Enzymatically induced alterations in the structure of rat serum lipoproteins

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Abstract Incubation of freshly isolated rat serum induces a large number of changes in the properties of the serum lipoproteins, especially the high density lipoproteins (HDL). The particle diameter of the HDL increases from about 10.4 nm to 12.3 nm and the protein content appears to increase by about 60,000 Daltons. Reactions catalyzed by lecithin:cholesterol acyltransferase (LCAT) lead to a marked decrease in cholesterol and phospholipid content, and an even greater increase in cholesteryl ester content. Especially noteworthy are the marked increases in apoE and apoA-IV which are found associated with HDL as a result of this process. Data indicate that the affinity of apoE and apoA-IV for the HDL particles may be influenced by the proportion of surface to core lipid and by the presence of products of the LCAT reaction. Changes in the apoprotein content of very low density lipoproteins are also observed, with A-I and A-IV appearing in this density interval. All of the above changes can be prevented by the inclusion of 5,5'dithiobis(2-nitrobenzoate) or *p*-chloromercuriphenylsulfonate during the incubation, or by heat treatment of serum at 56°C for 30 min; these treatments are known to inhibit LCAT activity. It is concluded that LCAT action is the major cause of the various changes in HDL structure that are observed and that alterations in apoprotein content occur to correct the resultant imbalance between core lipid and coverage of this core by amphiphilic components. Increased apoE association with cholesteryl ester-rich HDL may provide an efficient means for receptor-mediated removal of cholesterol from the circulation.—**Swaney, J. B., M. W. Orishimo, and A. Girard.** Enzymatically induced alterations in the structure of rat serum lipoproteins. *J. Lipid Res.* 1987. **28:** 982–992.

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The plasma lipoproteins, which are complex assemblies of multiple protein and lipid components, undergo a variety of structural alterations, both enzymatic and nonenzymatic, after secretion and prior to their removal from the circulation. In addition to covalent modification of individual protein and lipid species (1), there also occurs a redistribution of components of these noncovalent assemblies which generates subpopulations of the major lipoprotein species (2). In the case of the lipid moieties this can occur as a result of spontaneous and nonfacilitated transfer, as with cholesterol (3, 4), or may result from protein-mediated transfer, as with phospholipid and cholesteryl ester (5, 6). Furthermore, it appears that the apolipoproteins may transfer onto or off of lipoprotein particles, thus altering the surface composition (7, 8). It is not presently known, however, what physicochemical perturbations are responsible for the changes in apoprotein composition or other physical properties of HDL which occur during its residence in the blood.

Changes in the levels of high density lipoproteins (HDL) are of special interest because of the apparent importance of this lipoprotein in affording protection against coronary heart disease and because of the metabolic differences among subpopulations of the HDL (2, 9). Incubation of the serum from various species at 37°C has been shown to cause an increase in the apparent size of the HDL particles by electrophoresis (10–12). Investigations into the cause of these changes in the properties of HDL have produced a mixture of conclusions. Studies of pig and rabbit serum by Ha, Gorjatschko, and Barter (13) and by Rye and Barter (14) implicate a serum factor in this process, but tend to rule out the involvement of lecithin:cholesterol acyltransferase (LCAT) or the lipid transfer factor; similar conclusions are reached by Gambert et al. in studies of rabbit, human, and rat serum (11). Recently, a protein factor, distinct from LCAT, has been reported as solely responsible for most, if not all, of the conversion of smaller to larger HDL species (15).

Contrary to these results are the studies of pig and human plasma by Knipping et al. (16) in which no evidence was found supporting the existence of such HDL transfor-

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; SDS, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoate); (-D-I), serum maintained at 4°C with no addition of DTNB or other LCAT inhibitors; (-D + I), serum incubated with no LCAT inhibitors added; (+ D + I), serum incubated in the presence of DTNB; (+ D + P), serum incubated following the addition of DTNB and phospholipase A_2; LCAT, lecithin:cholesterol acyltransferase.

mation factors apart from LCAT. Furthermore, studies of human plasma by Nichols, Gong, and Blanche (10) indicate that inhibitors of LCAT can attenuate the conversion to larger particles. Studies of rat plasma by DeLamatre et al. (8), although not dealing with the issue of particle size, did report a role for LCAT in increasing the apoA-IV content of the HDL; however, changes in apoA-IV content of HDL upon incubation were not seen by Jansen, Schoonderwoerd, and Dallinga-Thie (17). In considering the properties of sera from different species, it is important to take into consideration factors such as the presence of a cholesteryl ester exchange protein in some species (e.g., man) and its absence in the serum of other species, such as the rat (18).

In an effort to resolve some of the discrepancies regarding the role of LCAT in modifying the properties of HDL and to provide a complete characterization of the totality of enzymatically induced changes which occur in the rat HDL during in vitro incubation, the following studies were undertaken.

MATERIALS AND METHODS

Serum treatment and HDL isolation

Serum was obtained from male Sprague-Dawley rats (270–350 g, Taconic Farms) by exsanguination from the abdominal aorta. In some cases 5,5'dithiobis(2-nitrobenzoate) (DTNB) in 0.075 M sodium phosphate buffer, pH 7.4, was added immediately upon collection (final concentration, 1.5 mM) to inhibit LCAT activity. All blood samples were allowed to clot on ice for 3 hr and the serum was recovered by centrifugation. Alternatively, LCAT was inhibited by the addition of *p*-chloromercuriphenyl sulfonate (final concentration, 2 mM) in lieu of DTNB, or by incubation at 56°C for 30 min. In studies of phospholipolysis, pancreatic phospholipase A_2 (final concentration, 15 units/ml serum) (Sigma, St. Louis, MO) and calcium chloride (final concentration, 21 mM) were added to heat-inactivated or DTNB-containing serum.

Incubations were performed at 37°C or 0°C for 18 hr. Efforts to exclude oxygen by the use of a nitrogen atmosphere did not influence the outcome of the experiments. Incubation was interrupted by cooling on ice and DTNB was added to the noninhibited samples to yield the same final concentration (1.5 mM). One portion of each was adjusted to d 1.21 g/ml with KBr and centrifuged at 36,000 rpm in an SW 41 rotor; these samples were subsequently used for electrophoresis or for density gradient centrifugation. The remainder of the serum was adjusted to d 1.063 g/ml with KBr and centrifuged for 20 hr at 150°C in an SW 41 rotor at 36,000 rpm. The bottom fraction was adjusted to a density of d 1.21 g/ml and centrifuged for 44 hr under similar conditions to obtain the HDL. This material was deliberately not "washed" at d 1.21 g/ml in order to minimize losses which are known to result from ultracentrifugation. The $d < 1.063$ g/ml material was dialyzed against 0.15 M NaCl, 0.27 mM EDTA, pH 7.5, and centrifuged for 20 hr to obtain the VLDL. The bottom was adjusted to d 1.063 g/ml and centrifuged for 20 hr to obtain the LDL fraction. All fractions were dialyzed against 0.15 M NaCl, 0.27 mM EDTA, 20 mM $NaCO_3$, pH 8.4, prior to analysis.

Time course of incubation

The time course of events resulting from incubation at 37°C was established using two different protocols. In one, a set of five identical aliquots of freshly isolated serum was placed in a 37°C water bath; at selected times DTNB was added to a concentration of 1.4 mM and incubation was continued for a total of 24 hr. In a second protocol, identical aliquots were placed in a water bath at staggered times and the samples were all removed at one time with the addition of DTNB. Total and free cholesterol levels in these sera were determined.

These sera were then subjected to ultracentrifugation at d 1.063 g/ml and d 1.21 g/ml to isolate the HDL fraction, or only once at d 1.21 g/ml and the top fraction was used for density gradient centrifugation. The HDL obtained by either approach was analyzed by pore limit electrophoresis, SDS gel electrophoresis, and chemical cross-linking, and compositional analysis was performed.

Effect of albumin on rat serum incubation

For study of the effect of albumin on enzymatic remodelling of the HDL, bovine serum albumin (BSA, essentially fatty acid-free, Sigma) was dissolved in 0.15 M NaCl, pH 7.5, containing 1 mg/ml EDTA and incubated 1 hr at 55°C with excess palmitic acid, containing a trace amount of radiactive palmitic acid (Amersham). Following dialysis and a hexane extraction, this preparation was found to possess 6.5 mol of palmitic acid per mol of albumin. A portion of this preparation was subjected to acid/charcoal defatting by the procedure of Chen (19); residual palmitic acid was found to be approximately 0.1 mol/mol of BSA. These two preparations are referred to here as FA-rich BSA and FA-poor BSA, respectively.

Rat serum was first incubated at 37°C for 18 hr to allow substantial LCAT reaction. This material was then divided into six portions, three of which were adjusted to 1.4 mM with DTNB. FA-rich BSA was added to two aliquots of serum (± DTNB) to increase the albumin concentration by 50 mg/ml; FA-poor BSA was added to two similar aliquots. All six samples were incubated at 37°C for an additional 6 hr, after which DTNB was added to the three portions that had not been previously so treated. These samples were then centrifuged to obtain the HDL fraction, which was analyzed by the battery of procedures described above.

Density gradient centrifugation

Into the bottom of an SW 41 centrifuge tube were placed sequentially the following KBR solutions: 1.2 ml of d 1.006 g/ml, 2.5 ml each of d 1.019 g/ml, d 1.040 g/ml and d 1.063 g/ml. An aliquot (2.4 ml) of the d < 1.21 g/ml fraction of serum was subsequently added to the bottom of the tube and followed with 0.5 ml of a d 1.25 g/ml KBr solution. The fluid level in the tube was adjusted to 2 mm below the top with a d 1.006 g/ml KBr solution. These samples were then centrifuged at 34,000 rpm for 24–42 hr. The VLDL was recovered by removing 0.6 ml from the top with a syringe; the remaining fractions were collected through an ISCO gradient fractionator with continuous monitoring of optical density at 280 nm. Protein was quantitated in collected fractions by the procedure of Bradford (20).

Pore limit electrophoresis

The size of HDL particles was determined using a 4–30% acrylamide gradient gel essentially as described by Anderson et al. (21) using a voltage of 150 V for 44 hr applied to Pharmacia PAA 4/30 gels (Piscataway, NJ), or gradient slab gels were prepared and run in a homemade apparatus. The sample volume was adjusted in each case so that 10 μg of protein was applied in each slot; following electrophoresis, the gel was stained with Coomassie Brilliant Blue G-250. Protein standards for estimating molecular dimensions were purchased from Pharmacia and the following values for Stokes' diameter were used: thyroglobulin, 17.0 nm; apoferritin, 12.2 nm; catalase, 10.2 nm; lactate dehydrogenase, 8.1 nm; and serum albumin, 7.1 nm (22). The relationship between molecular size and electrophoretic mobility in these gels was originally described by Felgenhauer (23).

SDS gel electrophoresis and chemical cross-linking

SDS slab gel electrophoresis was performed using 3–27% acrylamide gels as described previously (24). Sample volumes were adjusted so that 25 μg of protein was loaded in each lane of the gel. Gels were stained with Coomassie Brilliant Blue G-250 and destained in 7% acetic acid–5% methanol. In some cases HDL samples were cross-linked using dimethylsuberimidate (Pierce Chemical Co., Rockford, IL) (25) and were electrophoresed on SDS gels along with cross-linked apoA-I and hemocyanin (Sigma, St. Louis, MO) as molecular weight standards.

Chemical analyses

Protein content was determined by the method of Lowry et al. (26), as modified by Markwell et al. (27). Total and free cholesterol were measured enzymatically, using a reagent kit prepared by Fermco (Elk Grove Village, IL); a sample of control human serum (Control H, Sigma) was analyzed on each occasion to verify the reproducibility of results from day-to-day. The mass of cholesteryl ester was computed from the difference between total and free cholesterol by utilizing a factor of 1.7. Triglyceride was determined enzymatically using a kit prepared by Sigma. Fatty acisds were determined using enzymatic reagents (NEFA kits) prepared by Wako Chemical Co. (Dallas, Tx). Phospholipid phosphorus was quantitated by the method of Marinetti, Erbland, and Stotz (28). Phosphorus was corrected to mass of phospholipid by using a factor of 25 for intact phospholipid and 17 for lysophosphatidylcholine. Molar ratios of lipid components were calculated using the following molecular weights: cholesterol, 386; cholesteryl ester, 655; intact phospholipid, 775; lysophosphatidylcholine, 520; and triglyceride, 850.

Phospholipid species were determined by phosphorus determination of lipid spots separated by thin-layer chromatography. Folch extracts (chloroform–methanol 2:1) were spotted on silica gel 60 plates (E. Merck, Darmstadt) and chromatographed using chloroform-methanol-acetic acid-water 25:14:4:2 (v/v/v/v).

In some cases the relative amounts of apolipoproteins were determined by Laurell immunoelectrophoresis (29). Antisera were prepared in rabbits by the injection of purified rat apolipoproteins. Standard curves were obtained by running dilutions of pooled rat serum simultaneously with the unknown samples. Apolipoprotein measurements for both whole serum and the derived d < 1.21 g/ml fraction were determined relative to the pooled serum and expressed as a percentage of serum levels of the apolipoprotein contained in the d < 1.21 g/ml fraction.

Calculation of particle diameters from compositional data

Compositional data were utilized to compute the diameter of a hypothetical spherical lipoprotein, based upon the ratio of polar and neutral lipid components; comparison of the hypothetical diameter with an experimentally determined value allows for an evaluation of any aberrance in the ratio of surface to core lipid in modified lipoproteins. Based on a model for lipoproteins where the neutral lipid core, consisting of cholesteryl ester and triglyceride, is covered by phospholipid and cholesterol (but not by contiguous protein), Shen, Scanu, and Kezdy (30) derived the following equations:

$$68.5n_{pl} + 39.1n_c = 4\pi(r - 20.2)^2 \qquad Eq.\ 1)$$

where n_{pl} is the number of molecules of phospholipid per particle, n_c is the molecules of free cholesterol per particle, (r – 20.2) corresponds to the radius (in Ångstroms) of core lipid covered by these components, r corresponds to the radius of the particle,
and

$$1556n_{tg} + 1068n_{ce} = (4\pi/3)\ (r - 20.2)^3 \qquad Eq.\ 2)$$

where n_{tg} is the number of triglyceride molecules per particle, n_{ce} is the number of cholesteryl ester molecules per particle, and (r – 20.2) is the radius (in Ångstroms) of a sphere representing the exterior of the lipid core.

By dividing equation 2 by equation 1 and taking into account that the number of molecules of each component is equal to the total molecules of lipid times the mole fraction ($\bar{x}$) of each component, one can show that

$$\frac{1556\ \bar{x}_{tg} + 1068\ \bar{x}_{ce}}{68.5\ \bar{x}_{pl} + 39.1\ \bar{x}_{c}} = \frac{(r-20.2)}{3} \qquad Eq.\ 3)$$

In this equation (r – 20.2) corresponds to the radius of a hypothetical lipoprotein with the proportions of neutral and polar lipid used for its calculation.

RESULTS

Effect of serum incubation on the physical properties of rat HDL

Pore limit electrophoresis **(Fig. 1)** of HDL isolated from rat serum shows that overnight incubation at 37°C (lane 3) increases the apparent particle size relative to HDL that was maintained at 0°C overnight (lane 2); this increase corresponds to an increase in Stokes' diameter from 10.4 nm to 12.3 nm. No change in particle diameter is observed in the presence of the LCAT inhibitors, DTNB (lanes 4 and 5) or *p*-chloromercuriphenyl sulfonate or after heating serum to 56°C for 30 min (data not shown). It should be noted that rat HDL generally gave a single, albeit somewhat broad, band in pore limit electrophoresis. Stokes' diameters measured for d < 1.21 g/ml fractions centrifuged only once gave values similar to those obtained from HDL centrifuged at both d 1.063 and d 1.21 g/ml.

To evaluate whether the observed change in apparent particle size was accompanied by changes in protein content of the HDL particle, chemical cross-linking of the isolated HDL particles was performed **(Fig. 2).** Incubation of serum consistently resulted in an increase in the apparent protein mass of the particle of about 60,000 daltons (170,000 for lane 3 versus 110,000 for lane 2). This increase in protein mass was also prevented by DTNB addition (lane 4) or heat inactivation of LCAT (lane 5). Densitometric scanning of cross-linked samples showed a single, symmetrical peak for both the incubated and unincubated samples.

Since incubation appeared to increase the total amount of protein on the rat HDL particle, SDS gel electrophoresis was performed on these HDL fractions to evaluate changes in apolipoprotein distribution **(Fig. 3).** Incubation of rat serum at 37°C (lane 2) can be seen to yield an HDL with increased amounts of both apoA-IV and apoE,

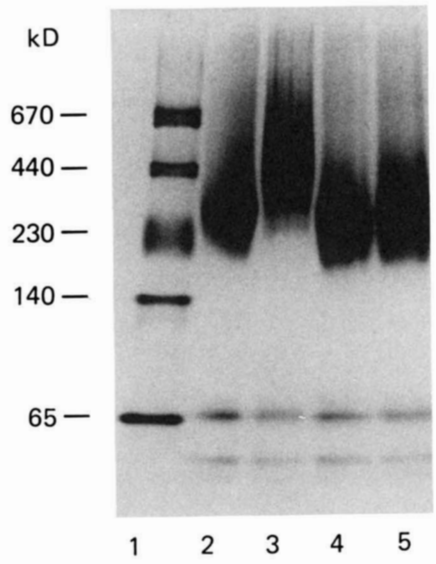

Fig. 1. Pore limit electrophoresis of HDL isolated from incubated rat serum. Lane 1, protein standards (thyroglobulin, apoferritin, catalase, lactate dehydrogenase, and albumin). Lanes 2–5, HDL from the following sera: lane 2, (-D-I); lane 3, (-D + I); lane 4, (+ D-I); lane 5, (+ D + I). All incubations were for 18 hr.

relative to apoA-I, when compared with the unincubated sample (lane 1). Inhibition of LCAT by the addition of DTNB (lane 3) or by heat inactivation (lane 4) yielded an HDL with particularly low levels of apoA-IV and apoE, relative to apoA-I. The source of the increased amounts of apo-E and apoA-IV were studied by performing rocket immunoelectrophoresis on the d < 1.21 g/ml and > 1.21 g/ml fractions of treated sera **(Table 1).** The proportion of apoE and apoA-IV in the lipoprotein-containing portion of serum increased markedly with incubation, whereas the amount of A-I was fairly constant in the

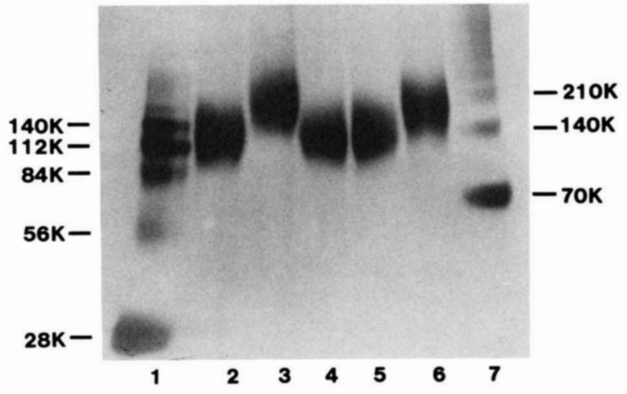

Fig. 2. Chemical cross-linking of HDL isolated from rat sera. Molecular weight standards: lane 1, self-associated forms of apoA-I; lane 7, oligomeric forms of hemocyanin. Lanes 2–6, cross-linked HDL isolated from the following sera: lane 2, (-D-I); lane 3, (-D + I); lane 4, (+ D + I); lane 5, heat-inactived sera incubated for 18 hr; lane 6, (+ D + P).

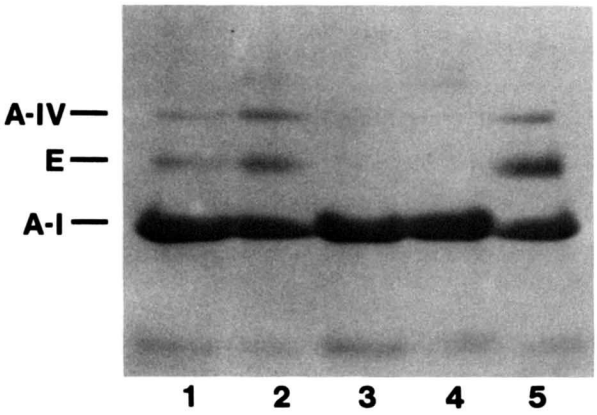

Fig. 3. SDS gel electrophoresis of HDL from rat sera. Lane 1, (-D-I); lane 2, (-D + I); lane 3, (+ D + I); lane 4, heat-inactivated, incubated sera; lane 5, (+ D + P).

d < 1.21 g/ml fraction. Although the d > 1.21 g/ml fraction included apoprotein lost from the lipoproteins due to centrifugation, each of the sera was treated identically so that differences in recoveries in the d < 1.21 g/ml fraction should not be influenced by this factor.

Treatment of rat serum by pancreatic phospholipase A_2

Since the initial reaction catalyzed by LCAT is a phospholipase A_2-type reaction yielding lysolecithin, we examined the effect of the addition of pancreatic phospholipase A_2 to DTNB-inhibited serum. Pore limit electrophoresis of HDL from sera treated with phospholipase produced a broad, indistinct protein band of larger size than that seen with untreated HDL. Density gradient centrifugation, followed by pore limit electrophoresis subsequently showed that this resulted from the production of multiple higher molecular weight species by phospholipase treatment (data not shown). However, cross-linking yielded a single, broad band for this HDL with a molecular mass comparable to that obtained with incubated serum in which LCAT was active (Fig. 2 lane 6); these data suggest that phospholipase digestion produced fragments resulting from varying degrees of phospholipid hydrolysis, but with a fairly constant protein mass. Similarly, SDS gel electrophoresis of HDL from phospholipase-treated serum yielded an apoprotein pattern enriched in apoA-IV and apoE (Fig. 3, lane 5); this pattern was comparable to HDL from serum in which LCAT was active (lane 2).

TABLE 1. Rocket immunoelectrophoresis determination of apoprotein recoveries in the d < 1.21 g/ml serum fraction as a percent of total serum level

	ApoA-I	ApoE	ApoA-IV
		% of total serum level[a]	
(-D-I)	88%	13%	52%
(-D + I)	87%	66%	75%
(+ D + I)	88%	26%	29%
(+ D + P)	72%	73%	87%

[a]Average of duplicate determinations.

Effect of incubation on HDL composition

In order to completely define the chemical changes in the HDL which resulted from either incubation with active LCAT or with phospholipase treatment, compositional data were obtained. **Table 2** presents the weight percentage of each component of the HDL resulting from various treatments.

Incubation of rat serum without inhibition of LCAT leads to a number of compositional alterations relative to unincubated serum or incubated serum containing an inhibitor of LCAT; these latter two samples yielded identical compositions, within experimental error. Consistent with the activity of LCAT, there was a marked reduction in free cholesterol and phospholipid and a substantial increase in cholesteryl ester content; triglyceride contents were not significantly different. Protein content remained the same or increased slightly, when expressed as a weight percent of the total mass. This is consistent with results of density gradient centrifugation (Fig. 4A) which indicated little or no change in density with incubation.

Determination of phospholipid species by thin-layer chromatography revealed that incubated HDL contained twice as much lysolecithin as control samples and a substantial decrease in phosphatidylcholine. The content of other phospholipid species remained essentially unchanged.

The phospholipase A_2 treatment of rat serum that we employed yielded a somewhat different constellation of changes in HDL composition. The total phospholipid content was reduced by about 60%, resulting from almost total hydrolysis of phosphatidylcholine. Phosphatidylinositol, which appeared not to be hydrolyzed by LCAT, was substantially degraded by phospholipase to lysophosphatidylinositol, which migrated with sphingomyelin. Although phosphalipase treatment did not alter the content of free cholesterol or triglyceride, a substantial increase in cholesteryl ester was observed. The content of protein was likewise increased.

In order to evaluate the effect of incubation on the relative proportions of polar and nonpolar substituents, the composition was recalculated on the basis of the molar percentage of each lipid component, and the diameter of a hypothetical lipoprotein containing this balance of substituents was calculated as described in the Methods section **(Table 3).** It can be seen that, with the unmodified rat HDL or with human HDL, there is fair agreement between the experimentally determined diameter and the value calculated from the lipid composition. On the other hand, after incubation with active LCAT, the lipid composition is grossly distorted such that the theoretical di-

TABLE 2. Composition of high density lipoprotein

	(-D-I) (5)[a]	(-D + I) (7)	(+D + I) (7)	(+D + P) (3)	Human HDL_2[b]
			weight % ± SEM		
Protein	34.5 ± 2.7	37.1 ± 2.2	32.6 ± 1.6	41.4 ± 1.7	41
Cholesterol	5.1 ± 0.5	1.6 ± 0.4	5.4 ± 0.6	5.5 ± 0.6	5.4
Cholesteryl ester	27.4 ± 1.0	37.9 ± 1.9	27.9 ± 1.7	38.6 ± 1.6	16
Triglyceride	2.0 ± 1.7	3.3 ± 1.6	2.9 ± 1.1	2.9 ± 0.9	4.5
Phospholipid[c]	31.1 ± 3.3	20.2 ± 2.2	31.2 ± 1.5	11.6 ± 1.5	30
			Percent of total phospholipid phosphorus		
Lyso PC	5.9 ± 0.5	13.5 ± 1.8	6.6 ± 0.7	75.0 ± 3.0	
SM[d]	10.1 ± 4.5	11.9 ± 0.8	7.8 ± 2.1	18.8 ± 3.3	
PC	75.0 ± 4.3	62.4 ± 1.2	76.3 ± 1.6	3.0 ± 0.5	
PI	7.7 ± 0.8	10.3 ± 1.5	8.3 ± 0.7	1.4 ± 0.7	
PE	1.3 ± 0.6	1.8 ± 1.1	1.1 ± 0.8	1.5 ± 0.5	

Abbreviations: lyso PC, lysolecithin; SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

[a]Number in parenthesis indicates the number of separate determinations, each of which was done in duplicate or triplicate.

[b]From reference 30.

[c]The value for phospholipid is corrected for the content of lysophospholipid, using values of 775 for the weight of intact phospholipid and 520 for the weight of lysolecithin.

[d]Lysophosphatidylinositol comigrates with sphingomyelin in this solvent system.

ameter is much larger than the measured value, even exceeding that expected for LDL. It is apparent that the incubated HDL (-D + I) has a marked shortage of polar lipid components.

Lipid distribution among lipoprotein classes

In order to quantitate the redistribution of lipid components among the lipoprotein classes as a result of incubation of serum where LCAT was not inhibited, the recovery of each component in the HDL, LDL, and VLDL fractions was determined and is expressed in **Table 4** in terms of mg/100 ml of serum. It can be seen that, although the total recovery of cholesterol plus cholesteryl ester is the same in both incubated and unincubated serum samples, there is a loss of free cholesterol from the LDL and VLDL fractions, as well as from the HDL. The free cholesterol which is lost from the various lipoproteins is relocated exclusively in the cholesteryl ester fraction of the HDL fraction, confirming that even with an 18-hr incubation there is little or no transfer of cholesteryl ester into the LDL or VLDL fractions.

Density gradient fractionation of sera

In order to detect changes in density distribution as well as component redistribution among subpopulations of HDL, the $d < 1.21$ g/ml fraction was prepared from incubated and nonincubated sera and these fractions were subjected to separation on a KBr density gradient. **Fig. 4** shows the protein distribution as a function of density. Essentially, the protein distribution of the incubated and

TABLE 3. Molar lipid composition of HDL and derived particle diameters

	(-D-I)	(-D + I)	(+D + I)	(+D + P)	Human HDL_2[a]
			mole %		
Cholesterol	13.6	4.4	14.0	15.0	16.9
Cholesteryl ester	42.6	62.8	42.7	62.0	30.4
Triglyceride	2.5	4.2	3.5	3.5	6.4
Lysolecithin	3.7	5.4	3.8	16.0	-
Other phospholipid	37.7	23.2	36.0	3.5	46.3
Hypothetical particle diameter (nm)[b]	12.8	24.8	13.4	-[c]	10.7
Experimentally measured particle diameter (nm)[d]	10.4	12.0	10.9	-[c]	10.2

[a]From reference 30.

[b]Calculated from the molar composition assuming the relationships between polar and core lipids from reference 30 as described in the Methods section.

[c]Values were not computed for phospholipase-treated HDL because of the high content of lysolecithin.

[d]As determined by pore limit electrophoresis.

TABLE 4. Serum concentrations of lipoprotein constituents

	HDL	LDL	VLDL	Total
		mg/100 ml serum		
(-D-I) Serum				
Protein	58.1	5.1	4.8	68.0
Phospholipid	58.2	7.3	9.3	74.8
Cholesterol	9.9	2.3	3.0	15.2
Cholesteryl ester[a]	30.1	5.0	2.0	37.1
(-D + I) Serum				
Protein	60.5	5.7	3.0	69.2
Phospholipid	61.1	6.4	8.0	75.5
Cholesterol	2.5	0.5	0.8	3.8
Cholesteryl ester[a]	42.2	3.3	2.1	47.6

[a]Cholesteryl ester is expressed as mg cholesterol/100 ml serum to facilitate comparison of cholesterol redistribution. The results are representative and are derived from one experiment in which all analyses were performed in duplicate on two separate occasions.

nonincubated samples is similar, except for a decrease in protein in the density interval of 1.04–1.06 g/ml. The apoprotein distribution across the peaks is very different, however, as seen from SDS gel electrophoresis of individual fractions **(Fig. 5).** With the unincubated sample the apoE is distributed mostly at the lower density portion of the HDL peak, with overlap into the LDL region (Fig. 5A). This probably corresponds to the fraction of HDL which has been termed HDL_1 (31). The apoA-IV, however, is located almost exclusively in the HDL fractions with the highest densities. Upon incubation, both the apoE and apoA-IV are found to be more uniformly distributed across the HDL fractions.

Individual fractions were also applied to pore limit electrophoresis gels to establish the HDL size distributions represented therein. The particle sizes obtained for the peak fractions containing the bulk of the protein corresponded to the values representative of the d 1.063–1.21 g/ml fraction (Table 3). The earlier, less dense, fractions

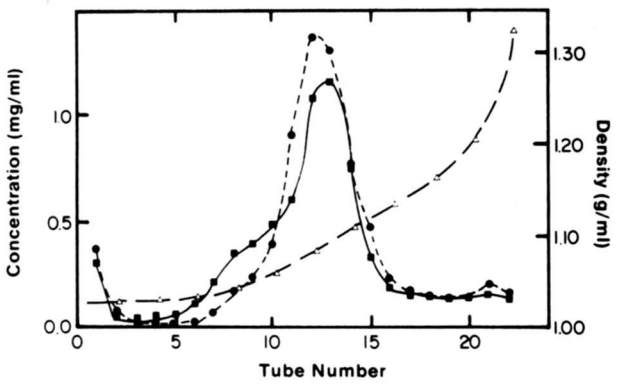

Fig. 4. Density gradient centrifugation of the d < 1.21 g/ml fraction of (-D-I) serum (■—■) and (-D + I) serum (●—●). Panel A gives the protein distribution; the density of every other tube was determined by weighing aliquots of known volume (△– – –△).

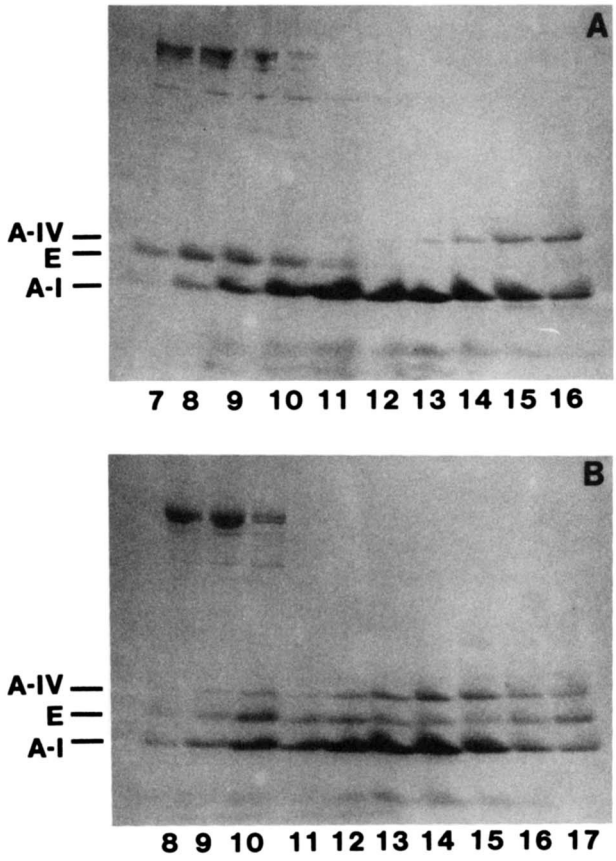

Fig. 5. SDS gel electrophoresis of individual fractions obtained from KBr density gradients (Fig. 4). Panel A: (-D-I) serum and panel B: (-D + I) serum. Numbers at the bottom edge correspond to the tube numbers in Fig. 4. For each example 25 μg of protein was applied to the gel.

gave bands with diameters larger than those of the major HDL fraction but smaller than for rat LDL. Crosslinking followed by SDS gel electrophoresis gave a similar pattern of protein mass distribution as a function of density (data not shown).

Time course of incubation

The effect of length of incubation on the properties of HDL was determined in order to ascertain the time course of the observed changes and to look for intermediate products at early time points. As shown in **Fig. 6,** the rate of cholesteryl ester formation was linear during the first 2 hr and reached a near-maximal value of 88% esterification by 24 hr. The LCAT activity in this serum was calculated from the loss of free cholesterol and was found to be 60 ± 5 μmol CE formed/liter per hr, corresponding to a fractional esterification rate of 10–12%/hr. This molar esterification rate is somewhat lower than in man, but is in reasonable agreement with other values for LCAT activity reported for the rat (32).

While the proportions of apoE and apoA-IV in HDL both increased with time, these proteins did not appear to

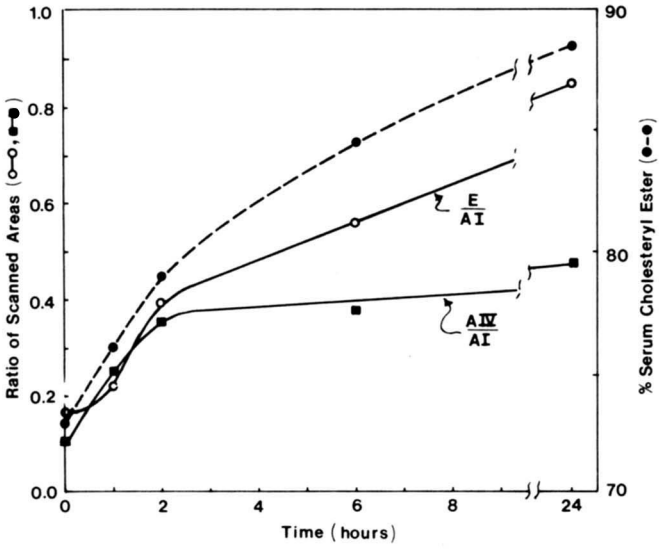

Fig. 6. Time course of LCAT effects. Samples of rat serum were incubated for various periods of time. The percentage of cholesterol in ester form is indicated by the dashed line. HDL was isolated from the sera by density gradient centrifugation and the tubes containing the main HDL peak were dialyzed and electrophoresed on SDS gels. Densitometric scanning of Coomassie-stained gels yielded relative areas of E/A-I (○---○) and A-IV/A-I (■—■).

follow the same time course (Fig. 6). Specifically, the proportion of apoA-IV to apoA-I does not continue to increase significantly after 2–3 hr whereas the proportion of apoE to apoA-I changes less at shorter time points, but continues to increase up to 24 hr. Pore limit electrophoresis of the HDL or of the $d < 1.21$ g/ml fractions from these sera show only small changes in particle size at short time intervals, but substantial changes at the 6- and 24-hr time points **(Fig. 7).** Thus, there appears to be a correlation between retention of apoE on the HDL and its increase in Stokes' diameter.

Densitometric scans of the pore limit electrophoretic gels did not reveal a reciprocal relationship with time between the smaller band and the larger molecular weight species which one would predict if a precursor–product relationship existed between these species (data not shown). Although the major band showed a continuous increase in size with time, the presence of shoulders on this band at intermediate time points suggests that this lipoprotein was not homogeneous in size. Surprisingly, densitometric scanning of bands obtained with cross-linked samples of HDL showed a relatively narrow and symmetric band of progressively increasing molecular weight as a function of incubation time. While the explanation for this is unclear , it may mean that there is a moderately fast apoprotein exchange among the HDL during the 60–90 min of the cross-linking reaction, resulting in an averaging of the protein masses on the individual lipoprotein species; the existence of equilibria between lipid-bound and unbound apoproteins has been documented and modelled by Ponsin and Pownall (33).

Effect of serum albumin

Since compositional data indicated that there is accumulation in the HDL of some lysolecithin, possibly due to saturation of endogenous serum albumin, we examined the effect of adding additional fatty acid-poor albumin after an 18-hr incubation of serum. A preliminary experiment indicated significant effects on the properties of HDL, with an especially marked increase in apparent particle size by pore limit electrophoresis (Fig. 7, lane 6). To examine this effect more carefully, an experiment was performed in which rat serum was incubated for 18 hr, allowing a substantial LCAT reaction, and either FA-rich BSA or FA-poor BSA was added and incubation was continued for 6 hr. In one set of samples, DTNB was added at the time of BSA addition. The additional incubation when LCAT was active made only minor changes in the properties of the HDL when compared to its mate which contained DTNB. This suggests that the albumin effect is not dependent upon additional LCAT activity, as would be the case with relief of product inhibition, but is more likely due to alteration on polar lipid/nonpolar lipid balance through removal of lysolecithin, which accumulates on the particle. This is supported by complete compositional analysis of lipid and phospholipid species which demonstrated that a major effect of adding FA-poor BSA was to cause a 40% reduction in the content of lysolecithin in the HDL.

Subsequent studies have shown that the free fatty acid content of rat HDL is approximately 1.4% by weight after incubation with active LCAT, as compared with a value approximately 0.5% when LCAT in inactivated with DTNB. The percentage of free fatty acid increases to

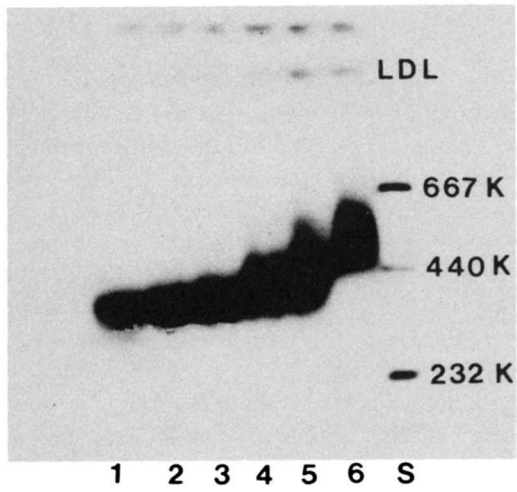

Fig. 7. Pore limit electrophoresis of the $d < 1.21$ g/ml fraction of rat sera incubated at 37°C for various periods of time. Times of incubation are: lane 1, 0 hr; lane 2, 1 hr; lane 3, 2 hr; lane 4, 6 hr; lane 5, 24 hr. The sample in lane 6 had fatty acid-poor BSA added to the serum after 18 hr of incubation. Lane S contains protein standards.

around 4% with the addition of phospholipase A_2 or after after injection of heparin to release lipase activity, but returns to below 0.5% when BSA is added at a concentration of 50 mg/ml. The addition of either FA-rich or FA-poor BSA did not have a material effect on the extent of cholesterol esterification either in whole serum or in the isolated HDL, even in the absence of DTNB.

The addition of fatty acid-poor BSA was found to increase the apparent particle diameter to 15.3 nm, compared with 12.3 nm for the incubated control and 10.4 nm for unincubated controls. The addition of fatty acid-rich BSA did not cause any increase in particle size. Similarly, protein mass as determined by cross-linking was increased by the addition of fatty acid-poor BSA but was relatively unaffected by fatty acid-replete BSA when compared with the control HDL.

Interesting effects were observed with the apoprotein distribution of these samples as revealed by densitometric scanning of SDS gels **(Table 5).** In this case a portion of each serum sample was centrifuged at d 1.21 g/ml after incubation and the top portion was separated on a KBr density gradient, similar to the separation shown in Fig. 4. The HDL was fractionated into three portions. The major fraction, containing approximately 60–70% of the HDL and corresponding to tubes 11–13 in Fig. 4, is referred to as pool II. Four fractions on either side of this peak were pooled and are referred to as pools I and III, respectively. The lighter density HDL fraction is seen to have little apoA-IV, but substantial amounts of apoE; conversely, the heavier HDL (pool III) contains the highest proportion of apoA-IV. The production of apoE in pools I and II seems most sensitive to the effects of added FA-poor BSA, showing significant reductions relative to apoA-I.

TABLE 5. Ratio of SDS gel apoprotein peaks for density gradient pools

	Pool I (d 1.04–1.07)		Pool II (d 1.07–1.10)		Pool III (d 1.10–1.16)	
Serum Treatment	E / A-I	A-IV / A-I	E / A-I	A-IV / A-I	E / A-I	A-IV / A-I
37°C	0.71	0.04	0.37	0.24	0.51	1.08
37°C + FA-poor BSA	0.24	0.03	0.17	0.29	0.49	1.19
37°C + FA-rich BSA	1.00	0.01	0.34	0.14	0.32	1.38
0°C			0.15	0.12		

DISCUSSION

The results of these studies document myriad consequences of rat serum incubation which appear to be largely, if not exclusively, attributable to the action of lecithin:cholesterol acyltransferase. Although a few reports have indicated that inclusion of sulfhydryl reagents to inactivate LCAT in rat serum did not prevent alterations in HDL size (11, 14, 15), our repeated and consistent results are to the contrary. Explanation for these differences may reside in the incomplete characterization of the products or of the extent of cholesterol esterification in previous studies. One laboratory has reported the existence of a conversion factor responsible for many of the physical alterations in HDL (15), although previous studies by this group did not find changes in HDL particle distribution upon 6 hr of incubation of rat serum in the presence of LCAT inhibitors (34). Our findings suggest that in the rat this conversion factor must play a minor role relative to LCAT.

The major effects of serum incubation seem to be on the HDL, with a resultant increase in size and protein content, altered lipid composition, and apparent redistribution of apolipoproteins. Although some of these changes are reminiscent of the putative transformation of HDL_3 to HDL_2 in man, there are significant differences. For one, the increase in particle mass appears to be only about 40–60%, even after 24 hr, compared with a 100% mass increase for human HDL_2 over HDL_3 (30). Furthermore, in the rat there is only a small shift in HDL density, if any, upon incubation. Some of these differences might be attributable to the absence of a cholesteryl ester transfer protein in the rat.

We were especially struck by the altered balance of HDL apolipoproteins in the incubated serum. We confirm the observations of DeLamatre et al. (8) that active LCAT leads to an increased presence of apoA-IV in the HDL, and that this appears to be derived from the lipoprotein-free fraction of serum. However, we also observe a large increase in the relative content of apoE on the HDL following incubation, and that most of this increase occurred after the increase in A-IV leveled off (Fig. 6). Although our data indicate that this apoE is derived from the $d < 1.21$ g/ml fraction (Table 1), it should be kept in mind that this material had been centrifuged once at d 1.21 g/ml. DeLamatre et al. (8) observed that apoE was not found in the $d < 1.21$ g/ml fraction, either before or after incubation. We interpret our data to mean that apoE is loosely bound to HDL of unincubated serum, and is lost to the $d > 1.21$ g/ml fraction during ultracentrifugation, but that it becomes more firmly bound to HDL as a result of the LCAT reaction. The increase in the proportion of apoE in the HDL particle appears to follow the LCAT reaction (Fig. 6) and may be due to the accumulation of products, such as lysolecithin. One line of data in support of this notion is that inclusion of fatty acid-free BSA simultaneously removes anions, including lysolecithin and fatty acids, and leads to a reduced content of apoE (Table 5).

In vitro incubation of rat serum leads to the production of HDL species with markedly altered lipid composition. We were surprised to find that the expected balance between surface and core lipids, as judged from the model

and algorithms derived by Shen et al. (30), was radically altered by incubation. It appears that in the LCAT-modified particle there is far too little phospholipid and free cholesterol to cover the surface of the nonpolar lipid core (Table 3). When similar calculations were performed using the data of Chen et al. (35) on the transformation of small, spherical HDL by LCAT, a similar discrepancy was found between polar and nonpolar lipids. Thus, it appears that under conditions where polar lipids are relatively unavailable, substantial reorganization of the HDL surface occurs which allows the hydrophobic domains of the apoproteins to substitute for the polar lipids in providing coverage for the hydrophobic core. It is of interest to note that with the lipoprotein lipase reduction of chylomicron core lipids there is thought to be a loss of surface apoproteins and lipids, whereas with the LCAT-mediated generation of a hydrophobic core in HDL there appears to be the acquisition of apoproteins.

One aspect that has proven difficult to elucidate is the exact mechanism of the transformation that LCAT effects in HDL. The increase in protein mass per particle observed by cross-linking or shown by increased apoE and apoA-IV content in SDS gel electrophoresis is incompatible with the small increase in protein content of the HDL fraction when expressed per 100 ml of serum (Table 4). These data suggest that particle fusion is taking place, but the compositional and size data are not compatible with a simple doubling of each component. Instead, we suggest that a gradual, yet dynamic, remodelling of the HDL takes place by a process that may involve one or more fusion events, followed by component disproportionation. Since we do not observe particles smaller than the original HDL at the same time as the larger particles, an observation also made by Rye and Barter (15) under conditions where LCAT is active, we presume that these fuse with other species or that the original fusion event involves more than two particles. A complex mechanism of this sort has also been invoked to explain the molecular transformations of small, spherical HDL by LCAT (35).

Regardless of the mechanism for the formation of the product HDL, it is important to consider whether the species formed by in vitro incubation bear any relevance to particles formed in vivo. Although the maximal changes require a lengthy (18–24 hr) incubation, data in Fig. 6 show that significant changes occur in periods as short as 2 hr. Furthermore, removal of lysolecithin and free fatty acids by albumin, which presumably would not be saturated in vivo, could promote the formation of the larger HDL by promoting particle fusion events. On the other hand, the apparent absence of the large, product HDL in freshly isolated plasma might be due to competing enzymatic processes that slow or reverse the effects of LCAT, such as the action of lipoprotein lipase or hepatic lipase; evidence for this latter process was recently reported by Lefevre, Chuang, and Roheim (36) and by Hopkins and Barter (37). It could also be true that the increased affinity of apoE for the large HDL species may lead to its rapid removal by a process involving the B, E receptor, such as has been suggested by Koo, Innerarity, and Mahley (38) in the canine model.

It should be noted that incubation of rat serum also yielded some alterations in the VLDL particles. Specifically, the incubated VLDL possessed substantial amounts of A-IV and A-I proteins, relative to the E protein, whereas the control VLDL had essentially no A-IV or A-I (data not shown). This might be attributable to the loss of VLDL surface lipid, with uptake of protein from the HDL fraction. This may also be an indication of fusion occurring with products generated during the disproportionation of the HDL.

In summary, our data support the concept that LCAT action in rat serum produces a wide range of effects, especially in the HDL fraction, which are manifested in apoprotein redistribution, increased particle sizes, and greatly altered lipid composition. These changes are important, not only because of the implied alteration in the mode of interaction between proteins and core lipids, but also because of potentially altered metabolism through interaction with the LDL (B,E) receptor. The physicochemical mechanism for the structural transformations remains to be elucidated.

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