Isolation and Partial Characterization of Chimpanzee Plasma High Density Lipoproteins and Their Apolipoproteins†

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ABSTRACT: Chimpanzee high density lipoprotein (HDL) fractions were isolated by preparative ultracentrifugation between d = 1.063–1.125 g/ml (HDL_2) and 1.125–1.21 g/ml (HDL_3). The HDL_2 and HDL_3 contain 42 and 58% protein and have the same lipid components although present in different amounts. The phospholipid composition in both subclasses is quite similar, but human HDL_2 contains more sphingomyelin than HDL_3. As in human HDL the unsaturated fatty acids, oleic and linoleic acid, are predominant in both HDL chimpanzee subclasses. The two chimpanzee HDL subclasses are very similar in amino acid composition and differ significantly from their human counterparts only in tryptophan and cystine content. As in man the chimpanzee $apoHDL_2$ and $apoHDL_3$ show Leu-Thr-Gln as the COOH-terminal sequence and aspartic acid as a major NH_2-terminal amino acid. The apolipoproteins of chimpanzee high density lipoproteins with a mol wt of 29,000 and 19,000 are similar to human apoLp-Gln-I and human apoLp-Gln-II. A fraction with a mol wt of ±19,000, and a mobility corresponding to human apoLp-Gln-II reveals the presence of a disulfide bond as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol. In chimpanzee $apoHDL_2$ there is a higher ratio of apoLp-Gln-I/apoLp-Gln-II than in $apoHDL_3$ and both are different from the human apoHDL classes, in a lower content of apoLp-Gln-II. Isoelectric focusing of the apolipoproteins yields three major fractions with a molecular weight and an amino acid composition similar to human apoLp-Gln-I. By gel filtration on Sephadex G-200 $apoHDL_2$ and $apoHDL_3$ are eluted into six and seven components, respectively. The main component has a mol wt of 29,000 with an electrophoretic mobility and an amino acid composition similar to human apoLp-Gln-I. A fraction with a mol wt of 19,000 and an amino acid composition comparable to human apoLp-Gln-II is also isolated. These studies demonstrate that chimpanzee and human high density lipoproteins have similar physical and chemical properties. Because of the similarities between the human and chimpanzee high density apolipoproteins, the use of the chimpanzee as a research animal for lipoprotein metabolism seems logical.

In the last years the composition, the function, and the structure of circulating plasma lipoproteins have been extensively studied. Most studies, however, were mainly confined to human lipoproteins (Oncley and Harvie, 1969; Scanu, 1971; Scanu and Wisdom, 1972; Shore and Shore, 1972; Fredrickson *et al.*, 1972). A few studies on plasma lipoproteins of the rat (Koga *et al.*, 1971), of the pig (Fidge, 1973; Jackson *et al.*, 1973), of the dog (Solyom *et al.*, 1971), and of the guinea pig (Puppione *et al.*, 1971) were reported.

Because of their close phylogenetic relationship to man the nonhuman primates are of interest as animal models in the study of atherosclerosis (Scott *et al.*, 1971). Properties and composition of the two major ultracentrifugal subclasses of serum lipoproteins from normal *Macacus rhesus* were recently studied by Edelstein *et al.* (1973). Inasfar as the lipid composition was considered we evaluated the use of nonhuman primates in experimental atherosclerosis (Peeters and Blaton, 1969; Blaton *et al.*, 1972). Previous investigations on the electrochromatographic separation and properties of plasma lipoproteins from baboon and chimpanzee showed many similarities of the main lipoprotein classes to human plasma lipoproteins (Blaton and Peeters, 1971). By feeding a high cholesterol diet in the presence of saturated fats we were able to induce plasma hyper-β-lipoproteinemia in the chimpanzee as well as in the baboon (Blaton *et al.*, 1970, 1974; Peeters *et al.*, 1970; Howard *et al.*, 1972). Changes in chimpanzee plasma lipoproteins similar to human hyper-β-lipoproteinemia were observed. However, the phospholipid subclasses of the chimpanzee react differently from the human patient with hyper-β-lipoproteinemia and from the baboon on an atherogenic diet. Since the phospholipid composition in the human lipoprotein classes is related to the polypeptide composition (Shore and Shore, 1972) we decided to investigate the apolipoproteins of the chimpanzee and the baboon.

The objective of this study was the isolation and partial characterization of the chimpanzee plasma high density lipoproteins HDL_2 and HDL_3,[1] in order to elucidate the relationship between the apolipoproteins from man and chimpanzee and to stress the usefulness of this animal in the study of metabolism, structure, and function of lipoproteins.

Materials and Methods

Animals. Four male chimpanzees (Pan Troglodytes Schweinfurti) about 12 years old were fed a standardized control diet (Blaton *et al.*, 1974). Fasting blood was taken into EDTA (1 mg/ml) from the femoral artery under Sernylan (Parke, Davies and Company) as described previously (Mortelmans,

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[1] Abbreviations used are: VLDL, very low density lipoproteins of d = 1.006 g/ml; LDL_1, low density lipoproteins of d = 1.006–1.019 g/ml; LDL_2, low density lipoproteins of d = 1.019–1.063 g/ml; TLDL, total low density lipoproteins; HDL, high density lipoproteins; apoHDL, protein moiety of HDL; HDL_2, high density lipoproteins of d = 1.063–1.125 g/ml; HDL_3, high density lipoproteins of d = 1.125–1.21 g/ml; $apoHDL_2$ and $apoHDL_3$, protein moieties of HDL_2 and HDL_3, respectively; apoLp-Gln-I, major polypeptide of HDL with COOH-terminal glutamine; apoLp-Gln-II, polypeptide of HDL with COOH-terminal glutamine; HSEtOH, β-mercaptoethanol.

1969). Plasma was obtained after centrifugation at 3000*g* and used within 6 hr for lipoprotein isolation.

Isolation of Lipoproteins. After an initial centrifugation at 9000*g* for 30 min at 4° (IEC ultracentrifuge, A 321 rotor) chylomicron free plasma was layered with $d = 1.006$ g/ml of NaCl and VLDL[1] was isolated at 70,488*g* for 26 hr at 16°. The pooled infranatant, adjusted to $d = 1.019$ g/ml by addition of solid NaCl, was centrifuged at 70,488*g* for 22 hr at 16°. The top fraction (LDL_1) was collected and the infranatant was adjusted to $d = 1.063$ g/ml with NaCl. LDL_2 was isolated under identical conditions as for LDL_1. The high density lipoproteins were obtained by ultracentrifugal flotation at $d = 1.125$ g/ml for HDL_2 and at $d = 1.21$ g/ml for HDL_3 at 114,480*g* for 22 hr and 25 min at 16°. The high density lipoproteins were recentrifuged at $d = 1.063$, and at $d = 1.125$ g/ml for HDL_2 and at $d = 1.21$ g/ml for HDL_3. Densities were obtained by adding NaCl–KBr solutions. The isolated HDL_2 and HDL_3 components were dialyzed against 0.1 M NaCl before delipidation.

Preparation of Apolipoproteins. Reagent grade urea (Merck) was filtered through a column of mixed bed resin (Amberlite MB-1). All other chemicals were reagent grade. The high density lipoproteins were delipidated according to Shore and Shore (1967). The aqueous phase, containing apoHDL, was dialyzed against 0.02 M Tris-HCl buffer (pH 8.5)–0.1 M NaCl and against 0.1 M Tris-HCl buffer (pH 9.0), in the presence of 8 M urea.

Agarose Gel Electrophoresis. Agarose gel electrophoresis was performed in Veronal buffer (pH 8.6), ρ 0.037, containing 3% albumin. Agarose gels (Seakem, 0.83%) were prepared in Veronal buffer (pH 8.6), ρ 0.05, with 1% albumin. Run conditions were 140 V for 2 hr 15 min. The gels were stained with Sudan Black. The agarose electrophoretic method of Hatch *et al.* (1973) was used for the quantitative evaluation of the lipoprotein pattern.

Antisera were prepared in male rabbits (2–3 kg) by intramuscular injection of 1–1.5 mg of $apoHDL_3$ emulsified with equal volumes of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). After 3 weeks the animals received an intramuscular booster injection and were bled 15 days later. The antisera were stored at −20° in 1:100,000 (w/v) merthiolate.

Analytical Polyacrylamide Gel Electrophoresis. Gels of 10.9% acrylamide and 0.3% *N,N'*-methylenebisacrylamide (2.7% cross-linking) were prepared in the presence of 8 M urea using the modified method of Davis (1964). Electrophoresis of the apolipoproteins was carried out in Tris-borate buffer (pH 8.8) in the presence of 8 M urea. After electrophoresis the gels were stained with Coomassie Brilliant Blue R 250.

Sodium Dodecyl Sulfate–Polyacrylamide Molecular Weight Determinations. Apolipoproteins were preincubated in 2% sodium dodecyl sulfate, 1% β-mercaptoethanol, and 0.01 M sodium phosphate (pH 7.0) at 37° for 2 hr and dialyzed against 0.1% sodium dodecyl sulfate–0.1% β-mercaptoethanol in the same buffer. Gels of 10% polyacrylamide and 0.3% *N,N'*-methylenebisacrylamide (2.7% cross-linking) in the presence of 0.1% sodium dodecyl sulfate were prepared according to Weber and Osborn (1969). Electrophoresis was carried out at 8 mA/gel for 3 hr 15 min with Bromophenol Blue as marker.

Standard proteins of known molecular weight were incubated in the presence of 1% sodium dodecyl sulfate, 1% β-mercaptoethanol, and 0.01 M sodium phosphate (pH 7.0) at 37° for 2 hr. From the standard proteins a calibration curve was obtained by plotting electrophoretic mobility *vs.* the logarithm of the molecular weight. Unknowns were computed from the calibration curve. The molecular weight standards cytochrome *c* (mol wt 13,500) and ovalbumin (mol wt 45,000) were purchased from Boehringer Mannheim and cytochrome *c* (mol wt 12,400), chymotrypsinogen (mol wt 25,000), myoglobin (mol wt 17,800), and bovine serum albumin (mol wt 68,000) were purchased from Serva Heidelberg.

Sephadex G-200 Gel Filtration. The apolipoproteins were fractionated by a modification of the procedure of Scanu *et al.* (1969). Two sequentially connected columns (2.5 × 100 cm) of Sephadex G-200 were equilibrated with 0.01 M Tris-HCl buffer (pH 8.6)–8 M urea–0.001 M EDTA in the presence of sodium azide (20 mg %). Urea solutions were freshly prepared and filtered through a column of mixed bed resin (Amberlite MB-1) before use. Protein (20 mg) was applied and the column was eluted at 15° with the equilibration buffer at a flow rate of 9.5 ml/hr. The effluent was continuously monitored at 280 nm (LKB 8300 A Uvicord). The fractions were pooled and were concentrated in an Amicon Diaflo ultrafiltration cell (UM 2 membrane; mol wt cut-off 1000). The concentrated fractions were freed of urea by dialysis or by Sephadex G-15 gel filtration and were lyophilized.

Preparative Isoelectric Focusing. Isoelectric focusing was performed according to the method of Vesterberg and Svensson (1966). pH and sucrose gradients were prepared essentially as described by LKB instruments. Preisoelectric focusing, without sample, was carried out in 6% ampholine (pH 4–8). Run conditions were 600 V for 64 hr at 25°. Aliquots were collected and the ampholine fractions between pH 5 and 6.8 were pooled. Protein samples were analyzed by isoelectric focusing in the presence of 8 M urea. Protein (10–15 mg) was applied to the column and the isoelectric focusing was carried out between pH 5 and 6.8 at 37° with an initial potential of 200 V for 15 hr, followed by 400 V for 3 hr, and finally by 700–800 V for 3 hr. The absorbance of the effluent was continuously monitored at 280 nm (LKB 8300 A Uvicord). Fractions of 2 ml were collected, the pH was measured with a Radiometer pH meter (Copenhagen), and the optical density was determined on a PMQ 2 Zeiss spectrophotometer at 280 nm. The appropriate fractions were pooled and freed of urea, sucrose, and ampholines by dialyzing against buffer at increasing salt concentration and by gel filtration on Sephadex G-15.

Amino Acid Analysis. For amino acid analysis about 2 mg of protein was hydrolyzed in 6 N HCl for 22 hr at 110°. After hydrolysis the excess of acid was removed by nitrogen evaporation at 40°. The dry samples were dissolved in 2.0 ml of sodium citrate–HCl buffer (pH 2.20).

Amino acid analyses were carried out according to Spackman *et al.* (1958) by ion-exchange chromatography on a Beckman Model 121 amino acid analyzer. Due to losses from hydrolysis correction factors of 10 and 15% were applied in estimating the serine and threonine content, respectively (Moore and Stein, 1963). Half-cystine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation of the protein (Hirs, 1967); tryptophan was determined after hydrolysis for 22 hr at 110° in *p*-toluenesulfonic acid according to Liu and Chang (1971).

NH_2-Terminal Amino Acid Analysis. Amino-terminal residues were determined by the dansylation procedure as described by Gros and Labouesse (1969). The dansyl amino acids were detected by thin-layer chromatography on silica gel plates according to Zanetta *et al.* (1970).

COOH-Terminal Amino Acid Analysis. Diisopropyl fluorophosphate treated carboxypeptidase A, obtained from Sigma, was prepared according to Ambler (1967). Instead of *N*-ethylmorpholine acetate buffer, sodium bicarbonate buffer (pH 8.5)

TABLE I: Human and Chimpanzee Plasma Lipids.[a]

Subject	No.	TC (mg/100 ml)	TG (mg/100 ml)	PL (mg/100 ml)	TC/PL
Human (25–45 years old)	93	223 ± 3	84 ± 3	205 ± 3	1.09
Chimpanzee	4	259 ± 21	60 ± 10	295 ± 18	0.88

[a] Mean values ± standard error; TC, total cholesterol; TG, triglycerides; PL, phospholipids; TC/PL, total cholesterol/phospholipid ratio.

was used. The apolipoproteins were dialyzed exhaustively against sodium bicarbonate buffer (pH 8.5). Dialysis membranes with a mol wt cut-off of 3500 (Spectrapor TM 3) were used. The ratio of protein to enzyme was 1/50 (w/w). The mixtures were incubated at 37° and at scheduled time interval the reaction was stopped by the addition of 1 vol of 10% trichloroacetic acid. The precipitate was removed by centrifugation and the supernatant was subjected to amino acid analysis. Blank levels were obtained by incubation of the carboxypeptidase A in the absence of the protein and by amino acid analysis of the dialyzed protein. The release of amino acids was quantitatively determined with the Beckman Model 121 amino acid analyzer. The conditions for the amino acid analysis were the same as for the analysis of physiological fluids. Lithium citrate buffers were used for the separation of asparagine and glutamine as described in Beckman Bulletin A-TB-044 (1967).

Lipid Analysis. Lipids were extracted from the lipoproteins with chloroform–methanol (2:1, v/v). Cholesterol, phospholipids, and triglycerides were determined according to the methods of Rozenthal *et al.* (1957), Peeters *et al.* (1970) and Van Handel and Zilversmit (1957). The fatty acid profile of the lipoproteins was obtained by gas–liquid chromatography as reported by Blaton *et al.* (1970).

Total Protein Determinations. Protein concentrations were determined by the method of Lowry *et al.* (1951). Bovine serum albumin (Poviet) was used as a standard.

TABLE II: Analytical Ultracentrifugal (UCF) and Quantitative Agarose Electrophoretic Values of Chimpanzee Plasma Lipoproteins.[a]

Method	$S_f > 400$ (mg/100 ml)	S_f = 20–400 (mg/100 ml)	S_f = 0–20 (mg/100 ml)	HDL (mg/100 ml)
Agarose	0	61	416	430
UCF	0	68	445	454

[a] Mean values of two determinations on two male chimpanzees.

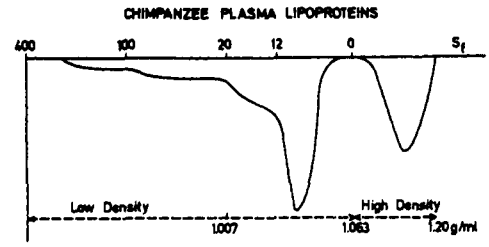

FIGURE 1: TLDL and HDL spectra for normal adult chimpanzees.

Results

Lipoprotein Spectra. Analytical ultracentrifugal analyses of plasma lipoproteins were performed as described by Ewing *et al.* (1965). Figure 1 shows the TLDL and HDL spectra for normal adult chimpanzees. Table I describes the lipid composition of human and chimpanzee plasma. The ultracentrifugal and the electrophoretic data of chimpanzee plasma lipoprotein, which are similar, are given in Table II. In comparison with the human data, chimpanzee plasma shows higher phospholipid and HDL concentrations.

Isolation and Chemical Analysis of HDL_2 and HDL_3. The purified HDL_2 and HDL_3 fractions give, respectively, a single precipitation line against antichimpanzee HDL_3. On agarose electrophoresis the individual lipoprotein fractions migrated as a single band. HDL_2 and HDL_3 have an average dry weight composition of, respectively, 42 and 58% protein, similar to human high density lipoproteins (Scanu, 1972). Both HDL subclasses have the same lipid components but differ in the relative distribution (Table III). In accord with previous reports on human high density lipoproteins (Skipsky *et al.*, 1967), chimpanzee HDL_3 shows a higher protein percentage and less cholesterol than HDL_2 and higher ratios of cholesterol esters to free cholesterol, phospholipid to cholesterol, and phosphatidylcholine to sphingomyelin (Table IV). In both HDL fractions phosphatidylcholine (80%) is the predominant phospholipid followed by sphingomyelin (13%) and phosphatidylethanolamine (6%). Minor phospholipid components are hydroxyphosphatidylcholine, phosphatidylinositol, and phosphatidylserine. Although the chimpanzee HDL have very similar values for sphingomyelin, human HDL_2 has somewhat more sphingomyelin (14.5%) than HDL_3 (9.2%). The fatty acid analysis of HDL_2 and HDL_3 reveals a quite similar pattern for both lipoproteins (Table V). The unsaturated fatty acids, oleic acid and linoleic acid, are major components, whereas palmitic acid is predominant among the saturated fatty acids.

TABLE III: Chemical Composition of Chimpanzee Plasma High Density Lipoproteins.[a]

Lipoprotein Fraction (Density Range, g/ml)	Protein	TG	Chol	CE	PL	Prot/ Lipid
HDL_2 ($1.063 < d < 1.125$)	41.8 ± 2.5	2.2 ± 0.9	6.5 ± 0.1	24.9 ± 2.1	24.6 ± 1.0	0.74
HDL_3 ($1.121 < d < 1.21$)	57.7 ± 3.8	2.9 ± 0.4	3.4 ± 0.2	17.0 ± 1.5	19.0 ± 2.4	1.30

[a] Mean values ± standard error of two determinations on two male chimpanzees; TG, triglycerides; Chol, free cholesterol; CE, cholesterol esters; PL, phospholipids.

TABLE IV: Per Cent Phospholipid Composition of Human and Chimpanzee High Density Lipoproteins.

Lipoprotein Fraction (Density Range, g/ml)	Subject	PC	OH-PC	Sph	PI	PS	PEt
HDL_2 ($1.063 < d < 1.125$)	Human[a]	73.8	2.0	14.5	2.4	0.9	3.3
	Chimp.[b]	80.2	0.4	13.2	0.2		6.0
HDL_3 ($1.125 < d < 1.21$)	Human	77.1	5.4	9.2	1.4	0.6	2.5
	Chimp.	79.1	0.5	11.3	1.5	0.6	7.0

[a] Skipsky (1971). [b] Mean value of two determinations on two male chimpanzees; PC, phosphatidylcholine; OH-PC, lysophosphatidylcholine; Sph, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PEt, phosphatidylethanolamine.

TABLE V: Fatty Acid Composition of High Density Lipoproteins Isolated from Chimpanzee Plasma.[a]

Lipoprotein Fraction (Density Range, g/ml)	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:3	20:4
HDL_2 ($1.063 < d < 1.125$)	2.7	23.8	3.9	8.4	19.3	28.2	0.5	1.4	2.4	9.4
HDL_3 ($1.125 < d < 1.21$)	1.6	25.9	3.6	7.8	17.3	29.5	0.8	1.7	2.1	10.0

[a] Mean values are per cent by weight of total fatty acids from duplicate analysis on two batches of lipoproteins.

Apoprotein Characterization of HDL_2 and HDL_3. By chemical analysis it was proved that the delipidation removed all lipids from the lipoproteins. Both chimpanzee apoproteins show a similar amino acid composition and the most significant differences to the human data are lower tryptophan and half-cystine values in the chimpanzee (Table VI). apoHDL_2 and apoHDL_3 were analyzed by polyacrylamide gel electrophoresis in a sodium dodecyl sulfate medium with and without the reducing agent β-mercaptoethanol (Figure 2). The peptide pattern of apoHDL_2 and apoHDL_3, obtained by densitometry of polyacrylamide gel, is given in Figure 3. The presence of a disulfide bridge in the fraction with a mol wt of 19,000 is established by following the per cent change of the peptide composition under the influence of β-mercaptoethanol. A similar peptide composition is obtained for both apolipoproteins. At least seven–eight peptides are separated. The major fraction with a mobility of human apoLp-Gln-I shows a mol wt of 29,000. The second major fraction composed of two bands on polyacrylamide gel electrophoresis has a mol wt of ~12,500. A fraction with a mol wt of 19,000 migrates with the same mobility as human apoLp-Gln-II. The minor components have molecular weights of, respectively, 10,000, 53,000, and 92,000.

COOH-Terminal Amino Acid Sequence. The release of amino acids from chimpanzee apoHDL_2 and apoHDL_3 as a function of enzymatic digestion time indicates that glutamine is a CO-

TABLE VI: Amino Acid Composition of Plasma High Density Lipoproteins.[a]

Amino Acid	mol/10^3 mol of Amino Acids			
	apoHDL_2		apoHDL_3	
	Chimp.	Human[b]	Chimp.	Human[c]
Lys	84	91	85	92
His	19	14	18	15
Arg	58	45	53	46
Trp	9	20	8	15
Asp	85	78	85	77
Thr	47	49	47	51
Ser	66	63	64	68
Glu	192	177	195	185
Pro	41	49	43	47
Gly	41	43	43	44
Ala	74	73	75	75
Half-Cys	3	8	3	7
Val	53	63	54	60
Met	20	11	19	15
Ile	7	7	6	8
Leu	143	138	140	134
Tyr	29	37	31	38
Phe	29	34	30	34

[a] Mean values of two determinations on two male chimpanzees. [b] Scanu *et al.* (1969). [c] Shore and Shore (1967).

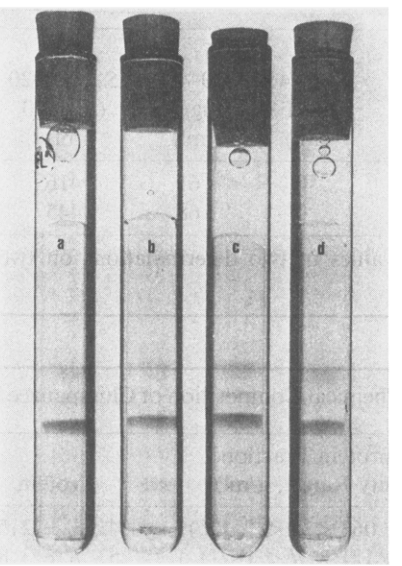

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of chimpanzee apoHDL_2 and apoHDL_3 with and without HSEtOH: (a) apoHDL_2 with HSEtOH; (b) apoHDL_3 with HSEtOH; (c) apoHDL_2; (d) apoHDL_3.

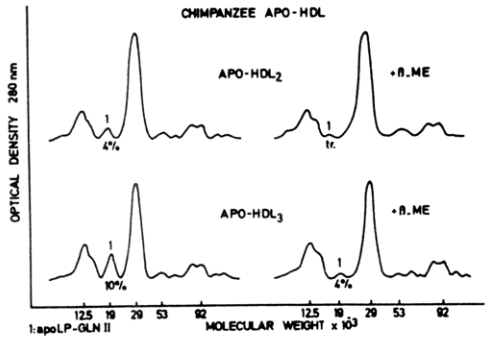

FIGURE 3: Densitometry of sodium dodecyl sulfate–polyacrylamide gels of chimpanzee apoHDL$_2$ and apoHDL$_3$.

OH-terminal residue in both apoHDL fractions (Figure 4a,b). The initial release of glutamine is higher than that of the other amino acids and liberation is almost complete after 10 min. Threonine is also rapidly released but complete liberation is not achieved after 60 min of digestion. Other amino acids present in significant amounts are asparagine and leucine. These amino acids are not C terminal but derived from the polypeptide chain.

NH_2-Terminal Amino Acids. Qualitative NH_2-terminal amino acid analysis of apoHDL$_2$ and apoHDL$_3$ by the dansylation method indicates aspartic acid as the major NH_2-terminal amino acid, although as for the human (Scanu *et al.*, 1972) there might be blocked NH_2 terminals present in the peptide mixture. Trace amounts of serine, threonine, and glutamic acid are detected in both apolipoproteins.

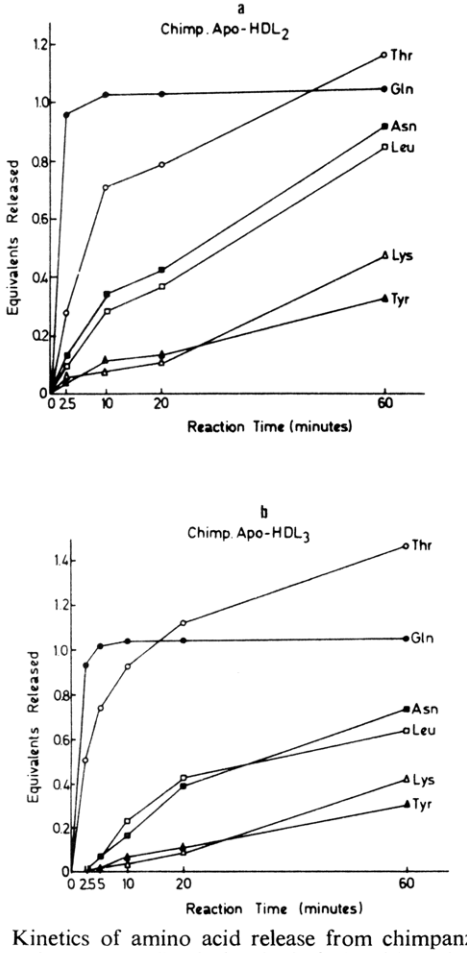

FIGURE 4: Kinetics of amino acid release from chimpanzee apoHDL$_2$ (a) and apoHDL$_3$ (b) during hydrolysis with carboxypeptidase A at 37°.

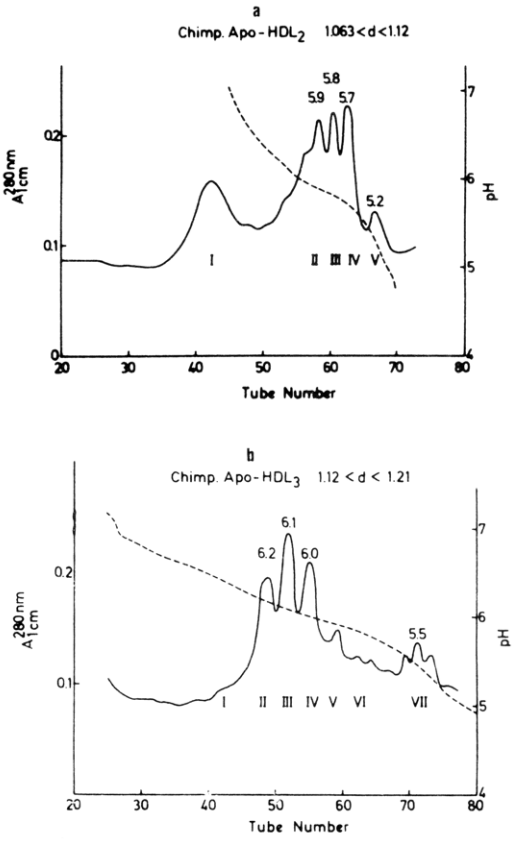

FIGURE 5: Optical density profile (–) and pH gradient (---) obtained by preparative isoelectric focusing of chimpanzee apoHDL$_2$ (a) and apoHDL$_3$ (b).

Isolation and Characterization of the Polypeptides of apoHDL$_2$ and apoHDL$_3$. ISOELECTRIC FOCUSING OF THE APOPROTEINS. After isoelectric focusing of apoHDL$_2$ and apoHDL$_3$ the patterns, shown in Figures 5a and 5b, are obtained. Four major components are fractionated in both lipoprotein subclasses and represented at least 95% of the protein applied to the column. Differences in p*I* values of the subfractions in apoHDL$_2$ and apoHDL$_3$ are not significant. The three major fractions (II, III, and IV) in apoHDL$_3$ with p*I* values around 6 show one major band on polyacrylamide gel electrophoresis in 8 M urea (Figure 6). Similar patterns were obtained for apoHDL$_2$. The fraction with a p*I* value around 5.5 is still heterogeneous on polyacrylamide gel electrophoresis. The amino acid composition of the main components in apoHDL$_2$ and apoHDL$_3$ are similar and the results for apoHDL$_3$ are given in Table VII. Similar results on human plasma high density

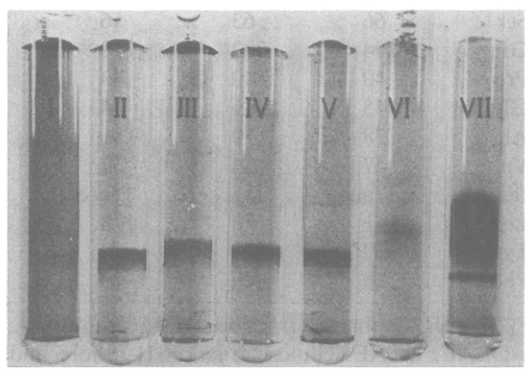

FIGURE 6: Polyacrylamide gel electrophoresis in 8 M urea of fractions isolated by isoelectric focusing of chimpanzee high density lipoproteins (HDL$_3$).

TABLE VII: Amino Acid Composition of Fractions Isolated by Isoelectric Focusing of Chimpanzee High Density Lipoproteins (HDL_3).[a]

Amino Acid	mol/10^3 mol of Amino Acids				
	apoHDL_3	II	III	IV	VII
Lys	88	86	94	92	96
His	19	21	22	23	11
Arg	55	69	73	74	31
Asp	88	94	88	87	86
Thr	48	42	43	42	64
Ser	66	66	66	63	69
Glu	201	194	191	195	190
Pro	44	40	43	41	50
Gly	44	45	40	37	49
Ala	78	78	79	78	72
Val	56	57	55	55	63
Ile	6	tr	2	2	16
Leu	144	151	151	157	124
Tyr	32	29	27	28	37
Phe	31	28	26	27	43

[a] Mean values of individual determinations of four runs. Trp, half-Cys, and Met are not determined.

apolipoproteins, fractionated by isoelectric focusing in the same manner as described above, were obtained (Blaton *et al.*, 1973). The amino acid composition of fraction II of human and of chimpanzee apoHDL_3 is compared with the human Sephadex fraction III according to Scanu *et al.* (1969), and with fraction II separated on DEAE-cellulose according to Shore and Shore (1968) (Table VIII). No differences are observed between human and chimpanzee fraction II. The amino acid composition is furthermore similar to the data of human apoLp-Gln-I. With our isoelectric focusing methodology we were able to isolate apoLp-Gln-I from human or chimpanzee high density apolipoproteins. Human and chimpanzee fraction VII with a p*I* value around 5.5 has an amino acid composition comparable with human apoLp-Gln-II isolated by gel filtration and DEAE-cellulose chromatography. Major differences are the presence of histidine and arginine, due to the presence of a component with mol wt 29,000 as shown by polyacrylamide gel electrophoresis.

TABLE VIII: Amino Acid Composition of the Major Apoprotein in Human and in Chimpanzee High Density Lipoproteins.

Amino Acid	apoHDL_3[c] F_{II}-E		apoHDL_3[a] F_{III}-Seph	apo-HDL_3[b] F_{II}-DEAE
	Chimp.	Human	Human	Human
Lys	86[d]	86	81	82
His	21	22	24	24
Arg	69	66	71	71
Asp	94	87	88	103
Thr	42	44	46	40
Ser	66	63	56	66
Glu	194	202	178	190
Pro	40	40	43	39
Gly	45	45	44	46
Ala	78	79	81	79
Val	57	54	57	53
Ile	Trace	Trace	Trace	0
Leu	151	156	178	155
Tyr	29	29	27	29
Phe	28	26	25	24

[a] F_{III}-Seph, Sephadex fraction III (Scanu *et al.*, 1969). [b] F_{II}-DEAE, DEAE-cellulose fraction II (Shore and Shore, 1968). [c] F_{II}-E, electrofocusing fraction (II). [d] Moles/10^3 moles of amino acid.

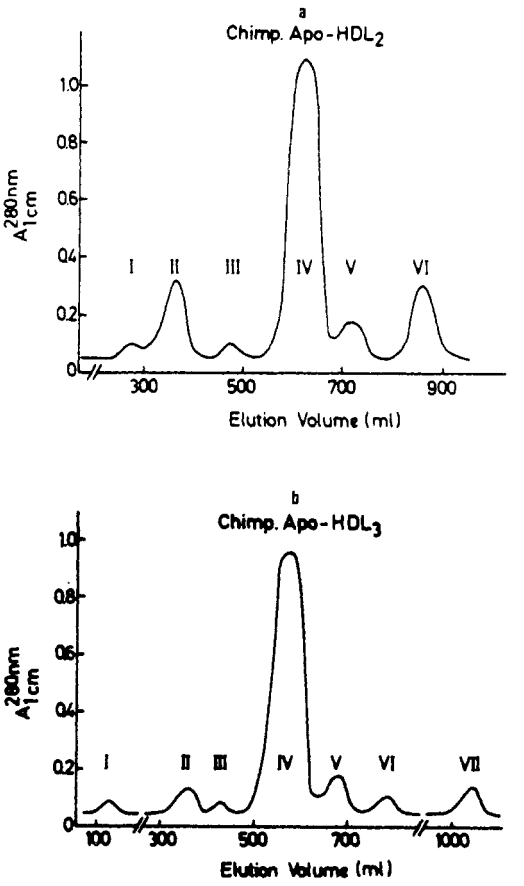

FIGURE 7: Gel filtration of chimpanzee apoHDL_2 (a) and apoHDL_3 (b) on Sephadex G-200.

FRACTIONATION OF APOPROTEINS BY GEL FILTRATION ON SEPHADEX G-200. Chimpanzee apoHDL_2 and apoHDL_3 were fractionated into six and seven components, respectively (Figure 7a,b). The approximate relative amounts of each component are estimated by ultraviolet (uv) absorption at 280 nm and are given in Table IX. Polyacrylamide gel electrophoreses in sodium dodecyl sulfate of the apolipoproteins and of the Sephadex fractions of apoHDL_3 are shown schematically in Figure 8. The major component of both HDL subclasses

TABLE IX: Percentages of Total Area under All Curves Obtained after Gel Filtration of Chimpanzee Plasma High Density Lipoproteins.

	Fraction						
	I	II	III	IV	V	VI	VII
apoHDL_2	2.4	14.7	2.8	59	6.8	14.3	
apoHDL_3	2.9	5.9	2.9	67.5	9.6	4.5	6.7

FIGURE 8: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fractions isolated by gel filtration of chimpanzee apoHDL$_3$.

(fraction IV) with the same mobility and molecular weight as human apoLp-Gln-I is slightly contaminated with peptides of higher molecular weight. Sephadex fraction V contains the peptide with a mobility of human apoLp-Gln-II, has a mol wt of 19,000, and is contaminated with apoLp-Gln-I. Although there are differences in the relative amounts of the various Sephadex fractions between chimpanzee apoHDL$_2$ and apoHDL$_3$, no differences in the amino acid composition are observed. The results for the Sephadex fractions of chimpanzee apoHDL$_3$ are given in Table X. The major component (fraction IV) has an amino acid composition similar to human apoLp-Gln-I. The amino acid composition of fraction V differs from human apoLp-Gln-II mainly in the histidine and arginine content, which can be explained by a contamination of Sephadex fraction IV as shown by polyacrylamide gel electrophoresis.

TABLE X: Amino Acid Composition of Fractions Isolated by Gel Filtration of Chimpanzee Plasma High Density Lipoproteins (HDL$_3$).[a]

Amino Acid	mol/10^3 mol of Amino Acids					
	apo-HDL$_3$	II	III	IV	V	VI
Lys	83	96	80	86	97	91
His	19	16	17	19	8	5
Arg	55	61	56	64	18	17
Asp	88	94	89	88	82	87
Thr	48	53	47	42	72	75
Ser	66	80	90	63	82	91
Glu	201	172	182	208	191	187
Pro	44	33	35	44	51	49
Gly	44	67	71	46	56	55
Ala	78	70	76	79	72	77
Val	56	55	50	54	61	61
Ile	6	15	12	2	15	14
Leu	144	130	129	154	111	101
Tyr	32	26	25	26	38	39
Phe	31	32	31	25	46	51

[a] Mean values of individual determinations of two runs. Trp, half-Cys, and Met are not determined.

Discussion

In previous work we have shown that the chemical composition of chimpanzee lipoproteins closely parallels that of human lipoproteins (Peeters and Blaton, 1972; Howard *et al.*, 1972; Blaton *et al.*, 1974). Therefore, chimpanzee plasma lipoproteins were fractionated by ultracentrifugation at the same densities employed for human lipoproteins.

The lipid and fatty acid compositions of the chimpanzee high density lipoproteins are similar to the lipid composition of the human high density lipoproteins. The phospholipid spectrum of both subclasses is quite similar. In comparison to the phospholipid distribution in human HDL the main difference is the higher sphingomyelin content in human HDL$_2$ over human HDL$_3$, which is not found in the chimpanzee. In spite of the small differences in lipid composition between chimpanzee HDL$_2$ and HDL$_3$, we decided to proceed with the study of each HDL subclass separately. However, the presence and composition of chimpanzee very high density lipoprotein were not investigated.

An identical amino acid composition is obtained for both subclasses. When compared to human apoHDL$_2$ and apoHDL$_3$ lower tryptophan and half-cystine contents are observed. Several peptides with distinctive mobilities are detected by polyacrylamide gel electrophoresis. The major fraction, present in apoHDL$_2$ and apoHDL$_3$ with a mol wt of ~29,000, exhibits a mobility corresponding to human apoLp-Gln-I. An apoprotein with a mol wt of 19,000 and with the same mobility as human apoLp-Gln-II is present in both HDL subclasses. The presence of disulfide linkages similar to human apoLp-Gln-II (Brewer *et al.*, 1972; Scanu *et al.*, 1971) was established using β-mercaptoethanol.

The data from carboxypeptidase A hydrolysis suggest that both apolipoproteins have the COOH-terminal sequence Leu-Thr-Gln consistent with the results of human apoHDL and apoLp-Gln-I (Kostner and Alaupovic, 1971; Edelstein *et al.*, 1972; Lux and John, 1972). As in the chimpanzee, in the human high density lipoproteins aspartic acid is the major NH_2-terminal amino acid.

The heterogeneity of the protein moiety is established by isoelectric focusing and gel filtration. After isoelectric focusing at least three main fractions show a near-identical amino acid profile to human apoLp-Gln-I and contain one major apoprotein with a mol wt of 29,000 and the mobility of human apoLp-Gln-I. The heterogeneity of human apoLp-Gln-I is reported by several authors. Shore and Shore (1969) first described the presence of three polymorphic forms in the major protein of human apoHDL$_2$ referred to as R_1-Thr, R_2-Thr, and R_3-Thr. Edelstein *et al.* (1972) clearly demonstrated the heterogeneity of human apoLp-Gln-I. DEAE-cellulose chromatography of Sephadex fraction III results in two major fractions slightly different in amino acid composition. Furthermore, Albers *et al.* (1971) separated Sephadex fraction III of human apoHDL$_2$ by isoelectric focusing into five components with different isoelectric points. Our results of isoelectric focusing prove also the heterogeneity of the chimpanzee mol wt 29,000 fraction, either due to polymorphism or to small differences in chemical composition.

By Sephadex chromatography and by isoelectric focusing we isolated a protein component with a mol wt of 19,000 with a disulfide bridge and with the same electrophoretic mobility as for human apoLp-Gln-II. The amino acid composition shows major differences in histidine and arginine content in comparison with human apoLp-Gln-II, which may be due to contamination with other components as demonstrated by polyacrylamide gel electrophoresis.

These results suggest certain conclusions about the protein structure of chimpanzee high density lipoprotein. The data are entirely consistent with the presence of a major apoprotein of mol wt 29,000 in chimpanzee HDL similar to the human apoLp-Gln-I. As in man the heterogeneity of the chimpanzee mol wt 29,000 fraction has been established. The presence in chimpanzee HDL of a second apoprotein with a mol wt of 19,000 similar to apoLp-Gln-II in the human HDL has been shown. The chimpanzee high density lipoproteins appear to differ from the human high density lipoproteins by a significant de-

crease of the relative content of the mol wt 19,000 fraction. This observation may be of considerable interest for elucidation of the structure and function of plasma lipoproteins. The close similarities in the lipoprotein data from human and chimpanzee indicate that these animals are valuable models in experimental atherosclerosis.

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Dynamics of Pyrene Fluorescence in *Escherichia coli* Membrane Vesicles†

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ABSTRACT: Pyrene was incorporated into both inner and outer membrane vesicles of *Escherichia coli* and excited by means of a 10-nsec pulse of ultraviolet light from a Q-switched ruby laser. The decay time of the excited state is subsequently measured by monitoring the fluorescence at 400 nm. The half-lives of the pyrene excited states are 85 and 125 nsec at 25° in the inner and outer membrane vesicles, respectively. Substantial differences in behavior between the inner and outer membranes are also illustrated by the temperature dependence of both the lifetime of the pyrene excited state and the absolute yield of pyrene fluorescence. Two structural transitions were observed for the outer membrane vesicles over the temperature range 0–35°. However, the inner membrane vesicles do not show phase transitions over the same temperature range. The rate of decay of the pyrene excited state increases in the presence of added quenchers such as O_2, iodide, and CH_3NO_2 which dissolve primarily in the water phase. The quenching experiments show that the inner membrane vesicles are more permeable than the outer membrane vesicles to these probes. Fluorescence polarization measurements of the microviscosity of the inner and outer vesicles give data which are in substantial agreement with the laser experiments.

Lipid dispersions have been extensively used as model systems to gain an insight into the functions of membranes, and in particular, specialized techniques have been developed to ascertain the conformational state of the membrane components (Cogan *et al.*, 1973; Hubbell and McConnell, 1968; Ladbrooke and Chapman, 1969). The technique of fluorescence polarization has been particularly useful in measuring the fluidity of the membrane systems (Cogan *et al.*, 1973), and recently, an application of the fluorescent probe technique has proved successful in analyzing several features of the factors controlling the permeability of simple micelles (Wallace and Thomas, 1973; Grätzel and Thomas, 1973) and phospholipid dispersions (Cheng and Thomas, 1974). In this latter technique the fluorescent probe such as pyrene is dissolved in the micelle or membrane and excited by means of a pulsed laser. The decay rate of the excited state is subsequently measured either by rapid absorption or emission spectroscopy. The aqueous phase of the system contains a suitable quencher which may penetrate the membrane and react with the excited state of the probe molecules. The rate of the quenching reaction or decay time of the excited state may be used as a measure of the ease of entry of the quenchers into the micelle or membrane and it is possible to discuss the factors which affect the movement of the quencher into the micelle.

In this paper, we report the application of the pulsed laser technique to *Escherichia coli* membrane systems. The microviscosity, the existence of phase transitions, and the structural organization in the hydrocarbon region of phospholipids have been investigated.

Experimental Section

Materials

Media for culture growth was prepared as follows: proteose peptone No. 3 (Difco) 1.0%, beef extract (Difco) 0.1%, and NaCl 0.5%. Galactose was obtained from General Biochemicals Inc. Na_2EDTA was from J. T. Baker Co. Lysozyme was three-times recrystallized from Calbiochem. Sucrose was obtained from Matheson Scientific.

Pure grade pyrene from Fluka was further purified by passage through a silica gel column in cyclohexane and then crystallized from the solvent. 2-Methylanthracene (Aldrich) was crystallized from ethanol. Laboratory distilled water from a Barnsted Still was redistilled from $KMnO_4$. The criterion of purity of the distilled water has been described previously (Cheng and Thomas, 1974), impurity levels are below 10^{-5} mol/l.

Methods

Bacterial Growth and Membrane Preparation. A mutant of *Escherichia coli* 0111:B_4, designated J-5, was used for all experiments. This strain lacks UDP-galactose 4-epimerase and therefore cannot use galactose as a carbon source (Elbein and Heath, 1965). When galactose is added to the medium, it is used solely for the synthesis of lipopolysaccharide. Thus, in the absence of galactose an incomplete lipopolysaccharide is synthesized which lacks a large portion of carbohydrate (Elbein and Heath, 1965; Levy and Leive, 1968). Cells were routinely grown in the presence of 2.5 mM galactose at 37° unless stated otherwise.

Inner and outer membrane vesicles were prepared from spheroplasts by osmotic lysis and isopycnic sucrose gradients

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