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STRUCTURE AND METABOLISM OF SERUM HIGH DENSITY LIPOPROTEINS

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STRUCTURE AND METABOLISM OF SERUM HIGH DENSITY LIPOPROTEINS

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CHAPTER I

INTRODUCTION

The serum or plasma of all vertebrates contains a variety of lipid compounds which include free and esterified cholesterol, mono-, di-, and tri-glycerides, nonesterified or "free" fatty acids, and a number of phospholipids or phosphatides. These lipids per se are essentially water-insoluble. In the serum they are bound to specific proteins and have been discribed as lipoproteins, which is the means by which lipids are generally solubilized. By corollary, the protein moieties of the different lipoproteins also act as vehicles for the transport of lipids in the serum.

The earliest suggestions regarding the existence of lipid-protein associations in the serum were advanced in the first two decades of this century by Nerking (1), Haslam (2), Chick (3), and Bang (4) who showed that phospholipids and cholesterol were present in certain sub-fractions of euglobulin. In 1929 Macheboeuf (5) first isolated a horse serum lipoprotein fraction which contained constant and characteristic amounts of cholesterol and phospholipids. Subsequently, the serum lipoproteins of other vertebrates and man were investigated by a variety of reliable techniques including salting-out with ammonium sulfate (5,6),

Cohn fractionation (7), moving boundary (8) and zone electrophoresis using paper and starch gel (9,10,11,12,13,14,15), precipitation by high molecular substances (16,17,18,19), immunochemical (20), turbidimetric (21), ultracentrifugal (22,23,24,25), and chromatographic (26) procedures. In particular the ultracentrifugal technique was exceedingly useful for the separation and identification of serum lipoproteins (27,28,29,30,31, 32,33).

Serum lipoproteins were found to be a heterogeneous group of compounds which differed in density and composition (34,35), sedimentation (36,37), and electrophoretic mobility (10).

The research work on serum lipoproteins suffered from its inception from the lack of a systematized classification and inevitably resulted in different, but fortunately interrelated, methods of classification as shown in Table I. The Committee on Lipid and Lipoprotein Nomenclature of the American Society for the Study of Atherosclerosis (38) recommended in 1956 that lipoprotein fractions be described according to the method of separation. Therefore, two major groups could be identified by density gradient studies. Those of density less than 1.063 g per ml comprised the low density lipoproteins, while those of density greater than 1.063 g per ml represented the high density lipoproteins. The former included several subfractions, all of which were distinctly different from the high density ones in several characteristics, including a lower protein content and a higher cholesterol/ phospholipid ratio (39,40).

The high density lipoproteins are composed of the alpha lipoproteins (αLP, d = 1.063-1.210 g per ml) i.e. HDL_2 and HDL_3. Lipo-

TABLE I

NOMENCLATURE OF LIPOPROTEIN FRACTIONS

Density g/ml	Cohn Fractionation (7)	S_f (36)	-S (37)	Starch Electrophoresis (10)	Other (56)
0.960-1.006	I + III	20-400	> 70	α_2 or 'origin' lipid	Chylomicrons and very low density LP
1.006-1.019	I + III	12-20	40-70	β_1	Low density βLP
1.019-1.063	I + III	0-12	25-40	β_1	β Lipoprotein
1.063-1.125	IV + V	HDL_2	20-25	α_1	α Lipoprotein (αLP)
1.125-1.210	IV + V	HDL_3	1-10	α_1	α Lipoprotein (αLP)
> 1.210	-	-	-	α_1	Very high density LP (VHDLP)

proteins of density greater than 1.210 g per ml include the very high density lipoproteins (VHDLP) and lipalbumin, the albumin bound fatty acids (41).

The term very high density lipoproteins is used to designate lipoproteins other than lipalbumin which sediment at 105,000 x g after 44 hours of centrifugation in a solvent of density 1.21 g per ml.

All subsequent discussion will deal mainly with human high density lipoproteins, unless otherwise specified.

The high density lipoproteins, excluding lipalbumin, have an electrophoretic mobility similar to that of α_1 serum globulin (42).

Studies by several researchers (43,44,45,46,47,48) using precipitation, absorption and agar diffusion techniques with heterologous antigenic sera, indicate that the high density lipoproteins are immunochemically distinct from other serum proteins and particularly from their lower density counterparts.

The amount of high density lipoprotein reported in human serum varies with the methods used with a mean value estimated between 250 and 450 mg per 100 ml serum (35,49,50). These reports also show pronounced sex differences in the amounts of high density lipoproteins which are significantly higher in the female than in the male at every age from 17 to 65 years. The clinical importance of this significant difference between sexes is not clear.

Of the high density lipoproteins, the alpha lipoproteins (αLP) have been extensively investigated. They are soluble in water and electrolytic solutions (51) and are relatively more stable to dialysis than the low density lipoproteins (52). The molecular

weights range between 165,000 and 450,000 (52,53,54). The αLP molecules are assumed to be ellipsoid in shape (53) to account for the intrinsic viscosity, 0.066, and the frictional ratio data. The latest calculations by Vandenheuvel (55) give a common short axis dimension of about 60 Å and long axis dimensions of 290 and 171 Å, which correspond to the molecular weights reported by Hazelwood (54) as well as permitting the lipids to be packed in correct proportion within a protein shell 10 Å thick and having a 15.7 $Å^2$ mean area per residue. These data are not totally reliable, however, because the actual geometric form of the αLP is uncertain.

Normally, the high density lipoproteins contain about 26 and 47 per cent of the total serum cholesterol and phospholipids, respectively with a cholesterol-phospholipid ratio of approximately 0.45 (56). The per cent composition of serum αLP is reported by Bragdon et al. (40) as follows:

	%
Protein	46.4
Triglycerides	8.1
Phospholipids	26.1
Free Cholesterol	2.0
Esterified Cholesterol	17.4

The αLP have been subdivided into two fractions of density, 1.080 and 1.15 g per ml (36), the latter containing a higher protein content (34,39).

There are small amounts, if any, of non-esterified fatty acids bound to αLP. Data on αLP fatty acids are difffcult to interpret because the lengthy procedures for the isolation and purification of αLP, as described later, lead to hydrolysis of triglycerides, and, therefore, elevated values for the fatty acids.

The phosphatide composition of αLP is similar to that of whole serum except for a higher percentage of lysolecithin and a lower percentage of sphingomyelin (57,58):

	Per Cent of Whole Serum Lipid P			
	Cephalin	Lecithin	Lysolecithin	Sphingomyelin
Whole Serum	4.8	68.2	8.0	18.9
>1.063 g/ml Lipoproteins	5.2	69.9	11.2	13.6

The fatty acid composition of the cholesterol ester, phospholipid and glyceride moieties of αLP, studied by gas-liquid chromatography (58.59), is similar to that of serum and β-lipoproteins. The principal fatty acids of cholesterol esters are linoleic, oleic and palmitic; those of phospholipids are palmitic, oleic, linoleic and arachidonic; and those of glycerides are oleic, palmitic and linoleic in order of decreasing amounts.

The protein moiety (αP) obtained by delipidation of αLP with ethanol and ether at -25°C was found to be homogeneous on the basis of physicochemical criteria which were applied for its characterization (60). There were no identifiable free -SH groups or cysteine (60,61) contrary to the earlier findings of Shore and Shore (62) in a study on the amino acid composition of the same protein. However, Scanu and Hughes (63) have found that the amino acid composition of several subfractions of αLP was similar and this was cited as supporting evidence for the homogeneity of the αP. The carboxyl and amino-terminal amino acids of αLP protein were shown to be threonine and

aspartic acid, respectively (53,64,65), and were present in a 1:1 ratio assuming a molecular weight of 75,000 for αP.

Moving boundary electrophoresis of the delipidized αLP revealed the presence of one peak with a rate of migration equal to 5.00×10^{-5} sq.cm./v./sec. (60).

The sedimentation coefficient, also reported by Scanu et al. (60), was $s_{20,w}$ 4.11 and the molecular weight about 75,000. Recently, Shore and Shore (66) obtained a subunit of αP by the addition of 0.08% sodium dodecyl sulfate to the protein solution. The sedimentation coefficient of the subunit was $s_{20,w}$ 2.3 to 2.6 with a molecular weight of 38,000 ± 1000. On the basis of the latter weight, one mole of amino-terminal aspartic acid was present per mole αP. In view of these findings Shore and Shore suggested that Scanu et al. (60) had isolated a dimer form of the protein.

The αP contains about 0.5 per cent hexose in the form of galactose and mannose, and 0.35 per cent hexosamine as glucosamine on dry weight basis (60).

The recombination of lipid-free protein with lipids in vitro (67) and in vivo (68) shows that αP retains its functional capacity to bind lipids. Furthermore, the biological half-time of the protein, labeled with I^{131} which is about 4 days, is similar to intact αLP (45,63,69). The serum αLP of dog (68) and rabbit (70) also have short half-times.

The αP is also capable of recombination with chylomicrons in vitro (67). The data on the carboxyl and amino-terminal amino acids (53,64), fingerprinting (71,72), sonic oscillation experiments

(73) and exchange of I^{131}-labeled chylomicron protein *in vitro* (74) indicate that one of the proteins present in the chylomicrons has the same characteristics as that of the αLP protein.

The αLP phospholipids exchange freely with lower density lipoproteins (75,76,77,78). On the other hand, *in vitro* (79) and *in vivo* (80) studies indicate that free cholesterol in serum, chylomicra and red blood corpuscles is readily exchangeable, with partial equilibration occurring within minutes. Turnover studies which involve αLP with labeled cholesterol or phospholipid, therefore, have not been popular because of these facts which render metabolic interpretations dubious.

The synthesis of αLP by rat-liver slices (81) and perfused rat liver (82) has been demonstrated, and the rate of synthesis of the protein is independent of cholesterol synthesis (82). No data is available on the synthesis of lipoprotein by tissues other than the liver although the small intestine is thought to be an active site for αP biosynthesis (72).

In vivo studies (69) indicate that the protein moieties of high and low density lipoproteins are metabolically distinct which is contrary to the interrelationships between exchangeable lipid components, as described earlier.

The serum levels of αLP are decreased in primary or secondary hyperlipidemic disease states (56) and by nutritional and hormonal factors (83). There is no evidence, however, that decreased serum αLP level *per se* is harmful.

The very high density lipoproteins (VHDLP) have not been

isolated yet as a chemical entity. The 1.210 infranatant of human serum contains 8-15% of the total serum lipid phosphorus but essentially no cholesterol (34,35,40,56.84). In the dog, chicken, rabbit and rat, the VHDLP contain 7, 9, 23 and 27-40% of the total serum phospholipids with no cholesterol (34,85). Turner et al. (86) have demonstrated the presence of a phospholipid-protein complex, free of cholesterol and accounting for about 60% of the total serum phospholipids. They have reported that, following the intravenous administration of P^{32} to human subjects, the specific activity is most pronounced in the phospholipid-protein complex (87); this complex was never isolated and purified.

The VHDLP were later described as a phosphorus-containing peptide (40,84,88). Kunkel and Trautman (42), who correlated the ultracentrifugal and electrophoretic properties of human serum lipoproteins by using starch block or a polyvinylresin (Geon 400x65), showed that the VHDLP are not specifically separated from αLP by electrophoresis. Phillips (89) showed by a silicic acid column chromatographic technique that the VHDLP contain phosphatides with the following distribution:

	Mean of Total Phosphorus %
Cephalin	6
Sphingomyelin	14
Lecithin	25
Lysolecithin	56

The lysolecithin in the VHDLP accounts for about 50 per cent of the total serum lysolecithin (89).

As yet, no metabolic role can be ascribed to the VHDLP.

The purpose of this dissertation is two-fold:

(A) Chemical and Physico-chemical Studies of Human and Horse αLP;

1. To compare the physico-chemical characteristics of human and horse serum lipids and αP;

2. to elucidate the behavior of αLP protein in electrolytic solutions containing urea, sodium dodecyl sulfate or succinic anhydride with regard to association-dissociation phenomena; and,

3. to identify the protein moiety of VHDLP;

(B) Recombination and Metabolic Study;

1. To demonstrate <u>in vitro</u> recombination of αP with serum lipids under a variety of conditions;

2. to study the time course of the plasma radioactivity following the injection of I^{131}-labeled αLP or αP in normal human subjects and patients with hyperlipidemia; and,

3. to show the excretion of I^{131}-labeled αLP in the urine of a patient with the nephrotic syndrome, following the intravenous administration of labeled αLP.

CHAPTER II

MATERIALS, SUBJECTS, AND METHODS

Materials

Human Serum or Plasma

Venous blood was obtained from the anticubital veins of young and apparently healthy fasting male and female individuals who were either medical students or employees of the University of Oklahoma Medical Center.

To obtain plasma, blood was collected in plastic containers (Pliapak, Panheparin 2115, Abbott) with heparin sodium as the anticoagulant. Plasma was then separated and processed within two hours after the venipuncture.

Serum was also obtained from blood collected from fasting individuals and allowed to clot in evacuated empty plasma containers (Abbott). Sera from several donors of the same sex were pooled in batches of 300 to 600 ml prior to ultracentrifugation.

Horse Serum

Blood was obtained from the jugular veins of 18 normal female Shetland ponies belonging to Mr. H.O. Pope and from an American saddle-bred gelding and a standard-bred trotter mare owned by Mr. M.A. McDearmon.

The blood was collected in empty, plasma containers (Abbott) by local D.V.M. Drs. T.G. Sewell of Guthrie, Oklahoma, and J.M. Brown of Oklahoma City.

Trypsin (Pancreas)

Grade A, activity 6.0 units per mg (Kunitz) was purchased from C.F. Boehringer & Soehne, Gmbtt, Manheim.

α-Chymotrypsin (Bovine Pancreas)

Grade A, crystalline, salt-free, activity 7.8 units per mg (Wu & Laskowski) was purchased from C.F. Boehringer & Soehne, Gmbtt, Manheim.

I^{131} and Ioresin

Both sterile sodium radio-iodide (I^{131}) solution and the anion exchange resin, Ioresin, were purchased from Abbott Laboratories, Oak Ridge, Tennessee, U.S.A.

Subjects

Three, healthy, male volunteers with normal serum lipid levels were utilized as control subjects. Their ages ranged between 26 and 56 years. Subject P.G. had idiopathic epilepsy since childhood but well controlled with dilantin and phenobarbital.

Six patients, four with primary hyperglyceridemia, one with idiopathic familial hypercholesterolemia, and one with hyperlipidemia of nephrosis associated with subacute glomerulonephritis, ranging in age from 14 to 65 years, were studied. Subjects S.H. and H.H. were siblings with familial hyperchylomicronemia, heparin-unresponsive and

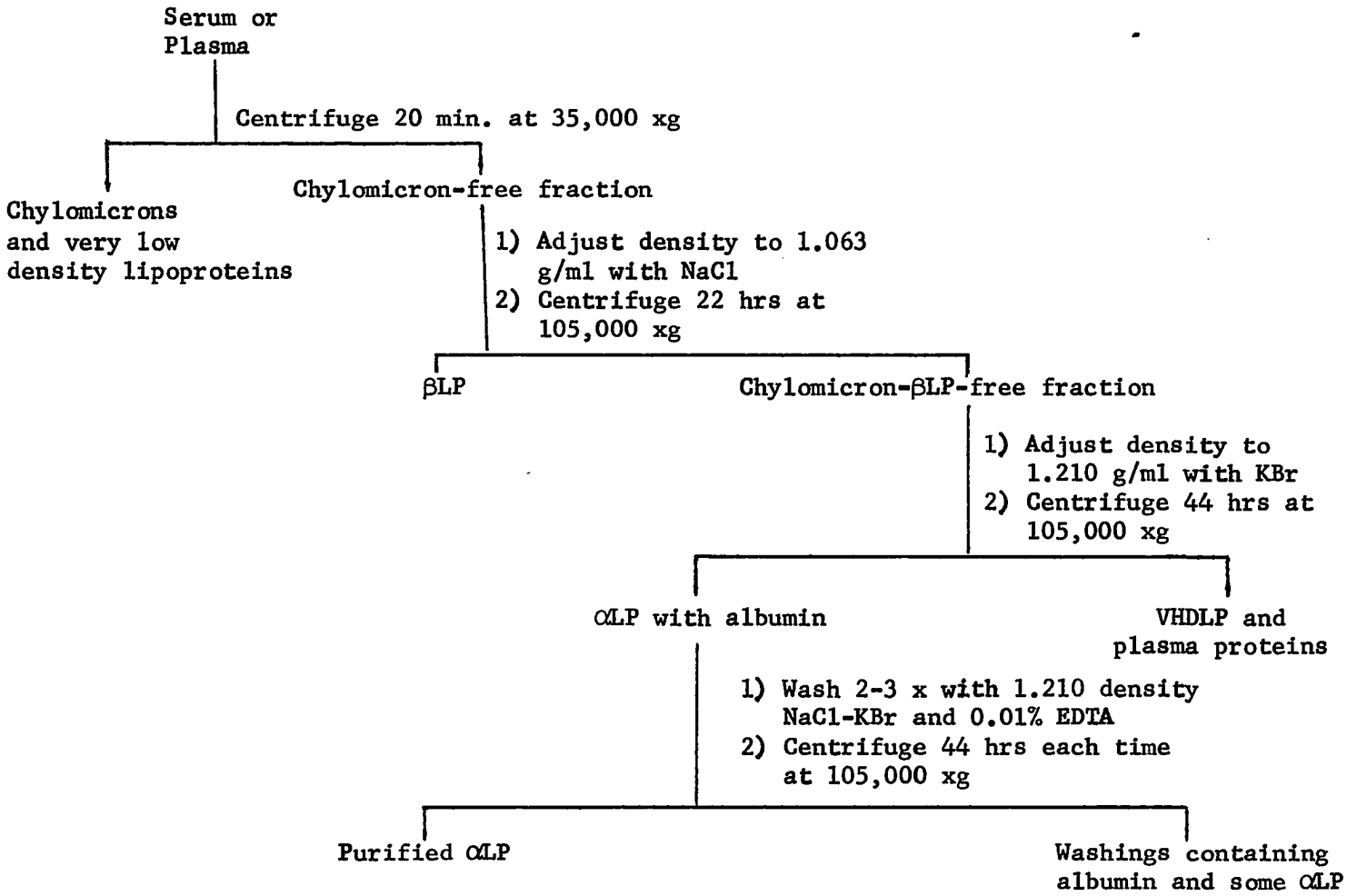
fat-accentuated hyperglyceridemia with little or no hypercholesterolemia or hyperphospholipidemia (90,91,92). Hepatosplenomegaly was present and a history was obtained of "abdominal crises" in both subjects, but there were no xanthomata, xantholasmata or arcus senilis. Subjects O.C. and O.W. had a dietary carbohydrate-accentuated and heparin-responsive (91, 92) hyperglyceridemia with evidence of old cerebrovascular accidents and roentgenologic evidence of calcification of the abdominal aorta. The patient with idiopathic hypercholesterolemia, J.C., had xanthomata and xantholasmata but no arcus senilis. Finally, the patient with the nephrotic syndrome, M.S., was hospitalized at the Children's Memorial Hospital, Oklahoma City, with the classical symptoms and signs of nephrotic syndrome. The kidney biopsy from this patient also showed findings of subacute glomerulonephritis. Of these 6 patients, S.H. and M.S. were the only female individuals.

Methods

Isolation and Purification of αLP

The isolation procedure, a modification of the methods of Gofman et al. (93) and Lewis et al. (33), is depicted in Figure I. Fresh serum was subjected to ultracentrifugation in Spinco cellulose tubes (1/2 x 3 1/2 in), at 0°C, in the No. 40 rotors of three Model L Spinco or Beckman ultracentrifuges. The chylomicrons and very low density lipoproteins were separated at 35,000 x g for 20 minutes. This step could be omitted when the procedure was applied only for the isolation of αLP. The density was then adjusted to 1.063 g per ml with NaCl and lipoproteins of density less than 1.063 g per ml were

Figure 1 - Isolation and Purification of αLP



separated at 105,000 x g for 22 or 17 hours. The solvent density was increased to 1.210 g per ml with potassium bromide and the αLP separated at 105,000 x g for 44 hours. The αLP were purified 2 or 3 times to remove traces of albumin by washing with 1.210 density sodium chloride-potassium bromide solution containing 0.01% EDTA and recentrifuging after each wash for 22 or 44 hours.

In all fractions centrifugation resulted in the flotation of lipoproteins (supernatant) of density less than the medium, while lipoproteins of density greater than the solvent sedimented to the bottom (infranatant) with a colorless zone remaining in between. The supernatant fraction was separated from the infranatant by slicing in the center of the clear zone using a Spinco tube slicer. The very high density lipoproteins sedimented along with all serum proteins in the 1.210 infranatant fraction.

Delipidation of αLP

The ultracentrifugally separated and purified αLP solution was dialyzed against 0.15 M sodium chloride solution at 1°C for two days with changes of the NaCl solution twice daily. Subsequently, lipid extraction was performed according to the method described by Scanu, Lewis and Bumpus (65) in a cold chamber at -30°C. One part of αLP solution was transferred into a flask containing 30 parts of redistilled ethanol and peroxide-free ether (3:1 v/v) previously cooled to -30°C. Gentle shaking performed at 15-minute intervals for 2 to 3 hours was followed by filtration (Whatman Filter Paper 41H) of the material in the same chamber and washing the precipitate with peroxide-free ether

The filter paper with the protein precipitate was transferred next to a modified Soxhlet apparatus that provided continuous extraction of any remaining lipid at -30°C, for 24 hours, with peroxide-free ether as the solvent. The protein remaining on the filter paper was placed over Drierite in an evacuated dessicator for 6 hours at room temperature and then stored in the refrigerator.

The alcohol-ether and ether extracts were evaporated to dryness and the lipids and protein (none present) contents determined.

A sample of the dry, delipidized ŒLP was analyzed for nitrogen content by the Kjeldahl method (94) and for any remaining lipid which could be extracted with Bloor's ethanol-diethyl ether (3:1 v/v).

The protein values were calculated by multiplying the nitrogen values by a factor of 6.25.

Lipid and Lipoprotein Determinations

Total and free cholesterol were determined by the method of Schoenheimer and Sperry (95), lipid phosphorus by the method of Fiske and Subbarow (96), triglycerides by the method of Van Handel and Zilversmit (97) and non-esterified fatty acids by the method of Dole (98).

Four classes of lipoproteins, namely, <1.006, 1.006-1.019, 1.019-1.063 and >1.063 g per ml, were partitioned by ultracentrifugation by a modification (99) of the method of Havel, Eder and Bragdon (35). The samples were adjusted to densities of 1.006, 1.019 and 1.063 g per ml using sodium sulfate solutions of varying densities and subsequently centrifuged in polyethelene tubes (1/2 x 3 1/2 in) at

105,000 x g and 0°C for 22 hours in No. 40 rotor of the Model L Spinco or Beckman ultracentrifuges. The tubes were subjected next to quick freezing at -70°C in a dry ice-acetone bath and sawed in the middle of the colorless zone into supernatant and infranatant fractions using a self-powered jig-saw (Sears, Roebuck and Co.). The two fractions were mixed with isopropanol, made up to a standard volume and filtered. The total cholesterol was determined by the auto-analyzer using the procedure of Zlatkis, Zak and Boyle (100) and the lipid phosphorus determined according to foregoing method (96).

Phosphatide Analysis by Silicic Acid Chromatography

For the lipid extraction, one volume of whole serum or αLP was extracted with twenty volumes of redistilled chloroform-methanol (1:1 v/v) according to a modification of the Sperry procedure (101).

The method of Marinetti and his associates (102) was used for silicic acid column and silicic acid impregnated paper chromatography. The silicic acid (Mallinckrodt AR, 100-250 mesh) was standardized according to the procedure by Horning et al. (103) and contained less than 10% moisture. A sample containing approximately 500-750 ug of lipid phosphorus was applied onto a 10-gram column (2.5 cm diameter). Neutral lipids were first eluted with chloroform. The phospholipids were next eluted in four fractions, using about 125 ml of each of 20%, 30% and 50% methanol in chloroform and finally absolute methanol. The lipid phosphorus in the fractions was analyzed; the recoveries were over 90%. Samples were applied in triplicates and the paper developed with diisobutylketone:acetic acid:water (40:25:5) at 20°C for about 18

hours. The papers were then dried; one section of the strip of paper stained with Rhodamine 6G and corresponding spots analyzed for phosphorus (104). The results of phosphatide analyses were reported as per cent of total phosphorus eluted from the column.

I^{131} Labeling of αLP and αP

A modification (105) of the method of Boursnell et al. (106) was used for labeling αLP and αP (delipidized αLP). The radioiodinated protein was passed twice through an anion exchange resin, Ioresin (Abbott), to remove the free iodine. The number of iodine atoms per protein molecule varied between 1:1 and 3:1 (105) with a concentration of 3 mg protein and 10 to 25 uc of I^{131} per ml.

The labeled αLP-I^{131} and αP-I^{131} were checked by agar and starch gel electrophoresis, as described below, and showed the same mobilities as their unlabeled counterparts. When the protein moiety was precipitated quantitatively with 50 per cent trichloroacetic acid, more than 95% of the total radioactivity was recovered in the precipitate. Scanu and Hughes (72) reported that 5 to 8 per cent of the total activity was in the lipid moiety of αLP-I^{131}.

In Vitro Recombination Studies

A tracer amount of the labeled αP-I^{131} was incubated with three 5 ml sera of normal or hyperlipidemic human subjects and of horse for 30 minutes at 20°C in three Spinco cellulose tubes. The densities of the samples were adjusted to 1.006, 1.063 and 1.21 g per ml by the addition of solid NaCl-KBr followed by ultracentrifugation at 105,000 x g for 22 hours, as described earlier. The supernatant and infranatant

fractions were separated by slicing the tubes and their radioactivity determined in a well-type, crystal scintillation counter (see below).

In Vivo Recombination and Turnover Studies

To block thyroidal uptake of radioiodine, all subjects in this study received Lugol's solution, 30 drops daily starting 2 days prior to αLP-I^{131} or αP-I^{131} injection and continued throughout the study period.

Approximately 25 µc of αLP-I^{131} or αP-I^{131} was administered intravenously to each subject after an overnight fast and after 30 minutes blood was obtained in heparinized syringes. Subsequently, blood samples were obtained at 24-hour-intervals for 8-10 days.

Six subjects were given during the study period a large fatty meal. Two and one-half hours later, a post-prandial blood sample was obtained, followed by an injection of 100 mg of heparin sodium intramuscularly, and half an hour later a second blood sample was collected. In both occasions blood was obtained for non-esterified fatty acids as well, with EDTA as the anticoagulant, and the samples were immediately iced to prevent in vitro hydrolysis of triglycerides.

Plasma was separated less than one hour after the blood was obtained. From each sample of blood, a 5 ml aliquot was counted in a glass vial for total radioactivity while 3 other aliquots were subjected to ultracentrifugation at 3 different densities as described under the in vitro recombination studies. The supernatant and infranatant fractions were similarly separated and counted for radioactivity.

The I^{131} activity of all serum samples were recorded initially

and at the conclusion of the study in a well-type sodium iodide-phthalate crystal scintillation counter (Picker) with an efficiency of 52 per cent and a background of 120-140 counts per minute. An I^{131} standard with the same volume was also counted daily to detect variations in counter sensitivity.

Twenty-four-hour urine collections were obtained and 10 ml aliquots counted for radioactivity.

Amino Acid Analysis

The protein moiety was dried to constant weight in vacuo over P_2O_5. The conditions for acid hydrolysis were constant boiling hydrochloric acid (1000 volumes) in an evacuated tube at 110°C for 20 hours. The tryptophane determination was performed after hydrolysis with 3.5 N barrium hydroxide (1000 volumes) in an evacuated tube at 100°C for 10 hours. The conditions for amide nitrogen hydrolysis were 1 N sulfuric acid (1000 volumes) at atmospheric pressure and 100°C for 3 hours.

The chromatographic analysis of the amino acids, as well as the phosphorus content of the protein, were determined by Analytic Corporation (New York).

Enzymatic Hydrolysis of Protein

The primary structures of the human and horse αP were compared by tryptic and chymotryptic hydrolysis (107) and the peptides separated by two-dimensional chromatography and high voltage electrophoresis (108).

The protein was dissolved in 6 M urea and 0.3 M ammonium carbonate buffer (pH 8.4), diluted with distilled water, 1:1.5 v/v, and kept in a hot water bath for 15 minutes, then cooled to room temperature.

One drop of phenol red and an aqueous solution of trypsin (ratio trypsin/protein 1:30) were added. The mixture was incubated in a water bath at 28°C for 1.5 hours. Subsequently, the same amount of chymotrypsin was added and the mixture treated similarly. The hydrolysate was transferred to a 1 x 6 cm Dowex 50W-X2 (Baker, chloride salt, 100-200 mesh) column. Urea was removed with 200 ml of water. The peptides were eluted with 4M ammonium hydroxide, lyophilized overnight, redissolved in water, and applied on Whatman No. 3 papers. The chromatograms were developed with the upper phase of a mixture of normal butanol:acetic acid:water (4:1:5) for about 7 hours and dried overnight at room temperature. The second dimension was developed by electrophoresis with acetic acid:pyridine: water (10:1:89) at 2000 volts (215 mA) for 50 minutes. The spots were developed with a 0.5% methanolic ninhydrin solution and heated for 10 minutes at 80°C.

Terminal Amino Acid Determination

The dinitrofluorobenzene (DNFB) method was used (109). To 1 volume of protein (2.5 mg) solution containing 2% sodium bicarbonate, 2 volumes of 2.5% DNFB in ethanol were added and the solution allowed to stand at room temperature for 2 hours. The pH was adjusted to 2 with 0.1 N HCl, the mixture extracted with peroxide-free ether, and the dinitrophenol-protein dried in a dessicator over P_2O_5. The dinitrophenol-protein was hydrolyzed with 7.5 N redistilled HCl for 24 hours under reflux, diluted with water and extracted four times with 5 ml portions of peroxide-free ether. The ether extracts were combined, the solvent was removed *in vacuo*, and the residue transferred quantitatively

to the paper with acetone. Chromatograms were developed with tertiary amyl alcohol saturated with 0.05 M potassium acid phthalate (pH 5.9) for 12 hours. After drying the paper, the DNP-amino acid spots were eluted with 4 ml of 1% sodium bicarbonate solution and the optical density determined in a Beckman DU spectrophotometer at 360 mμ. A known sample of a chemically pure DNP-amino acid (the same as that found in αP) was used simultaneously to correct for losses due to the procedure.

Starch Gel Electrophoresis

The gel was processed according to the procedure of Smithies (110) using hydrolyzed starch (Connaught) placed in fiber glass molds (1 x 14 x 15 cm) and left standing overnight before use. A cut was made in the gel at a distance of 3 cm from the cathode end. Samples for electrophoresis were applied on small pieces of filter paper (Whatman No. 1) and placed in the slit. Electrophoresis was performed at a potential gradient of 6 volts per cm at room temperature using a Heathkit power supply. Subsequently, the gel was sectioned into two layers to allow for a separate staining with Amido Black B for protein and Oil Red O for lipid. When radioactive material was checked by electrophoresis, the stained section was sliced in equal pieces and radioactivity measured in a well-type crystal scintillation counter.

Agar Gel Electrophoresis and Immunoelectrophoresis

The technique of Grabar and Williams (111) was used. A 1 per cent agar (Difco) solution in a sodium veronal buffer (pH 8.6) was spread evenly over dry glass plates (10 x 8 cm) pretreated with ethanol

to render them fat-free. Slits (1 x 3 mm) were cut for the application of samples 1 cm from the cathode end. Electrophoresis was performed in 4 to 6 hours at 6 volts per cm and 10 milliamperes. The gel was stained with Amido Black B or Oil Red O. For the immunoprecipitin study, a trough (3 x 60 mm) was cut perpendicular to the original slit at a distance of 5 mm and filled with horse anti-normal human serum (Immunology, Inc., Chicago).

Determination of Sedimentation Coefficients

The equation of Degeles and Gutter (112) was used to determine the apparent sedimentation coefficient (^{s}app.):

$$s_{app.} = \frac{2.303 \quad (d \ \log x \ / \ dt)}{60 \quad (\omega^2)} \qquad (1)$$

where x is the distance of the boundary from the center of the rotation at time t (in seconds), and ω is the angular velocity of centrifugation.

The schlieren pattern was developed on photoplates (Eastman Kodak) automatically in the Spinco Model E ultracentrifuge at 8 or 16 minute intervals. A micro-comparator giving an accuracy of 1×10^{-4} cm was used to determine the position of sedimenting boundaries. The d log x / dt was obtained by plotting d log x versus t.

The $s_{20,w}$ (sedimentation coefficient under standard conditions of 20°C and water as solvent) was obtained from $s_{app.}$ using the Svedberg and Pedersen equation (113).

CHAPTER III

RESULTS

Isolation and Purification of αLP

The sera or plasma of fasting healthy human donors and horses were analyzed for total lipids; the results are presented in Table II. The ages of the male and female subjects ranged between 22 and 35 years, and the total cholesterol, phospholipid and glyceride serum levels fell within the normal range.

Half of the mares were pregnant, but there were no significant differences in the serum lipids or lipoprotein patterns between the pregnant and non-pregnant mares. The total serum cholesterol level for horses was below 100 mg per 100 ml and the total serum lipids (two-thirds to three-fourths of which were αLP) averaged about 290 mg per 100 ml corresponding to approximately half the average lipid level in normal human serum.

After separating the very low density and β-lipoproteins, the αLP were isolated by sequential preparative ultracentrifugation. The purity of the separated αLP was determined by standard procedures, and the findings will be presented later in connection with similar characterization results obtained for delipidized αLP.

The purified αLP was analyzed for lipid and protein content; the

TABLE II

SERUM LIPIDS OF NORMAL DONORS AND HORSES

Source and Number	Age	Sex	Total Cholesterol	Phospho-lipids	Triglycerides
			mg/100 ml	mg/100 ml	mg/100 ml
Human (8)	23-35	Females	189±24	267±19	42±10
Human (4)	22-28	Males	224±12	269±19	79±15
Horses (18)	1-6	Mares	84±27	182±36	24±13

results are shown in Table III. The values of several preparations were similar and dialysis did not alter the composition. The αLP of both male and female individuals had the same composition. Compared with human serum αLP, the αLP of horse serum contained a higher protein content and a lower cholesterol/phospholipid (C/P) ratio.

TABLE III

PER CENT COMPOSITION OF PURIFIED HUMAN AND HORSE SERUM αLP

Source of αLP	Lipid Protein	Total Cholesterol	Phospho-lipid	C/P Ratio
	%	%	%	
Human Male	49.1	21.3	29.6	0.73
Human Female	44.4	22.6	32.8	0.69
Horse	55.8	13.9	30.3	0.46

Phospholipids of Human Serum, Human αLP and Horse Serum

The phospholipid composition of purified αLP was determined by a combined silicic acid column and silicic acid impregnated paper chromatography. Each volume of αLP was extracted initially with twenty volumes of redistilled chloroform and methanol (1:1 v/v) and washed three times with distilled water.

The phosphatide composition of human αLP was compared with the average phosphatide distribution of human and horse sera (Table IV). The per cent distribution of sphingomyelin in human serum αLP was

TABLE IV

PHOSPHOLIPID COMPOSITION OF PURIFIED αLP, NORMAL HUMAN AND HORSE SERUM

Sample	Source	Per Cent of Total Lipid P					
		Lecithin	Sphingo-myelin	Lyso-lecithin	Cephalin	Phospho-inositol	Unknown
Serum	Human	72.1	19.2	1.0	2.5	4.8	1.0
αLP	Human Male	79.2	13.8	1.0	2.5	3.4	1.3
αLP	Human Female	77.1	15.0	0.2	2.8	3.6	0.9
Serum	Horse	80.9	8.8	4.6	3.8	0.5	1.4

lower than in human serum but higher than in horse serum.

The αLP of male and female individuals had similar phosphatide composition with lecithin and sphingomyelin as the principal phospholipids. Further subfractionation of the αLP between the densities 1.063-1.125 and 1.125-1.210 g per ml showed no significant change in the phosphatide distribution. Lysolecithin was partly soluble in water which probably accounted for the low value in αLP. Unwashed αLP contained about 5 per cent lysolecithin. The plasmalogens were not separated from the cephalin and lecithin.

Normal horse serum contained minute amounts of inositol phosphatide compared with human serum and αLP.

Delipidation of αLP

The purified αLP, dialyzed against 0.15 M NaCl, was delipidized by extraction with ethanol-diethyl ether (3:1 v/v) followed by diethyl ether at -30°C in a Soxhlet apparatus. The extracts were analyzed for lipid and protein content and found to contain more than 90 per cent of the original lipid components but no protein (Table V). The lipid-free protein was recovered in an 81 per cent yield. However, this efficient method of extraction requires a modified Soxhlet apparatus for the continuous ether extraction.

Purified αLP were submitted, therefore, to a repeated batch-wise extraction with a mixture of redistilled ethanol-diethyl ether (3:1 v/v) for three days. The analyses of lipid extracts, shown in Table VI, gave recoveries of lipids and protein residue comparable to those obtained by a more elaborate method of continuous extraction.

TABLE V

LIPID AND PROTEIN IN EXTRACTS AND RESIDUE FOLLOWING DELIPIDATION OF PURIFIED HUMAN SERUM αLP

Sample	Cholesterol	Phospho-lipid	Trigly-cerides	Lipid Protein
	mg	mg	mg	mg
αLP	19.3	33.4	4.3	55.6
Alcohol-ether extract	17.8	27.7	3.7	-
Ether extract	3.3	2.3	0.1	-
Protein residue	-	-	-	45.0
Percent recovery	94	90	88	81

TABLE VI

LIPID AND PROTEIN IN EXTRACTS AND RESIDUE FOLLOWING DELIPIDATION OF PURIFIED HORSE SERUM αLP

Sample	Cholesterol	Phospho-lipid	Lipid Protein
	mg	mg	mg
αLP	21.2	46.0	85.0
Alcohol-ether extract I	18.0	40.7	-
Alcohol-ether extract II	0.4	1.0	-
Alcohol-ether extract III	0.1	0.1	-
Protein residue	-	0.5	78.1
Percent recovery	87.3	90.9	91.9

The protein obtained after drying over Drierite in a dessicator appeared grayish-yellow and amorphous and remained stable for as long as a year when kept in a stoppered glass tube at 10°C.

The protein was soluble in water and electrolytic solutions, but its solubility decreased at lower temperature and acid range of pH. Solubility values in a 0.15 M NaCl, phosphate buffer (pH 6.8, u 0.1), sodium barbiturate (pH 8.6, u 0.1), and carbonate-phosphate (pH 9, u 0.1) have been reported (65) previously. The protein was readily soluble at pH values between 9 and 11 as well as in aqueous solutions containing 1 to 8 M urea, 0.04 to 0.1% sodium dodecyl sulfate (S.D.S.) or 0.1% succinic anhydride.

In solution, the protein was not as stable as in the dried form and showed evidence of denaturation within a few days. The denaturation process could be delayed by using deionized water and by keeping the protein in dilute base at 0° to 3°C in stoppered, clean test tubes.

The delipidized αLP contained less than 2 per cent phospholipid; there were no neutral lipids.

One sample of human serum αP was completely free of phosphorus after lipid extraction with methanol-chloroform (1:1 v/v).

After dessication in vacuo to constant weight, human and horse αP contained about 89 per cent protein nitrogen. Most of the remaining eleven per cent of the dry weight was assumed to consist of structural water.

Homogeneity of αLP and αP

The purity of human and equine serum αLP and αP was investigated

by several methods:

Analytical Ultracentrifugation

The purified human and equine serum αLP, dialyzed against 0.15 M sodium chloride solution, was subjected to ultracentrifugation at 52,640 r.p.m. and 20°C. The schlieren pattern showed a single sedimenting boundary in both instances and was not altered by change in concentration or pH.

The homogeneity of the delipidized αLP was similarly established by analytical ultracentrifugation. Both human and horse serum αP, dissolved in 0.15 M sodium chloride or phosphate buffer (pH 8.0, u 0.1), showed only one sedimenting boundary. Representative samples of the schlieren patterns obtained for both αLP and αP are provided in Figures 2, 3, 4, and 5.

Starch Gel Electrophoresis

A single protein band which migrated towards the anode was found in the electrophoresis of purified human and horse serum αLP and αP on starch gel. The protein band stained for lipid in the case of αLP but not αP.

In electrolytic solutions human serum αLP migrated approximately midway between a reference albumin band and the origin, while horse serum αLP migrated nearer to the albumin band. Human and horse αP migrated equally as an immediately postalbumin band. The difference in migration between αLP and αP was of value in studying recombination of αP with lipids.

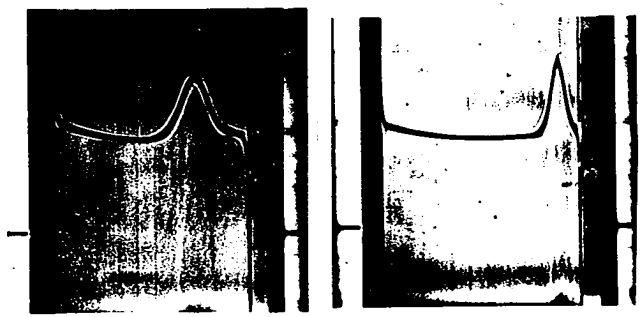


Figure 2. Human Serum αLP at 30 (right) and 72 (left) minutes.

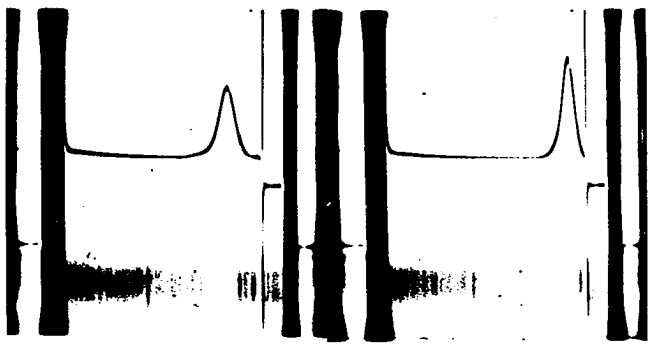


Figure 3. Horse Serum αLP at 19 (right) and 43 (left) minutes.

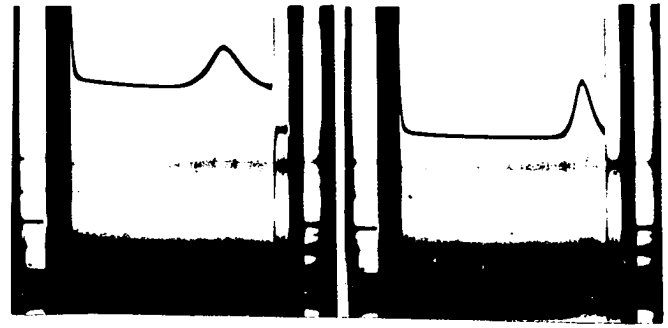


Figure 4. Human Serum αP at 25 (right) and 57 (left) minutes.

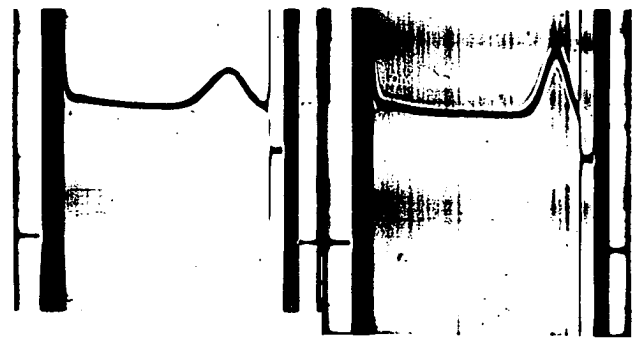


Figure 5. Horse Serum αP at 28 (right) and 52 (left) minutes.

Concentration of Proteins in Sedimentation Patterns Approximately 7 mg per ml; All Runs at 52,640 r.p.m., Temperature at 20°C.

Agar Gel and Immuno-electrophoresis

Only one protein band was observed in the electrophoresis of human and equine αLP and αP on agar gel. The αLP band also stained for lipid, but not αP.

The migration of αLP was similar to albumin on agar gel while αP migrated one-third of that distance from the origin.

Immunoprecipitation of human serum αLP and αP with anti-human horse serum showed only one narrow precipitation line in the same region where the protein band appeared. Neither the αLP nor αP of horse serum gave any cross-reaction with the above antiserum.

Because anti-human horse serum contained antibodies against human serum αP, the latter was antigenic and, therefore, not identical with horse serum αP.

Terminal Amino Acid Residues

The amino-terminal amino acid is aspartic acid for both human and horse serum αLP and αP. The quantitative recoveries are shown in Table VII. Horse serum αP contained approximately two umoles of DNP-aspartic acid per umole of protein, assuming a molecular weight of 70,000 for αP. The human serum αP contained similar amounts of DNP-aspartic acid for the same molecular weight.

Preliminary experiments using the carboxypeptidase method as described by Fraenkel-Conrat et al. (109) suggest that threonine is the carboxyl terminal amino acid of both human and horse αP. The quantitative yield of the amino acid has not been calculated.

TABLE VII

AMINO-TERMINAL AMINO ACIDS OF HUMAN AND HORSE αP

	Mole DNP-Aspartic Acid per Mole of αP *
Human αP	1.5
Horse αP	1.8

* Assumed Molecular weight of 70,000

Biological Activity

The peptide moiety of human αLP and αP was labeled with I^{131}, passed through a sterilized ultrafine filter (Pyrex), then reinjected into normal human individuals. The radioactivity disappearance rate for ten days after injection was plotted on semilog paper and half-times determined graphically. The half-times for both αLP-I^{131} and αP-I^{131} were approximately 3.5 days as shown in Figure 9.

Recombination of αP with Lipids

The I^{131}-labeled αP was added in trace amounts (0.1 to 1.0 mg per 100 ml) to native serum and recombination with lipids established by preparative ultracentrifugation and starch gel electrophoresis after an incubation period of 30 minutes at about 20°C in vitro.

When αP-I^{131} was subjected to ultracentrifugation at 105,000 xg, at 0°C for 22 or 44 hours in a NaCl-KBr solution of density 1.210 g per ml, approximately 4 per cent of the radioactivity was present in the supernatant fraction and the remaining 96 per cent was in the infranatant. However, when αP-I^{131} or αLP-I^{131} was added to normal serum

of solvent density 1.210 g per ml and centrifuged under the same conditions, about 70 per cent of the radioactivity after 22 hours of centrifugation and 85 per cent after 44 hours were found in the supernatant. This indicates that αP-I^{131} recombined with serum lipid to form labeled αLP of density less than 1.210 g per ml and therefore underwent flotation upon centrifugation.

Starch gel electrophoresis was employed also to demonstrate recombination of αP with serum lipids. Following the electrophoresis of αP-I^{131}, the starch gel was stained with Amido Black B, cut into 1 cm sections, and radioactivity of each section determined in a well-type crystal scintillation counter. Radioactivity was detected only in the region of the αP band i.e. immediately post-albumin region, as indicated previously. However, when a tracer amount of αP-I^{131} was first added to normal serum and then subjected to electrophoresis, the radioactivity was detected in the αLP region. This again indicates that αP-I^{131} recombined with serum lipids to form the slower moving αLP molecules.

These experiments were performed on both human and horse αP and the results were the same within experimental error.

Furthermore, human serum αP-I^{131} added to horse serum *in vitro* recombined with serum lipids to form labeled αLP. Similarly, horse serum αP-I^{131} recombined with human serum lipids *in vitro* to form labeled αLP.

From these experimental findings, it may be concluded that both αLP and αP of human and horse serum were electrophoretically homogeneous and that the delipidized αLP was biologically active.

Amino Acid Analysis

The amino acid composition of human αP resembled that of the horse αP (Table VIII). The values referred to protein moiety dried to constant weight in a vacuum dessicator over P_2O_5. Both protein residues showed only traces of one-half cystine and had similar values for amide nitrogen and ammonia. The amino acid residues per smallest theoretical unit were different, although glutamic acid, leucine, lysine, aspartic acid, arginine and alanine were the principal amino acids. The smallest human αP unit (calculations based on isoleucine) gave a molecular weight of about 20,000; this is in agreement with the findings of Shore and Shore (66). The smallest horse αP unit (calculations based on methionine) gave a weight of 28,500.

These results show that the structures of human and horse αP may differ in distribution of amino acids as well as in size of the smallest theoretical unit.

Enzymatic Hydrolysis of αP

Aliquots of salt-free tryptic and chymotryptic hydrolysates of human and horse serum αP containing approximately 1.5 mg of peptide material were developed chromatographically in one dimension and by high voltage electrophoresis in the second dimension, and peptide spots were identified with the ninhydrin reaction.

The peptide patterns of tryptic digests of human and horse serum αP were different (Figure 6). Spots 1, 2, 3, 17, and 18 of the human serum αP pattern were absent from the horse αP pattern. On the other hand, spots 9, 10, 17, and 18 in the latter were missing from the

TABLE VIII

AMINO ACID ANALYSIS OF HUMAN AND HORSE SERUM αP

	Human		Horse	
	mg/100 mg	Residues/unit*	mg/100 mg	Residues/unit*
Glutamic acid	17.77	26	18.94	36
Leucine	11.73	17	15.35	30
Lysine	9.29	13	9.71	19
Aspartic acid	7.45	11	9.02	17
Arginine	6.39	9	7.36	14
Alanine	4.91	7	5.78	11
Serine	4.91	7	4.62	9
Valine	4.34	6	4.71	9
Tyrosine	4.31	6	3.29	6
Threonine	4.06	6	5.06	10
Proline	3.15	5	3.05	6
Phenylalanine	3.03	4	3.66	7
Glycine	2.2	3	2.65	5
Histidine	1.79	3	2.4	5
Tryptophane	1.76	3	2.47	5
Methionine	1.18	2	0.52	1
Isoleucine	0.69	1	0.98	2
1/2 Cystine	trace	trace	trace	trace
Ammonia	1.13	2	1.33	3
Amide Nitrogen	0.8	1	1.08	2

*Smallest theoretical unit of amino acid residues of serum αP

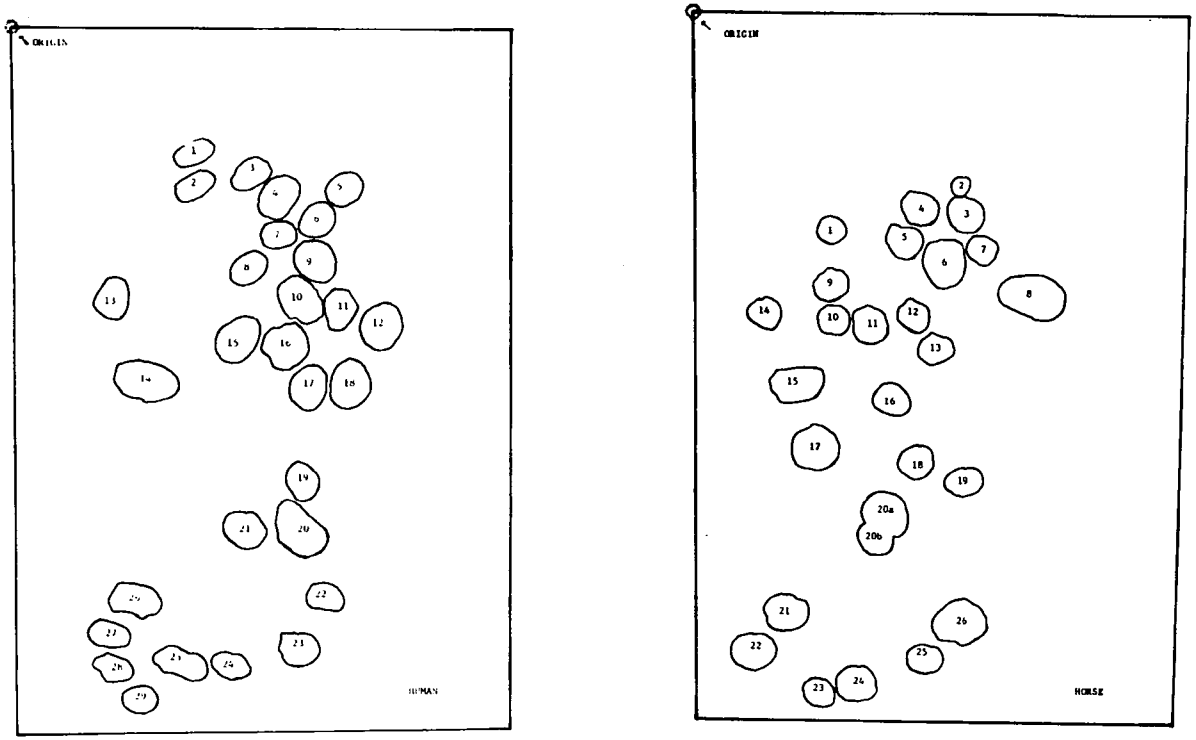


Figure 6. Peptide Pattern of Human and Equine Serum αP Digests Obtained with Trypsin.

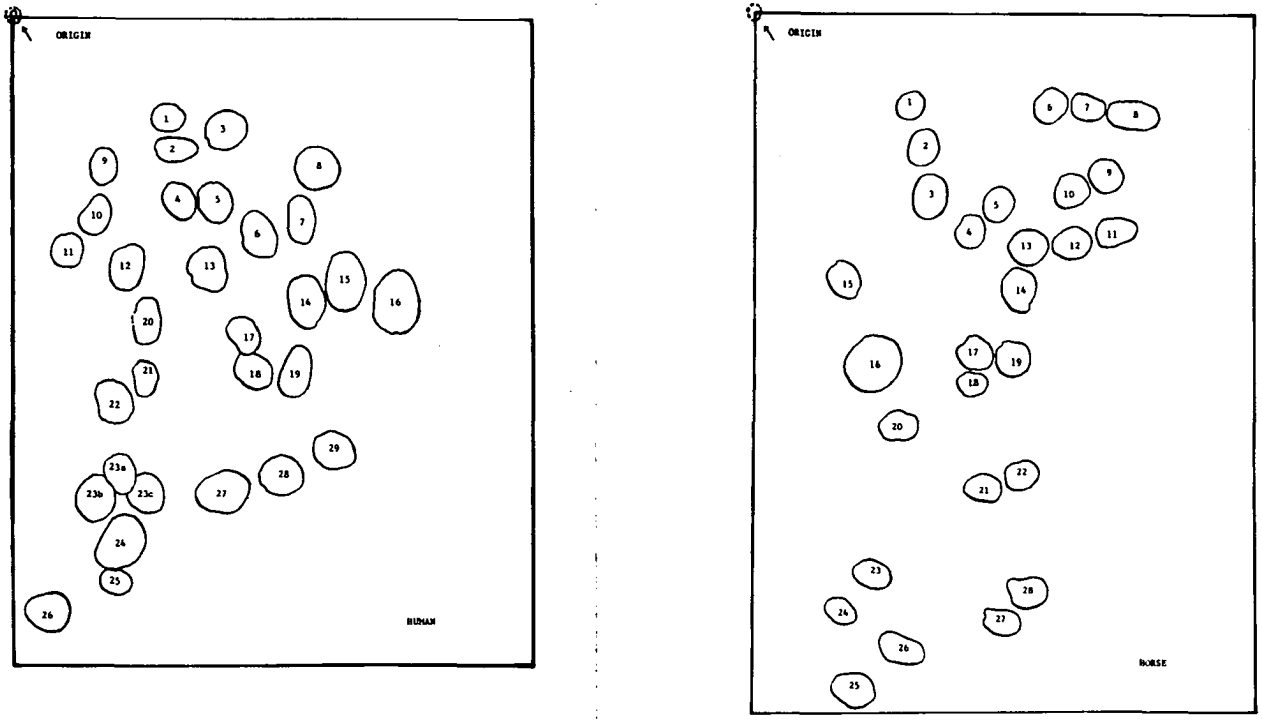


Figure 7. Peptide Pattern of Human and Equine Serum αP Digested Successively with Trypsin and Chymotrypsin.

former. Both human and horse serum αP, however, appeared to have similar peptides in the lower half of the patterns.

The successive hydrolysis of human and horse αP with trypsin and chymotrypsin also resulted in dissimilar peptide patterns (Figure 7). Spots 9, 10, 11, 14, 15, 16, and 29 in the human serum αP pattern were not seen on the horse serum αP pattern.

These results suggest that the amino acid sequence of human and horse serum αP are not the same.

Behavior of Human and Horse Serum αP in Various Aqueous Solutions

Several methods were used to investigate the reversible dissociation of αP in aqueous solution. The findings are summarized in Table IX and X.

Human serum αP dissolved in electrolytic solutions, pH 7 to 8, appeared as two adjacent protein bands and two schlieren boundaries within 48 hours (Figure 8). The major boundary had a sedimentation coefficient of 4.1 S and the minor boundary a value of 7.7 S. Only the major boundary and a single electrophoretic protein band on starch gel electrophoresis appeared, however, following the addition of urea (1 to 8 M) or increasing the pH to 11.0 by addition of alkali (Figure 8). The minor boundary did not reappear upon dialysis of the urea against 0.15 M NaCl solution or after readjusting the pH to 7.0 or 8.0.

Sodium dodecyl sulfate (0.1% or less) caused the dissociation of human and horse αP to a protein unit with sedimentation coefficient of about 2.5 (Figure 8). Only one protein band appeared on starch or agar gel with an electrophoretic mobility twice that of the original αP in

TABLE IX

BEHAVIOR OF HUMAN αP IN VARIOUS AQUEOUS SOLUTIONS

Media and Additions	Protein Bands on Starch Gel	Boundaries in Schlieren Pattern	S	Mobility on Agar Gel
Sodium chloride (0.15 M); pH 7	1 or 2	1 or 2	-	Regular (R)
Phosphate buffer (u 0.05, 0.1); pH 8.0	1 or 2	1 or 2	4.1 and 7.7	R
Barbiturate buffer (u 0.1); pH 8.0	1 or 2	1 or 2	-	R
NaCl (0.15 M); alkali to pH 11.0	1	1	-	R
Phosphate buffer (u 0.1); alkali to pH 11.0	1	1	4.0	R
Urea (1-8 M); pH 6.0	1	1	-	R
Urea (2 M); dialysis against NaCl (0.15 M)	1	1	-	R
NaCl (0.15 M); urea (2-6 M)	1	1	-	R
Phosphate buffer (u 0.1); urea (1-8 M)	1	1	4.0	R
Sodium dodecyl sulfate (S.D.S. 0.04-0.1%)	1	1	-	Increased
Phosphate buffer (u 0.1); S.D.S. (0.08%)	1	1	2.5	Increased
S.D.S. (0.08%); dialysis against NaCl (0.15 M)	1	1	3.9	R
Succinic anhydride (0.1%); alkali to pH 9.0	1	1	-	Increased

TABLE X

BEHAVIOR OF HORSE αP IN VARIOUS AQUEOUS SOLUTIONS

Media and Additions	Protein Band on Starch Gel	Boundary in Schlieren Pattern	S	Mobility on Starch and Agar Gel
Phosphate buffer (u 0.1); pH 8.0	1	1	3.9	Regular
Phosphate buffer (u 0.1); alkali to pH 11.0	1	-	-	"
Urea (1-2 M)	1	-	-	"
Sodium Dodecyl Sulfate (0.08%)	1	1	2.9	Increased
S.D.S. (0.1%); dialysis against NaCl (0.15 M)	-	2		-
Succinic anhydride	1	2		Increased

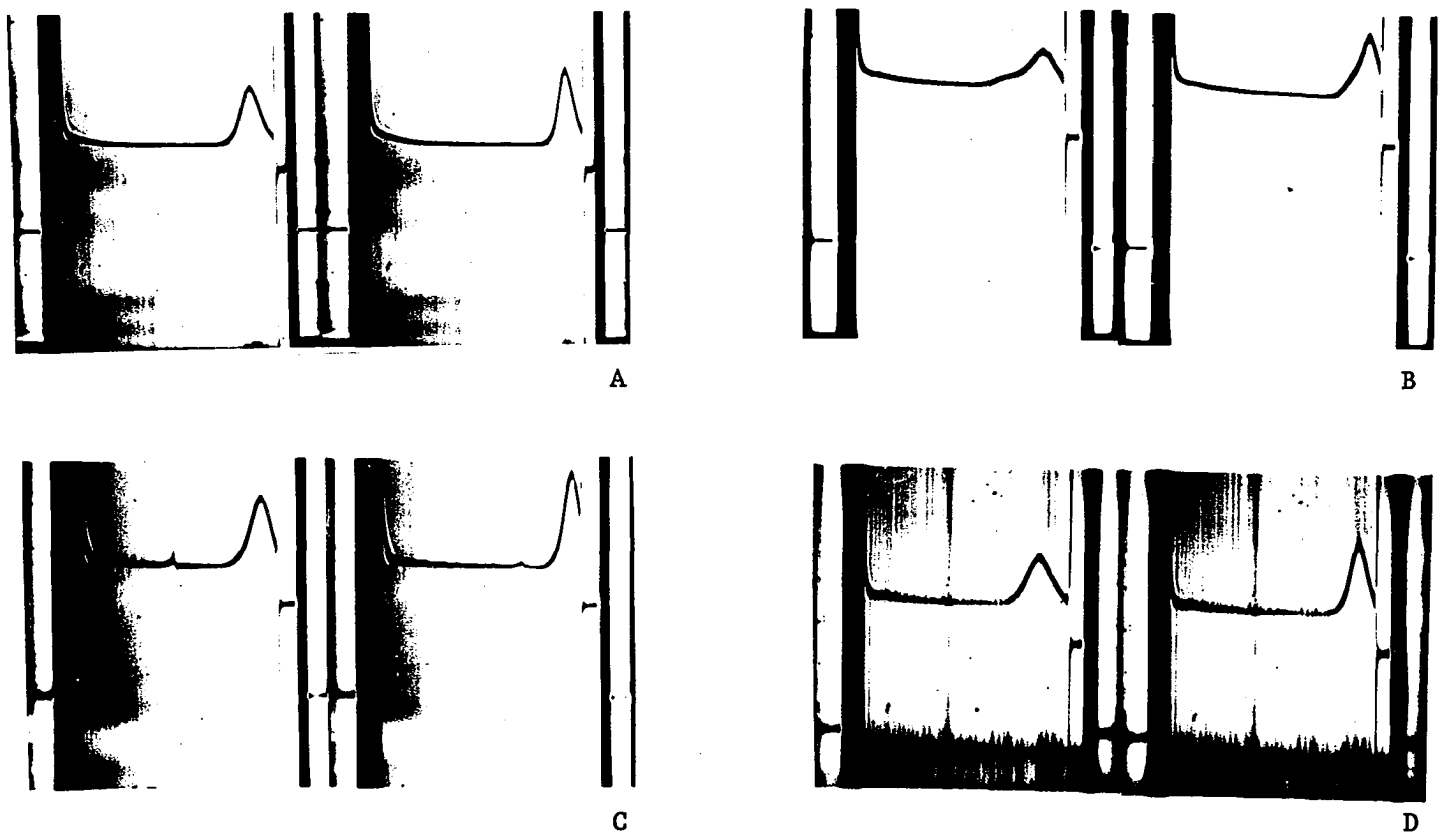


Figure 8. Sedimentation Patterns of Human αP in Phosphate Buffer (μ 0.1) at (A) pH 8.0, (B) pH 11.0, (C) Plus Sodium Dodecyl Sulfate (0.1%), and (D) After Removal of Sodium Dodecyl Sulfate by Dialysis. Interval Between Photographs was 16 Minutes, Protein Concentration 7 mg per ml, Centrifugal Speed 52,640 r.p.m. at 20°C.

phosphate buffer under similar conditions.

The addition of succinic anhydride (0.1%) to an aqueous solution of human or horse αP increased the electrophoretic mobility of the protein moiety on agar gel. Succinylation did not decrease the binding capacity of αP, nor the immunologic response of human serum αP.

After the removal of sodium dodecyl sulfate from the solution by dialysis against 0.15 M NaCl, the human αP reassociated to form the protein unit with the initial electrophoretic mobility and sedimentation coefficient of about 4.0 S (Figure 8). Following the removal of sodium dodecyl sulfate, horse αP appeared as two boundaries showing that reassociation might have occurred.

The in vitro recombination technique showed that human and horse αP-I^{131} dissolved in any of the described aqueous solutions at high pH or containing urea, sodium dodecyl sulfate or succinic anhydride maintained the biological function of binding serum lipids. It was demonstrated also by agar immunoelectrophoresis that human serum αP-I^{131} dissolved in various buffers at pH 7 to 11, in urea, sodium dodecyl sulfate (0.08%) or succinic anhydride (0.1%) solutions, gave a single, distinct precipitation line with horse anti-serum.

It was concluded from these results that human and horse αP underwent a reversible dissociation with anionic detergent, and that at least human serum αP formed aggregates in electrolytic solution which were disrupted with increased pH or addition of urea without loss of biological activity.

Behavior of αLP in Sodium Dodecyl Sulfate

The addition of sodium dodecyl sulfate to human and horse αLP

did not alter the electrophoretic mobility nor the sedimentation coefficient (4.5 to 5.0 S).

As in the case of αP, addition of sodium dodecyl sulfate (0.1%) to human serum αLP did not block the immunoprecipitin reaction with horse anti-serum on agar gel.

Finally, αLP treated with sodium dodecyl sulfate was not delipidized by the detergent. Starch gel electrophoresis showed the αLP band to stain positively for protein and lipid.

Using the in vitro recombination procedure, αP-I^{131}, incubated with normal serum containing sodium dodecyl sulfate (0.1%), recombined with the serum lipids to form labeled αLP to the same extent as in the absence of the detergent i.e. about 85% of the radioactivity of αP-I^{131} was found in the 1.210 g per ml supernatant following centrifugation at 105,000 x g, 0°C for 44 hours.

These results indicated that the biological and physical characteristics of αLP were not altered significantly by sodium dodecyl sulfate.

Identity of Protein Moiety of Human Serum VHDLP

The sera of normal male and female subjects, incubated with αP-I^{131} or αLP-I^{131} for 30 minutes at 20°C, were subjected to ultracentrifugation (105,000 x g for 44 hours at 0°C) after adjusting the solvent density to 1.210 g per ml and subsequently separated into two fractions with the Spinco tube slicer, as described previously. The infranatant fraction (VHDLP) contained 8 to 12 per cent of the total serum lipid phosphorus and 10 to 15 per cent of the total radioactivity

of αP-I^{131} or αLP-I^{131} in the original incubation mixture, but no cholesterol.

The lipid phosphorus in VHDLP, extracted with chloroform-methanol and analyzed by silicic acid column and paper chromatography, was identified as phospholipids. Lysolecithin, the principal component, accounted for about half the total lipid phosphorus of VHDLP in two separate determinations (Table XI).

TABLE XI

PER CENT PHOSPHATIDE COMPOSITION OF VHDLP

Chloroform-Soluble ug P	% Eluted from Column	% Total Lipid P					
		Lyso-lecithin	Leci-thin	Sphingo-myelin	Cepha-lin	Ino-sitol	Unknown
155	93	47	22	5	3	18	3
401	94	56	28	10	2	2	2

The phospholipid-containing VHDLP were present, therefore, along with αP-I^{131} in the 1.210 g per ml infranatant fraction. An attempt was made to show that the phospholipids might be bound to αP.

First, it was already demonstrated that a trace amount of αP-I^{131} added to serum was completely bound to lipids.

Second, the 1.210 g per ml infranatant was concentrated by repeated ultracentrifugation and a sample containing the VHDLP was stained with Sudan Black B, according to the procedure described by Zakelj and Cross (114) and applied on starch gel. The migration of the prestained VHDLP was in the immediate post-albumin region and corres-

ponded with the starch gel section where the radioactivity of the $\alpha P\text{-}I^{131}$ was detected.

Third, an α-protein-phospholipid complex was obtained by partial delipidation of the purified αLP with ether at 0°C for 24 hours (70). The electrophoretic mobility was similar to those of prestained VHDLP as well as $\alpha P\text{-}I^{131}$ in the 1.210 infranatant.

Finally, the proteins in the 1.210 g per ml infranatant fraction were treated with radioiodine and incubated with unlabeled, purified αLP for 30 minutes at 20°C. Subsequently, the added αLP separated and purified ultracentrifugally at 105,000 x g was found to be labeled.

The results of the above experiments indicated that αP could be the protein moiety of the VHDLP.

In Vivo Recombination and Turnover Studies

The time course of plasma radioactivity after the intravenous injection of $\alpha LP\text{-}I^{131}$ or $\alpha P\text{-}I^{131}$ to the three normal subjects and the patient (J.C.) with idiopathic hypercholesterolemia were similar; the half times averaged 3.5 days. In the patients (H.H. and S.H.) with hyperchylomicronemia (dietary fat-induced hyperglyceridemia), the half-times were 1.7 and 1.9 days, while in patients (O.W. and O.C.) with dietary carbohydrate-induced hyperlipemia, the half-times were 2.5 and 2.7 days, respectively.

In plotting plasma radioactivity against time, the 30 minute post-injection values fell in the period of mixing and rapid decline in radioactivity and were not plotted in Figure 9. The period of rapid decline was complete within 24 to 48 hours after injection, and only

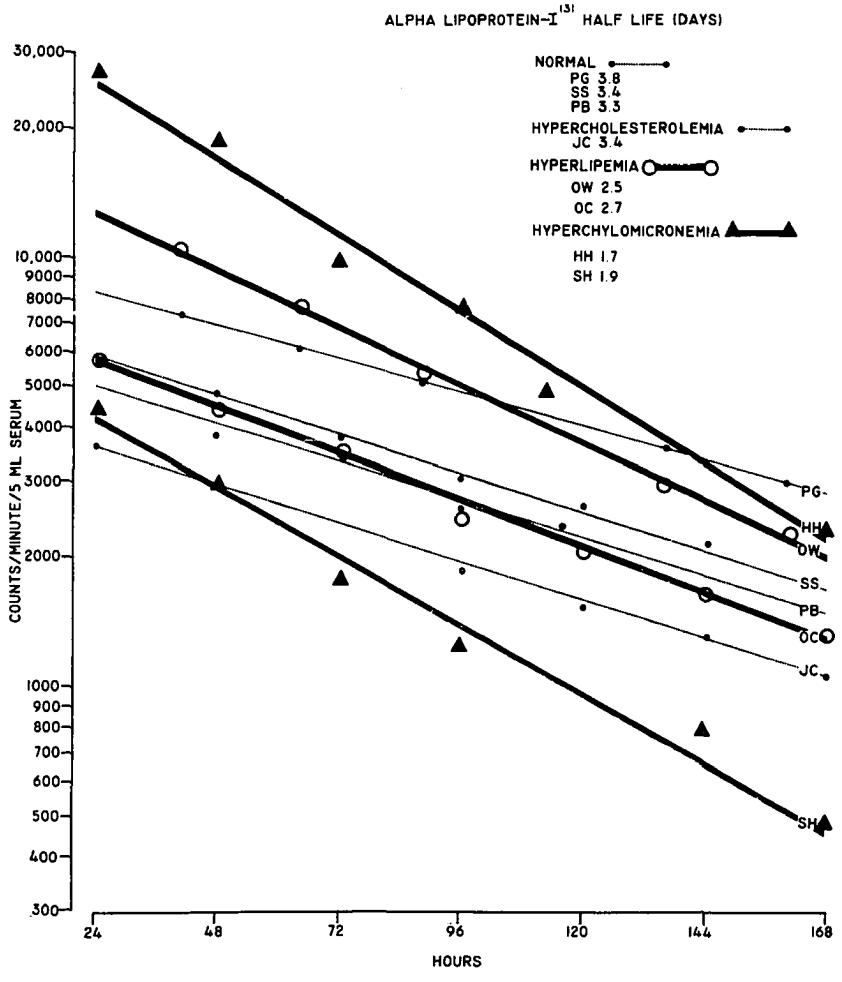


Figure 9. Time Course of Plasma Radioactivity Following αLP-I^{131} or αP-I^{131} Injection in Normal and Hyperlipidemic Individuals. See text.

the linear plots (Figure 9) which followed the latter period were used to calculate half-times.

Plasma samples, obtained from normal and hyperlipidemic subjects after an overnight fast, two and one-half hours post-fatty meal, and half an hour after a subsequent heparin injection, were separated ultracentrifugally into supernatant and infranatant fractions at three different densities and the radioactivity of each fraction determined. The plasma was also analyzed for total cholesterol, phospholipids, triglycerides and free fatty acids. The results are presented in Table XII.

The values for total cholesterol were not influenced by a fat diet or heparin. Phospholipids were increased by the diet in subjects S.H. and H.H. while the triglycerides increased in all subjects. Following heparin injection, the postprandial triglyceride level dropped and the free fatty acid level was elevated, presumably through release of lipoprotein lipase. However, subjects S.H. and H.H. were relatively less responsive to heparin administration (Table XII).

The distribution of αLP-I^{131} radioactivity in the 1.006 g per ml fractions is shown in Table XIII. In the normal subject (P.G.), the hyperlipidemic subjects (O.W. and O.C.) and the hypercholesterolemic subject (J.C.), neither the ingestion of a fatty meal nor subsequent heparin administration had any significant effect on the distribution of radioactivity in the 1.006 g per ml supernatant and infranatant fractions. In three studies with subject H.H. and two studies with subject S.H., an increase in the radioactivity of the 1.006 g per ml supernatant was observed two and one-half hours after a fatty meal.

TABLE XII

EFFECT OF FAT MEAL AND SUBSEQUENT HEPARIN INJECTION ON SERUM LIPIDS OF NORMAL AND HYPERLIPIDEMIC SUBJECTS

Subject	Age	Sex	Total Cholesterol			Phospholipids			Triglycerides			Free Fatty Acids		
			F*	PP**	PH***	F	PP	PH	F	PP	PH	F	PP	PH
			mg %			mg %			mg%			uEq/1		
P.G.	56	M	244	-	-	300	316	312	57	345	58	-	318	1236
H.H.	27	M	288	264	252	345	360	342	3112	3580	3000	-	-	-
H.H.	"	"	256	264	278	296	350	372	2420	2525	2285	588	596	761
H.H.	"	"	252	248	226	310	346	345	2185	2525	2625	456	378	723
S.H.	15	F	96	101	102	140	148	200	478	790	689	-	350	525
S.H.	"	"	96	110	115	162	210	205	818	1058	988	395	271	723
O.W.	51	M	228	-	-	328	320	318	636	889	605	-	312	667
O.C.	65	M	244	238	256	350	385	402	709	1093	872	-	408	2384
J.C.	34	M	424	440	436	326	392	377	92	228	126	485	761	1312

*F = Fasting; **PP = Postprandial; ***PH = Postheparin

TABLE XIII

EFFECT OF FAT MEAL AND SUBSEQUENT HEPARIN INJECTION ON DISTRIBUTION OF αLP-I^{131} RADIOACTIVITY IN CENTRIFUGE FRACTIONS

Subject	Percent Radioactivity in 1.006 g per ml Fractions					
	Fasting		Postprandial		Postheparin	
	Supernatant	Infranatant	Supernatant	Infranatant	Supernatant	Infranatant
S.S.	3	97	-	-	-	-
P.B.	6	94	-	-	-	-
P.G.	5	95	6	94	6	94
H.H.	6	94	31	69	16	84
H.H.	15	85	24	76	22	78
H.H.	9	91	30	70	18	82
S.H.	10	90	13	87	14	86
S.H.	14	86	19	81	20	80
O.W.	8	92	10	90	10	90
O.C.	6	94	7	93	7	93
J.C.	6	94	7	93	7	93

In subject H.H. heparin injection resulted in reduction of radioactivity in the 1.006 g per ml supernatant to values closer to fasting levels.

The radioactivity in the 1.063 g per ml supernatant and infranatant fractions of all sera was similar to that of the 1.006 g per ml fractions.

The 1.210 g per ml supernatant fractions contained in all sera about 70 per cent of the total radioactivity per sample after 22 hours of centrifugation at 105,000 x g. This is consistent with the in vitro findings already described.

Subject H.H. also received radioiodinated human serum albumin (Abbott) intravenously, and it was observed that less than 7 per cent of the radioactivity of the serum could be accounted for in the 1.210 g per ml supernatant, and that neither a fatty meal nor subsequent heparin injection had any effect on the distribution of radioactivity.

Urinary Excretion of Lipids and αLP-I^{131} in a Patient with the Nephrotic Syndrome

The αLP-I^{131} was administered intravenously to a 14-year-old female patient with the nephrotic syndrome associated with renal biopsy findings of subacute glomerulonephritis. The blood urea nitrogen ranged between 23 and 32 mg per 100 ml and the intravenous pyelogram showed both kidneys with good excretory function. The patient had hypoalbuminemia, proteinuria, and hyperlipidemia as shown in Table XIV. The total serum cholesterol, phospholipids, triglycerides and free fatty acids were increased. The serum lipoprotein distribution was determined on two samples and the average values presented in Table XV. There was an increase in the lipoproteins of all classes of low density

TABLE XIV

SERUM AND URINE LIPIDS AND PROTEINS OF A PATIENT WITH NEPHROTIC SYNDROME

Date	Sample	Cholesterol Total mg	Cholesterol % Ester	Phospholipid LPx25 mg	Triglycerides mg	NEFA uEq/l	Protein Gm Total	Protein Gm A/G Ratio
3-6-63	Serum*	755	78	670	528	-	3.8	0.7/3.1
3-10-63	Serum	640	-	655	480	803	-	-
3-22-63	Serum	861	67	743	534	-	5.0	0.4/4.6
3-6-63	Urine**	11.1	-	8.1	3	-	9.8	7.2/2.6
3-7-63	Urine	8.2	41	6.1	-	-	-	-
3-22-63	Urine	6.0	-	6.7	-	-	10.9	8.3/2.6

*Values reported per 100 ml serum
**Values correspond to 24-hour excretion in urine

lipoproteins and a decrease in the per cent distribution of αLP.

Following αLP-I^{131} injection, blood was obtained after 10 minutes, 12 and 24 hours, and urine was collected at 3-hour-intervals for 24 hours and subsequently two 24-hour urine collections.

TABLE XV

LIPOPROTEIN DISTRIBUTION IN A PATIENT WITH NEPHROTIC SYNDROME

	Lipoprotein Lipid per 100 ml of Serum							
	<1.006		1.006-1.019		1.019-1.063		>1.063	
	mg	%	mg	%	mg	%	mg	%
Cholesterol	130	18	105	13	468	61	62	8
Phospholipids	141	21	88	13	384	58	50	8

The total radioactivity of serum and urine was recorded in a well-type crystal scintillation counter. Urine was passed through Ioresin (Abbott) to remove free iodine, and an aliquot was counted for protein-bound iodine i.e. αP-I^{131}.

Approximately 47 per cent of the radioactivity disappeared from serum 24 hours after injection. Of this, 38 per cent was present in the urine. The second and third 24-hour urine collections contained 10 and 5 per cent of the initial dose of radioactivity, respectively.

In all urine fractions collected during the first 72 hours after injection, there was αP-I^{131} averaging about 9 per cent of the total urine radioactivity. None of the urine αP-I^{131} was found in the 1.063 g per ml supernatant fraction, approximately 25 per cent was present in

the 1.210 g per ml supernatant, and the remaining 75 per cent was in the 1.210 g per ml infranatant. The urine αP-I^{131} had an electrophoretic mobility on starch gel similar to native serum αP-I^{131}.

The urine contained small amounts of cholesterol and phospholipids, as shown on Table XIV. Following in vitro incubation of urine with a tracer amount of αP-I^{131} and ultracentrifugation, the 1.210 g per ml supernatant contained 30 per cent of the radioactivity.

From the results of the above experiments, it was concluded that αLP-I^{131} was excreted as such in the urine and that urine lipids of nephrotic patients could bind with αP-I^{131} to form labeled αLP.

CHAPTER IV

DISCUSSION

Human and Horse Serum αLP

The protein moiety of human serum αLP was successfully isolated by Scanu, Lewis and Bumpus (60) and the homogeneity was partially established by analytical ultracentrifugation, moving boundary and starch gel electrophoresis, solubility, and N-terminal amino acid (60). The results of our experiments on the separation and characterization of human serum αLP and αP using identical methods and delipidation procedure are in agreement with those reported by Scanu et al.

The first lipoprotein fraction of constant composition to be isolated by Macheboeuf (5) was a high density lipoprotein obtained from horse serum. However, there is very little information regarding the chemical composition and physico-chemical characterization of this lipoprotein or its protein moiety. Since horse αLP can be obtained in high yield and might serve as a readily available model substance for binding studies, it was of interest to study its properties and compare them with those from similar studies of human serum αLP.

The horse αLP (d 1.063-1.210 g per ml) and its lipid-free protein moiety were characterized by analytical ultracentrifugation, electrophoresis, end-group analyses, and recombination of the radioio-

dinated αP toward lipids. The results of these studies provide partial evidence for the homogeneity of these preparations.

It was of interest to find that human and horse αP which have similar lipid transport functions in plasma shares several characteristics such as sedimentation and electrophoretic mobilities, terminal amino acids, recombination capacity towards lipid, and behavior in aqueous media.

However, human and horse αP were immunochemically distinct. They differed further in respect to amino acid composition, molecular weights for the smallest theoretical unit of amino acid residues, and sequences of amino acids in the peptide chains as demonstrated by the dissimilar peptide patterns of the enzymic digests with chymotrypsin and trypsin.

These results indicated that, in spite of some chemical differences, horse αP is sufficiently similar, in respect to biological properties, to human αP to justify its use as a compound for future binding studies and for the evaluation of the types of noncovalent bonds that exist between the lipid and protein moieties. Basic information regarding size, composition and physico-chemical properties of the lipoprotein would be required to elucidate the complex structure of the lipid-protein association.

In this regard, observations of several researchers (71,72, 83) support an hypothesis which suggests that the protein is placed at or close to the surface of lipid spheres, or that a lipoprotein model consists of a lipid core which is partially surrounded by substructural protein or lipoprotein units (115). The calculated protein coverage

of human serum αLP (15.7 Å squared) allows the lipids to be packed in correct proportions inside a 10 Å thick shell of protein (55). These calculations, however, assume a prolate ellipsoid shape for αLP.

Because of the ease with which the lipid and protein moieties of serum αLP could be separated by treatment with organic solvents, the lipid-protein association was thought to involve, among others, London-van der Waals' dispersion forces (115,116,117) and hydrogen bonding. However, the ionic groups of phospholipids, the carboxylate groups of esters and the hydroxyls of cholesterol could form a network of organized forces which influence the protein-lipid association and configuration (55). The lipid to lipid bonding would not yield a reproducible macromolecular structure of αLP (115); this makes αP important for the solubilization of lipids and for maintainance of αLP structure.

Behavior of αP in Solution

Human and horse αP in solution could be dissociated by the addition of sodium dodecyl sulfate into smaller protein units as shown by the change in the sedimentation coefficient and electrophoretic mobility on agar gel with no effect on its immunologic response or lipid recombining capacity.

Shore and Shore (66) also showed that a subunit of 36,500 molecular weight, containing one mole amino-terminal aspartic acid per 38,000 g protein, was produced by the addition of sodium dodecyl sulfate to αP solution. The molecular weight of the subunit corresponded to approximately half the value reported by Scanu et al. (60)

for αP in electrolyte solution.

The αP subunit reassociated to yield the 'dimer' form upon dialysis to remove the sodium dodecyl sulfate from solution by dialysis.

Human αP formed aggregates in 0.15 M NaCl and phosphate buffer at pH 7 to 8 which could be dissociated by increase in pH or addition of urea with no change in the in vivo or in vitro recombining capacity of αP towards serum lipids (118).

Preliminary light scattering measurements performed by Dr. J. Kratohvil (119) on the same human αP, prepared in this laboratory, in the concentration range from 7.7×15^{-5} to 4.7×10^{-4} g per ml, in phosphate buffer solution (u 0.1, pH 8), indicated that polymerization of αP was a concentration dependent process. The addition of sodium dodecyl sulfate caused a decrease in the observed turbidities, in agreement with the lowering of the sedimentation coefficients.

Dissociation-association phenomena similar to those of αP were recently described for mitochondrial structural protein (121) and for several enzyme proteins (120).

The use of detergents and solvents to disrupt the inter-protein bonds inferred that mainly hydrophobic and hydrogen bonds contributed to the stability of the protein aggregates. Electrostatic forces, London forces or stabilization by electron delocalization across hydrogen bonds would probably be less prominent contributors to the stability of the αP structure(s) (117).

The mitochondrial lipoprotein might be closely related to αP. It should be feasible for the αP, which might be synthesized in the

microsomes, to travel to the mitochondria, pick up lipids from mitochondrial lipoproteins, and then leave the cell as α-lipoprotein molecules (81,82). This scheme would assign αP another function, namely, intracellular lipid transport.

The presence of αP aggregates in vitro would indicate that the protein molecules assume the most thermodynamically favored configuration under a particular set of experimental conditions. It was also speculated that αP polymerization could occur in vivo (118) to explain the two molecular weights reported, i.e., 175,000 and 375,000 (54). However, it would be difficult to say, as pointed out by Shore and Shore (66), whether αP chains interact directly or whether they were joined together by lipid 'bridges' into αLP molecules.

The dissociation of enzymes into subunits (120) result in loss of enzymatic activity which was regained upon reassociation of the subunits into their native form, which suggests that the high degree of folding is necessary for the enzymic substrate interaction.

The recombination of αP with serum lipids in the presence of urea, sodium dodecyl sulfate, and succinic anhydride may indicate that a higher degree of folding of the αP molecule could not be the major stabilizing factor in the protein lipid association and that succinylation (122) of the ϵ-NH_3^+ group of lysine does not inhibit recombination of αP with serum lipids.

Human and Horse Serum Phospholipids

The distribution of sphingomyelin in horse serum phospholipid was lower than in human serum or human serum αLP. Ordinary horse

diet, low in cholesterol, might decrease sphingomyelin distribution, but no data on horses could be obtained in the literature to that effect. In rabbits, Van Handel (123) showed that cholesterol feeding, but not soy bean phosphatides, raised total plasma phosphatide level and increased sphingomyelin distribution.

In human, serum and low density lipoproteins were richer in sphingomyelin than αLP (57,58). The presence of two-thirds to three-fourths of the horse serum lipids as αLP could be an additional factor which results in the decreased sphingomyelin distribution.

Human Serum VHDLP

The quantitative lipids and phosphatide analyses of human serum VHDLP were in agreement with those reported by Phillips (89) showing lysolecithin as the principal phosphatide.

Evidence which may suggest that the protein moiety of αLP and VHDLP are the same was obtained by ultracentrifugal and electrophoretic analyses of $\alpha P\text{-}I^{131}$ or $\alpha LP\text{-}I^{131}$, and by the identification of $\alpha P\text{-}I^{131}$ in the 1.210 g per ml infranatant fraction of serum from subjects who received $\alpha P\text{-}I^{131}$ intravenously. Final proof, however, would await isolation and characterization of the protein moiety of VHDLP.

The presence of high amounts of lysolecithin in VHDLP would suggest that electrostatic forces could be involved to a great degree as well as van dar Waals' forces in maintaining the structure of VHDLP.

The difference in electrophoretic mobility of αLP and VHDLP on starch gel was due to the difference in the amount of neutral

lipids bound, which affect the diffusion of the lipoprotein due to change in molecular size.

Metabolism of Human Serum αLP

The time course of the plasma radioactivity following the injection of αLP-I^{131} or αP-I^{131} in normal subjects indicated that the half-times in both instances were similar, averaging 3.5 days. This was in agreement with the results of Gitlin et al. (69), using αLP-I^{131} in healthy adult men. The latter investigators noted that the half-times were similar whether obtained from measurements of radioactivity in whole body or in plasma and that the injected αP-I^{131} traveled in the plasma along with the lipoproteins of density greater than 1.063 g per ml, a finding with which our data were in agreement.

The shorter half-time of αP-I^{131} in the hyperlipemic and hyperchylomicronemic patients than in the hypercholesterolemic and normal subjects would suggest a more rapid αLP turnover in the former individuals. This might be related to a smaller αLP pool size in these subjects in view of the reduced amounts of αLP lipids in their serum (90,91).

The greater radioactivity noted in the d <1.006 lipoprotein fraction in the hyperchylomicronemic siblings after the ingestion of a fatty meal probably is related to the fact that the excess low density lipids in these subjects were in the form of chylomicrons (S_f >13,000) as demonstrated by Gustafson et al. (123) in this laboratory.

It was mentioned earlier that αP was capable of recombination

with chylomicrons in vitro. According to results of Shore (53), Avigan et al. (64), Rodbell and Fredrickson (71,72), Furman et al. (73), and Hofmann (74), one of the proteins present in the chylomicrons possesses characteristics of the αP.

The redistribution of radioactivity, particularly in subject H.H., following heparin, would suggest that the postheparin lipoprotein lipase effected rapid hydrolysis of chylomicron triglycerides and disrupted the αP-chylomicron association.

Lipiduria and α-lipoproteinuria in the Nephrotic Syndrome

Lipiduria occurs in a variety of diseases (125,126) that affect the kidneys and lead to albuminuria. In nephrotic syndrome, urinary lipids are excreted either in the form of anisotropic bodies consisting mainly of cholesterol esters and some free cholesterol (127) or in a solubilized form, presumably bound to a protein. This solubilized lipid is capable of binding with αP-I^{131} in vitro to form αLP-I^{131}.

Gitlin et al. (69) has found protein-bound I^{131} in urine of nephrotic patients following the injection of αLP-I^{131}, a finding that is in agreement with our results. We also find that a fourth of the protein-bound I^{131} is present as αLP, the remaining three-fourths of the radioactivity remains in the 1.210 g per ml infranatant.

Studies by Gerard (128), Smetana and Johnson (129) on open nephron of Salamander indicate the occurrence of double atherocytosis (reabsorption of protein and cholesterol in different sections of the open nephron). In nephrotic patients, only αLP could pass through but not low density lipoproteins (69). The αLP are either partially de-

lipidized during the process of filtration through the glomerulus or, as currently believed, partially delipidized in the proximal tubule with preferential removal of cholesterol esters (130). The latter precipitate intracellularly in the proximal tubules leading to death and desquamation and appears as "Maltese-cross" bodies or aval bodies which are anisotropic.

Our results support the hypothesis that lipoproteinuria occurs in the nephrotic syndrome, but the cause of the lipoid changes in the proximal tubule remains a matter for speculation.

CHAPTER V

SUMMARY

The protein moiety (αP) of highly purified human serum α-lipoprotein (αLP, density 1.063-1.210 g per ml) has been isolated and its homogeneity established by ultracentrifugal, electrophoretic, end-group and biological analyses. The purpose of this dissertation has been to compare the chemical, physico-chemical, and biological characteristics of normal human and horse serum αLP and αP, to identify the protein moiety of very high density lipoproteins (VHDLP, density >1.210 g per ml) and to investigate the metabolism of radioiodinated human αLP and αP in normal and hyperlipidemic subjects.

Human and horse sera, analyzed for lipids and lipoproteins, were subjected to ultracentrifugation at 105,000 x g for 22 hours in a solvent density of 1.063 g per ml to remove lower density lipoproteins. The solvent density was adjusted to 1.210 g per ml and ultracentrifugation repeated to obtain αLP. The latter was washed 2 to 3 times with NaCl-KBr solution of density 1.210 g per ml and subjected to ultracentrifugation after each washing to remove traces of albumin. Subsequently, the αLP was delipidized at -30°C with redistilled ethanol and diethyl ether.

Horse serum contained less total lipids, greater proportions

of αLP and a lower percentage distribution of sphingomyelin and phosphoinositol than human serum. Human and horse serum αLP and αP dissolved in electrolyte solutions appeared as single boundaries during analytical ultracentrifugation and single protein bands after electrophoresis on agar or starch gel. The sedimentation rates and electrophoretic mobilities of human serum αLP and αP were comparable with those of horse serum αLP and αP. Human and horse serum αP had the same N-terminal amino acid (aspartic acid) and C-terminal amino acid (threonine). Adding sodium dodecyl sulfate to lipid-free αP, but not αLP, produced dissociation into a subunit whose reassociation could be obtained by dialysis. In vitro studies showed radioiodinated human and horse αP to be undenatured and capable of recombining with serum lipids in electrolyte solutions, urea, sodium dodecyl sulfate or succinic anhydride. Human and horse serum αP were immunochemically distinct and had different amino acid residues and peptide patterns following hydrolysis with chymotrypsin and/or trypsin. Human serum αP dissolved in 0.15 M NaCl, phosphate or barbiturate buffer solutions formed aggregates which were dissociated by increasing the pH or adding urea.

It was concluded that horse serum αP has physico-chemical and biological characteristics sufficiently similar to those of human serum αP to permit its use in studying the recombining capacity of αP with lipids.

Studies on human serum VHDLP other than lipalbumin were performed using αP-I^{131} and starch gel electrophoresis and indicated that the protein moieties of αLP and VHDLP are probably identical.

An α-lipoproteinuria was demonstrated in a patient with the

nephrotic syndrome following the injection of αLP-I^{131}.

The half-times of highly purified αLP-I^{131} or αP-I^{131} administered intravenously to normal subjects were similar, averaging 3.5 days. The half-time of αP-I^{131} was normal in one hypercholesterolemic subject, 1.7 and 1.9 days in two siblings with hyperchylomicronemia, and 2.5 and 2.7 days in two patients with essential hyperlipemia. The lipoprotein fraction of density >1.063 g per ml contained over 94 per cent of the injected radioactivity in studies during fasting on all subjects except two hyperchylomicronemic siblings in whom an increased radioactivity was observed in the chylomicron fraction (density <1.006 g per ml). The marked accentuation of chylomicron radioactivity, obtained in one of the siblings following a fatty meal was partially reversible by injecting heparin, which stimulates lipoprotein lipase in vivo.

These observations suggest that (1) αP-I^{131} catabolism is accelerated in hyperlipemia and that (2) in hyperchylomicronemia an association of chylomicrons with αP occurs in vivo.

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