and esterified/unesterified cholesterol are shown in the lower part of the figure. Minimal value used in calculating both ratios was 0.04 μmole/sample.

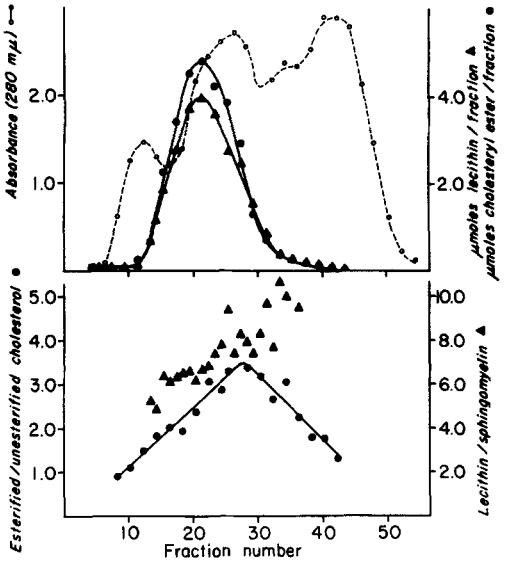

FIG. 5. Gel filtration of HDL from a normal, fasting woman. LDL were removed from 40 ml of plasma by chromatography on hydroxylapatite, and the remaining plasma proteins were applied to a size A Sephadex column. The concentrations of total protein and lipoprotein were determined as in Fig. 3, except that esterified cholesterol was also measured (upper part of figure). The ratios of lecithin/sphingomyelin and esterified/unesterified cholesterol (lower part of the figure) were calculated from minimum, measured values for phospholipid and cholesterol as in Fig. 4.

Because the HDL and LDL were not completely separated under the conditions employed for gel filtration, in some further experiments the LDL were removed by chromatography on hydroxylapatite prior to gel filtration. Fig. 5 shows the result of an experiment of this type. The LDL peak is absent but the HDL peak emerges in the usual volume. As indicated in the lower part of the figure, the lecithin/sphingomyelin and esterified/unesterified cholesterol ratios showed the same general trends as in the whole plasma experiments.

Fig. 6 shows an experiment performed with baboon plasma. The LDL were removed by chromatography on hydroxylapatite prior to gel filtration. In this experiment cholesterol ester concentration in the effluent was used as a measure of HDL content. As with human plasma, the HDL peak emerged just before the second major protein peak. The ratios of esterified to unesterified cholesterol are shown in the lower part of the figure. They are lower than the corresponding ratios for the human HDL shown in Fig. 5, but show a similar change with effluent volume.

Fig. 7 shows another type of experiment performed with human HDL. The LDL were again removed by chromatography on hydroxylapatite prior to gel filtration. The HDL peak was determined by measurement of total cholesterol (upper part of Fig. 7). The contents of the tubes corresponding to the stippled areas were combined into two fractions and each was ultrafiltered.

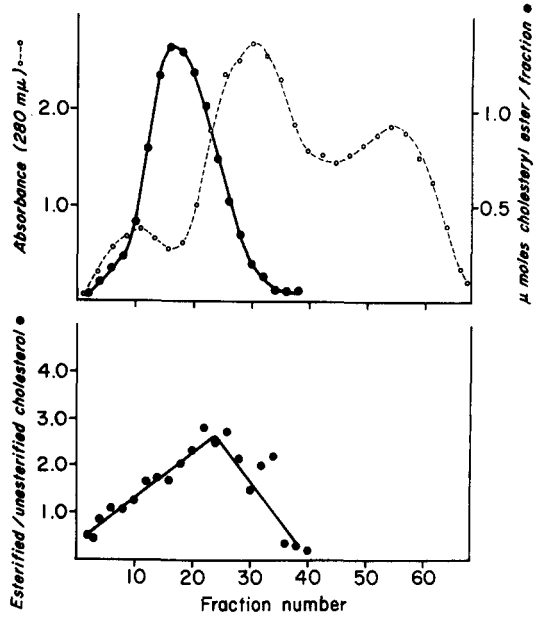

FIG. 6. Gel filtration of HDL from a fasting male baboon. LDL were removed from 20 ml of plasma by means of hydroxylapatite, and the remaining proteins were applied to a size A Sephadex column. The position of the HDL peak was determined by measurement of esterified cholesterol (upper part of figure). The ratio of esterified/unesterified cholesterol is shown in the lower part of the figure. The micro method was used for the cholesterol determinations (see Methods); and the minimum measured value used for the ratio calculations was 0.01 μmole/sample.

Aliquots from the two fractions were then mixed and passed through a 2.5 × 120 cm (size B) Sephadex column. The two partially separated peaks containing cholesterol shown in the lower part of the figure provide additional evidence of the heterogeneity of the HDL. Recombination and filtration of the flanks of a homogeneous peak would have been expected to yield a single peak.

The heterogeneity shown in the above filtration experiments involves three components of the HDL that are directly affected by the plasma acyltransferase reaction, viz. lecithin, unesterified cholesterol, and esterified cholesterol. Therefore, it is possible that the transferase may be an important cause of this heterogeneity. Additional evidence in support of this possibility is presented below.

Incorporation of Labeled Cholesterol into HDL Subfractions

In the experiment shown in Fig. 8 human whole plasma was incubated for 1 hr at 37°C with emulsified cholesterol-^{14}C; the LDL were removed by chromatography on hydroxylapatite; and the HDL-containing fraction was ultrafiltered and applied to a Sephadex column. The cholesterol ester content of the effluent is shown in the upper part of the figure, the specific activity of the esterified cholesterol in the lower part. The continuous change in specific activity across the HDL peak suggests that the individual HDL molecules comprising the peak vary in their action as substrates in the acyltransferase reaction. Two other possibilities, which seem unlikely at present, are as follows. The first is that the rate of esterification of the various HDL molecules is similar, but that the newly formed ^{14}C-cholesterol esters are diluted to differing degrees by preformed cholesterol esters. This seems unlikely, since the proportion of the total cholesterol in the esterified form also increases over a large part of the HDL peak as a function of the effluent volume (compare Figs. 5 and 8). The second possibility is that the specific activity of the unesterified precursor increases across the peak. Two experiments performed with baboon plasma indicate that this explanation is also unlikely (compare Fig. 6 with Figs. 9 and 11 below).

In the experiment shown in Fig. 9, baboon whole blood was incubated with cholesterol-^{14}C for 75 min at 37°C; plasma was prepared by centrifugation; LDL were removed by chromatography on hydroxylapatite; and the HDL-containing fraction was filtered through Sephadex. The change in cholesterol ester specific activity with elution volume resembles that found in the human HDL shown in Fig. 8, except for the plateau across the middle part of the peak. Now the specific activity of unesterified

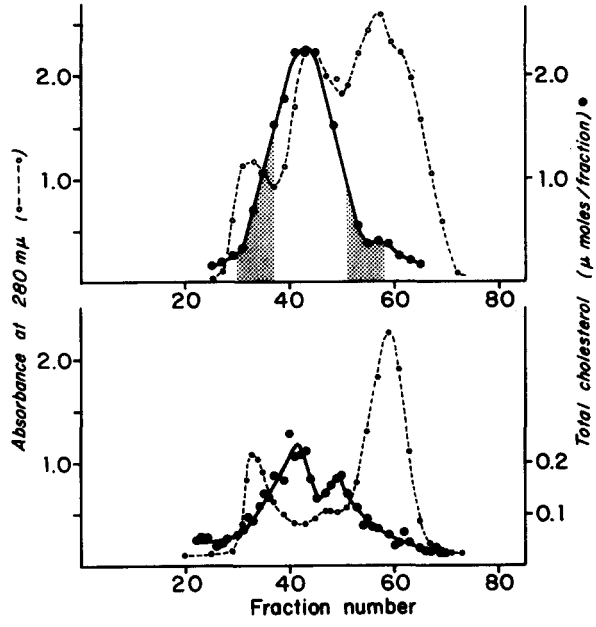

FIG. 7. Refiltration of HDL subfractions on Sephadex G 200. LDL-free plasma from a normal human male, 30 ml, was fractionated on a Sephadex G 200 (size A) column. Aliquots were analyzed for total cholesterol content (upper graph), and the effluent corresponding to the stippled areas was pooled to form two fractions. After ultrafiltration, these fractions were combined and applied to a second Sephadex G 200 column (size B = 2.5 × 120 cm) (lower graph). The total amount of protein applied was approximately $^{3}/_{8}$ of that in the corresponding effluent of column A.

cholesterol either decreased or remained the same across those areas of the HDL peak where that of esterified cholesterol increased. In this experiment partial equilibration of the unesterified cholesterol-^{14}C among the various HDL molecules had probably occurred by the end of the 75 min incubation. The initially high values for the specific activity of the unesterified cholesterol are probably due to the presence of residual quantities of emulsified cholesterol, since control experiments indicated that the latter emerges from Sephadex G 200 columns with the macroglobulin peak.

In the experiment shown in Figs. 10 and 11, mevalonate-^{14}C was injected intravenously into a 7.4 kg fasting male baboon. At the times indicated in Fig. 10, 5–8 ml of blood were withdrawn. Se-par-aid (Uni-Tech Chemical Mfg. Co., Panorama City, Calif.) was used to facilitate the rapid centrifugal separation of plasma from the cells, and the plasma lipoproteins were immediately chromatographed on columns of hydroxylapatite. This part of the procedure required less than 15 min. Fig. 10 indicates that the specific activity of both the esterified and the unesterified cholesterol of the HDL exceeded that of the LDL during the first 3 hr of the experiments. The data for the cholesterol esters are in accord with the observations of Gidez and Eder (22) and Goodman (4) in man. The initial difference between the specific activities of the HDL and LDL unesterified cholesterol has not been observed previously.

In this same experiment a large blood sample was withdrawn at 60 min: 30 ml of plasma was obtained, chromatographed on hydroxylapatite, and applied to a

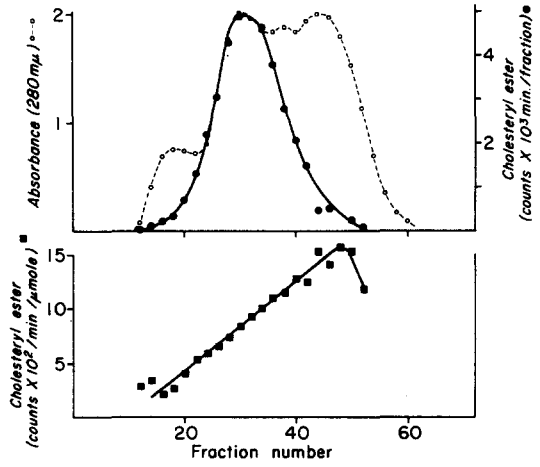

FIG. 8. Analysis of the HDL fraction from human plasma incubated with cholesterol-^{14}C. Of the labeled cholesterol 10^5 cpm/ml was added to 30 ml of plasma. After incubation for 60 min at 37°C, LDL were removed by chromatography on hydroxylapatite and the remaining proteins were fractionated on a size A Sephadex column. The position of the HDL peak was indicated by measurement of esterified cholesterol (upper part of figure). The specific activity of the esterified cholesterol is shown in the lower part of the figure.

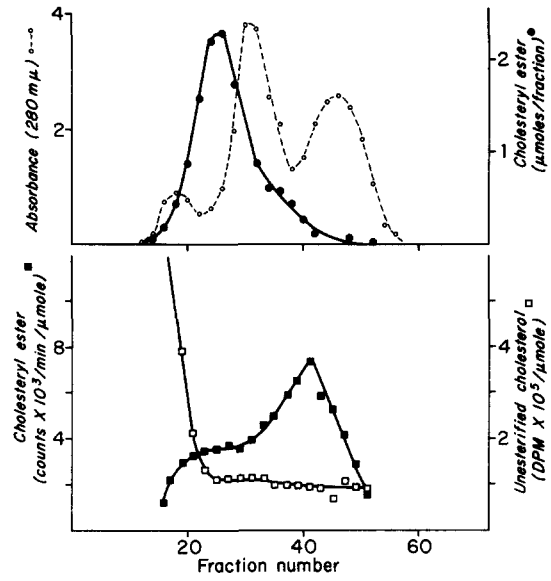

FIG. 9. Analysis of the HDL fraction from whole baboon blood incubated with cholesterol-^{14}C. Cholesterol, 10^5 cpm/ml, was added to fresh blood (containing 0.005 μmole/ml EDTA) from a male baboon. After incubation for 75 min at 37°C, the blood cells were removed by centrifugation, NEM was added, and the HDL fraction obtained by chromatography on hydroxylapatite was filtered through a size A column of Sephadex G 200. The position of the HDL peak was indicated by measurement of esterified cholesterol (upper part of figure), and the specific activity of the esterified and the unesterified cholesterol were determined (lower part of figure).

Sephadex column. The emerging lipoprotein was determined by analysis of esterified cholesterol (Fig. 11, upper part), and the specific activities of the esterified and unesterified cholesterol were determined across the HDL peak. The cholesterol ester specific activity curve was very similar to that obtained in the in vitro experiment shown in Fig. 9. This similarity between the in vivo and the in vitro experiments can be considered additional evidence in support of the physiological importance of the plasma acyltransferase reaction.

The specific activity of the unesterified cholesterol varied across the main part of the HDL peak; the values for the initial fractions are low compared to those in the experiment shown in Fig. 9 because of the absence of emulsified cholesterol. Furthermore, since the change in specific activity of the unesterified cholesterol does not parallel that of the esterified cholesterol, the increase in cholesterol ester specific activity across the peak is probably not due to an increase in the specific activity of the unesterified cholesterol in the same HDL molecules. Instead, it seems likely that those HDL molecules with the highest cholesterol ester specific activities are the preferred substrates of the plasma acyltransferase both in vivo and in vitro.

Another in vivo experiment is shown in Fig. 12. In this experiment 6.0 mc of DL-mevalonate-5-^{3}H was injected

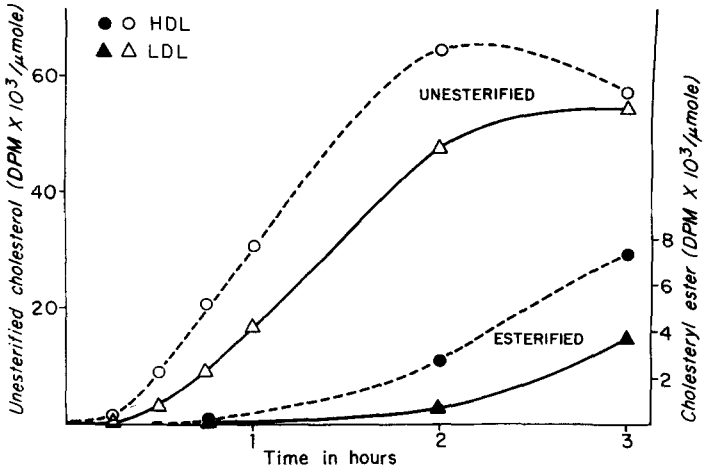

FIG. 10. Incorporation of labeled cholesterol into baboon plasma lipoproteins in vivo. DL-Mevalonate-2-^{14}C, 0.5 mc, was injected intravenously into a 7.4 kg fasting male baboon, and blood (5–8 ml) was withdrawn at the times indicated. Plasma lipoproteins were separated by hydroxylapatite chromatography. The lipids were extracted by chloroform–methanol and separated by thin-layer chromatography. Areas corresponding to unesterified and esterified cholesterol were scraped from the plates, saponified, and precipitated by addition of digitonin. The specific activities of the precipitated material are shown. Minimum values for cholesterol were as in Fig. 9.

into a 9.9 kg fasting male baboon. Blood was withdrawn 1 hr after injection and the 30 ml of plasma obtained was applied directly to a Sephadex column. Lipoprotein was determined by measurement of esterified cholesterol (upper part of Fig. 12). The specific activity measurements for unesterified cholesterol were technically unsatisfactory. The cholesterol ester specific activity curve is shown in the lower part of the figure, however, and resembles that shown in Fig. 11 for the area of the curve corresponding to the HDL peak. The specific activity of the LDL cholesterol esters decreases with increasing effluent volume. This may be due to the presence of VLDL in the initial part of the peak. Goodman (4) has observed that in man the specific activity of the VLDL cholesterol esters initially exceeds that of the LDL cholesterol esters, and the VLDL would be expected to emerge in the initial part of the LDL peak because of their large size (23).

DISCUSSION

In this investigation two principal types of experiment have been performed in order to obtain evidence concerning the role of the plasma lecithin:cholesterol acyltransferase in the metabolism of HDL. A comparison has been made between cholesterol esterification in plasma lipoproteins in vitro and in vivo, and an attempt has been made to identify HDL substrates and products of the acyltransferase reaction.

The in vitro experiments in which the initial rates of cholesterol esterification in HDL and LDL were compared suggest that HDL play a considerably more important role in the acyltransferase reaction than was inferred earlier (3). At that time the lipid composition of lipoproteins from fresh plasma was compared with that of lipoproteins from plasma which had been incubated for 24 hr at 37°C, and the greatest changes were found in the LDL fraction. In the present experiments similar changes were obtained after incubation for 24 hr. However, initially the rate of esterification was more rapid in the HDL than in the LDL fraction. Subsequently, the rate of change in amount of HDL esterified and unesterified cholesterol decreased (see Fig. 2). The reason for this has not been established, nor is it known why the rate of change in unesterified cholesterol decreased more rapidly than that of esterified cholesterol, although a redistribution of LDL unesterified cholesterol may be involved. Unesterified cholesterol is known to equilibrate rapidly among the plasma lipoproteins (24). Since the transferase reaction initially causes a relatively greater decrease in vitro in the unesterified cholesterol of the HDL than in that of the LDL, subsequent equilibration could cause a net migration of unesterified cholesterol from LDL to HDL. That a relative deficit in HDL unesterified cholesterol may also exist at zero time is suggested by gel filtration experiments (Glomset, J., unpublished observations) in which there was a relatively greater uptake of labeled unesterified cholesterol by HDL (those parts of the HDL curve not contaminated with emulsified, radioactive cholesterol) than by LDL in plasma

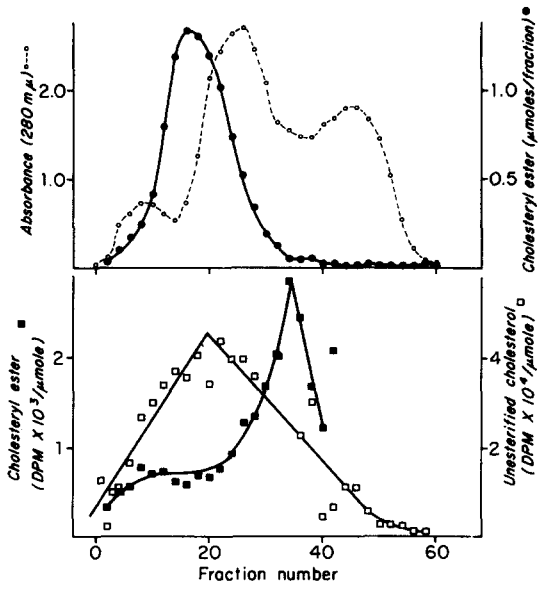

FIG. 11. Incorporation of labeled cholesterol into baboon plasma HDL fractions obtained by gel filtration. Plasma, 30 ml, was obtained 1 hr after injection of labeled mevalonate (same experiment as in Fig. 10) and applied to hydroxylapatite. The first fraction, containing the HDL, was subfractionated by filtration through Sephadex G 200, size A column. The position of the HDL peak was indicated by measurement of esterified cholesterol (upper part of figure). Esterified and unesterified cholesterol specific activities are shown in the lower part of the figure. Minimum values for cholesterol were as in Figs. 9 and 10.

containing NEM. Finally, the baboon experiment shown in Figs. 10 and 11 provides evidence of the existence of a similar deficit in vivo.

The relatively rapid esterification of labeled cholesterol by HDL in vitro (see Fig. 1) is comparable to that shown to occur in vivo. Goodman (4) injected labeled mevalonate into fasting humans and found that the specific activity of HDL cholesterol esters initially was considerably higher than that of VLDL or LDL cholesterol esters. Although his results do not permit quantitative evaluation of the differential rates of lipoprotein cholesterol esterification in vivo, they probably reflect these rates in much the same way as the data shown in Fig. 1 reflect those shown in the upper part of Fig. 2.

The similarity between our results and those of Goodman and the very similar esterification of the cholesterol of HDL subfractions in vitro and in vivo (compare Figs. 9 and 11) provide strong support for the possibility that the transferase reaction is the major source of the cholesterol esters of HDL. The cholesterol ester turnover times based on this possibility (see Table 1) suggest that the cholesterol esters of the HDL turn over approximately twice a day, while those of the LDL may turn over every 3–5 days. Since the corresponding turnover times (25) of the protein of the HDL and LDL are 5.1 and 4.5 days, respectively (26, 27), the HDL cholesterol esters may turn over independently of the protein moiety, while the LDL cholesterol esters and protein turn over as a single unit.

The measurements of lecithin, sphingomyelin, unesterified cholesterol, and esterified cholesterol in the gel filtration experiments support the concept of Oncley (7) that HDL, as a class, comprise a spectrum of molecules of different composition. They also seem consistent with the concept that the acyltransferase is an important cause of this heterogeneity. Thus, one provisional hypothesis might be the following. The HDL with the highest lecithin/sphingomyelin ratios, viz. those toward the end of the HDL peak, are those that have most recently entered the plasma. The transferase acts on all HDL molecules but has a particular affinity for those of relatively high lecithin content. (Those HDL molecules toward the very end of the peak, as in Figs. 9 and 11, would have to be admitted as an exception.) Action of the transferase causes a decrease in the ratio of lecithin to sphingomyelin and an increase in the ratio of esterified to unesterified cholesterol. The resultant loss in polar lipid causes an increase in the degree of aggregation of the lipoprotein complexes. [The lysolecithin resulting from the reaction probably leaves the lipoproteins (3).] While in the plasma or in the extracellular fluid, the partially reacted HDL molecules pick up unesterified cholesterol either from cell membranes (28) or from the LDL. Finally, with increasing size of the HDL aggregates the esterified cholesterol is either transferred to other lipoproteins (29) or is hydrolyzed by cellular cholesterol

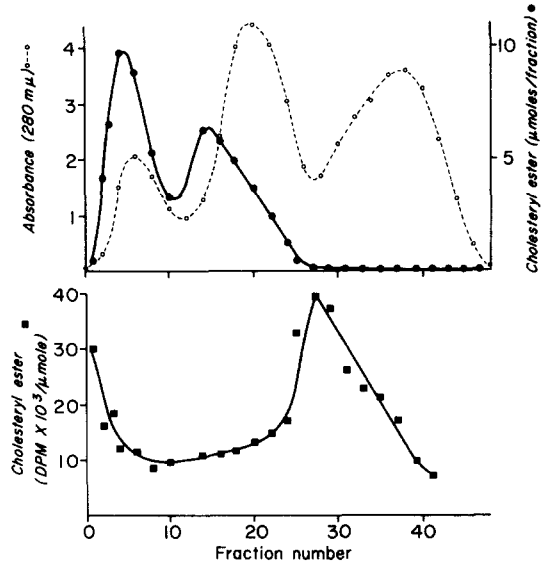

FIG. 12. Incorporation of labeled cholesterol into baboon plasma LDL and HDL. DL-Mevalonate-5³H, 6.0 mc, was injected into a 9.9 kg fasting male baboon. Plasma, 30 ml, was obtained 1 hr after injection and applied directly to a size A Sephadex G 200 column. Lipoprotein was determined by measurement of effluent esterified cholesterol (upper part of figure). The specific activity of the esterified cholesterol is shown in the lower part of the figure. Minimum values for cholesterol were as in Figs. 9–11.

esterases, with a resultant decrease in the ratio of esterified cholesterol.

This hypothesis notwithstanding, additional possible causes of the heterogeneity should be kept in mind. First, the question of artifact cannot be ruled out entirely. Levy and Fredrickson (9) noted that ultracentrifugal flotation seemed to cause the breakdown of HDL aggregates of lower density into products of higher density and that aging of the preparation alone seemed to have a similar effect. Therefore, it might be argued that the gel filtration procedure could also cause HDL breakdown, particularly since a single filtration experiment usually required at least 2–3 days to complete. HDL of densities <1.125 tend to emerge from the Sephadex columns before those of densities >1.125 and >1.21 (Janssen, E.T., unpublished observations). Therefore, a preparative artifact analogous to that described by Levy and Fredrickson might predictably cause a diminution in the HDL making up the beginning of the peak and an increase in those making up the end. However, the fact that the lecithin/sphingomyelin ratios differ in these regions (Figs. 3–5) does not seem to support this possibility. Other possible causes of the heterogeneity in the gel filtration experiments are the secretion of different types of primary HDL molecules into the circulation and the variable interaction of these with VLDL (30). Evaluation of these possibilities will have to await further investigation.

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