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Research Article

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A New Method for Isolating the Nonidentical

Protein Subunits of Human Plasma α-Lipoprotein

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ABSTRACT Human plasma alpha lipoprotein (αLP) was totally delipidated by gel filtration on Sephadex LH-20 in a medium of 2 butanol: acetic acid: H_2O, 4 : 1 : 5. The resulting alpha protein (αP) exhibited two major bands, labeled C and D, on acrylamide-gel electrophoresis in 5.0 M urea at pH 8.8 or 4.0. Minor bands labeled A and B, also present, were shown to be aggregates of C which form when the latter is lyophilized. The C and D components were isolated in pure form from αP (prepared by LH-20 chromatography of αLP) by gel filtration of this protein on Sephadex G-200 in a medium of 1.0 N acetic acid: the C component emerged with a distribution coefficient (K_d) of 0.4, and the D component with a coefficient of 0.7. From each 100 mg of αP, 68 mg of C and 22 mg of D were isolated. 3 mg of a minor fraction with K_d 0.1, containing A and B components as well as C, were also obtained. D but not C reacts with rabbit antiserum to human αLP. C and D differ substantially in content of arginine, histidine, ½-cystine, isoleucine, and tryptophan.

INTRODUCTION

The protein moiety of human plasma α-lipoprotein (αLP) (d 1.063–1.21), which will be termed αP, is currently under study in several laboratories. The lipoprotein has been delipidated by treating the aqueous suspension with ethanol–diethyl ether (1) or with ethanol-acetone (2) or by lyophilizing the lipoprotein and then removing the lipid by ethanol-acetone extraction (3). The resulting H_2O-soluble preparations of αP exhibit on ultracentrifugation in aqueous buffers two peaks, with $s_{20, w}$ values of 2 and 4–10 depending on pH (4) ; but they sediment as a single homogenous peak in 0.05 M sodium dodecyl sulfate with molecular weight estimated as 23,710 by Scanu (4) and as 31,000 by Shore and Shore (5). These preparations, furthermore,

move as a single band in zone electrophoresis in paper (6), agar gel (4), or cellulose acetate (3). Therefore, until recently it was thought that αP consisted of a single peptide subunit, mol wt 23,000–31,000, with a marked tendency towards aggregation.

Recently Shore and Shore (7) reported the presence of both glutamine and threonine as C-terminal residues in αP. Furthermore, they observed that αP was resolved into two major components by acrylamide-gel electrophoresis in 8.0 M urea at pH 8.8. When αP was chromatographed on a column of DEAE-cellulose in pH 8.0 Tris buffer containing 8.0 M urea, two minor and three major fractions were obtained. The first of the major chromatographic fractions represented in apparently pure form the more rapidly moving of the two components seen on disc electrophoresis. The next two major fractions obtained by chromatography appeared to contain both electrophoretic components with the slower moving one predominating. By rechromatography of the second major fraction on DEAE-cellulose in 8 M urea, the slow electrophoretic component could also be isolated in pure form (8). Glutamine proved to be the C-terminal residue of the rapid component (termed R-gln), and threonine that of the slow component (R-thr). R-gln and R-thr were found to differ substantially in amino acid composition. Shore and Shore concluded that αP is a mixture of two non-identical peptides, and calculated from the yields of C-terminal glutamine and threonine per gram of αP, that each peptide has a molecular weight of about 15,000 (7, 8).

In this laboratory, evidence of a different type for the presence of two nonidentical subunits in αP was also recently obtained (3). αP was prepared by extracting the lyophilized αLP with ethanol-acetone, dissolving the residual protein in 1.0 N acetic acid, and relyophilizing. This material was resolved by gel filtration on Sephadex G-200 in 1.0 N acetic acid into three fractions with distribution coefficient (K_d) values 0.12, 0.49, and 0.64. The amino acid composition and tryptic fingerprints of

TABLE I
Amino Acid Composition of αP,

	αP			Fraction I	
Amino acid	Prepa-ration 1	Prepa-ration 2	Prepa-ration 3	Prepa-ration 1	Prepa-ration 3
Lysine§	94.0	80.5	90.1	91.9	93.1
Histidine‡	16.2	14.7	15.3	17.1	15.6
Arginine‡	46.3	47.2	48.5	59.5	60.5
Aspartic acid‡	76.7	81.3	79.5	82.9	84.7
Threonine‖	48.2	46.9	50.1	39.0	38.3
Serine‖	62.3	60.7	65.5	60.4	62.9
Glutamic acid‡	191.2	206.3	189.3	199.5	200.1
Proline‡	41.7	40.2	45.3	40.7	36.8
Glycine‡	47.7	44.5	44.1	41.5	43.5
Alanine‡	74.1	73.0	76.2	75.1	74.0
½-Cystine¶	4.3	5.4	5.2	0	0
Valine§	57.8	60.6	60.4	54.8	55.6
Methionine¶	12.0	13.3	13.1	14.2	14.7
Isoleucine§	7.1	7.7	7.3	0	0
Leucine‡	135.3	133.4	131.7	146.5	151.3
Tyrosine‖	29.6	27.1	26.9	23.8	22.4
Phenylalanine‡	32.6	33.4	34.1	23.8	26.1
Tryptophan**	23.1	22.4	22.0	30.4	28.4

Values represent moles of each residue/1000 moles of amino acids. Preparations labeled 1 were derived from αLP of subject L.A.G.; those labeled 2, from subject A.D.R.; those labeled 3, from subject S.A.

* From Shore and Shore (ref 8).

‡ Values obtained by averaging results of 24-hr, 48-hr, and 72-hr hydrolyzates.

§ Average of two highest values among 24-, 48-, and 72-hr hydrolyzates.

‖ Values calculated by extrapolation to zero time.

¶ Determined after oxidation with performic acid.

** Determined colorimetrically.

the three fractions were different, and only the third (K$_d$ 0.64) reacted with antiserum to αLP. It was concluded that αP contained two different subunits, that one of these subunits was the principal component of the third fraction, and that the other subunit (which appeared to have a greater tendency to aggregate in 1.0 N acetic acid) was the principal component of the first two fractions.

Thus it appears established that αP is a mixture of two nonidentical peptides, but methods for totally separating the peptides and isolating each in pure form are still inadequate, in part due to the tendency towards aggregation of the peptides after the lipid has been removed. We have therefore investigated different methods of delipidating αLP and separating the two major subunits. Cellular lipoproteins have been successfully delipidated by chromatography in organic solvents on the recently introduced Sephadex LH-20, an alkylated derivative of Sephadex G-25 (9). We have now found that human plasma αLP can be totally delipidated by the same technique. The resulting αP exhibits two major

components on disc electrophoresis in 5.0 M urea, pH 8.9 buffer. Furthermore, αP prepared in this manner is resolved by gel filtration on Sephadex G-200 into two major fractions, one containing only the slow electrophoretic component, and the other containing only the fast component. The two components, now readily available in pure form, have been shown to differ in quantitative amino acid composition, and in reactivity with antiserum to αLP. Their electrophoretic mobilities and amino acid compositions suggest that they correspond to R-gln and R-thr of Shore and Shore.

METHODS

αP was prepared from the plasma of fasting normal male subjects, 20–40 yr old, as described previously (3). The purity of each preparation was documented, as in the previous study, by its movement as a single band on cellulose acetate membranes at pH 8.6 and pH 3.9, by formation of a single line of precipitation with rabbit antiserum to human αLP in Ouchterlony immunodiffusion, and by failure to react in immunodiffusion with antisera to human serum albumin, β-lipoprotein, transferrin, fibrinogen, γ-globulin, or α₂-haptoglobin.

Fraction I, Fraction II, and Fraction III

| Fraction II | | | Fraction III | | | | |
Prepa-ration 1	Prepa-ration 2	Prepa-ration 3	Prepa-ration 1	Prepa-ration 2	Prepa-ration 3	R-gln*	R-thr*
90.1	99.1	95.6	118.1	129.7	120.5	115.0	77.7
17.1	16.0	17.5	3.7	3.6	3.8	0	22.6
57.0	61.0	62.8	9.4	9.0	9.8	0	67.6
88.2	85.2	83.5	55.1	55.3	52.0	40.6	98.0
40.5	39.0	37.9	69.7	70.0	71.3	81.9	38.0
59.4	60.6	62.6	72.5	70.0	72.4	82.3	62.5
198.6	187.2	190.0	203.6	193.0	191.8	197.6	181.0
38.1	40.6	41.7	50.4	50.5	53.4	52.3	36.9
43.1	44.5	45.5	45.5	44.7	46.5	42.5	44.3
77.8	69.2	71.5	66.3	69.0	70.5	68.1	75.8
0	0	0	13.3	12.8	12.1	15.0	0
49.8	57.1	55.8	73.1	74.8	70.7	76.8	51.3
13.1	14.4	15.0	12.7	13.0	14.0	14.8	15.0
0	0	0	20.0	17.7	19.3	15.0	0
145.9	150.3	150.9	98.8	95.6	99.9	103.2	148.1
24.9	22.0	22.6	42.6	38.9	40.6	45.2	28.4
25.7	23.3	26.1	48.8	49.0	50.1	49.9	22.9
30.0	30.4	29.5	0	0	0	0	30.0

Two types of column chromatography were employed, both at room temperature. (*a*) αLP was chromatographed on Sephadex LH-20 (Pharmacia). The gel particles were swollen and washed, with removal of fines, in 2 butanol: acetic acid:water, 4:1:5; a slurry of the concentrated gel was packed by gravity in a 3 × 100 cm column. A sample of αLP containing 40–70 mg of lipoprotein-protein was dissolved, as described in the experimental section, in 10 ml of the 2 butanol:acetic acid:water solvent system and introduced into the column; the column was eluted with the same solvent, at a flow rate of 40 ml/hr. 10-ml fractions were collected, 0.5-ml aliquots of each were dried at 80°C *in vacuo*, hydrolyzed in 2.5 N NaOH at 90°C, and analyzed with the colorimetric ninhydrin reagent (10). To analyze each fraction of the column for lipid content, 0.5-ml aliquots were dried at 80°C *in vacuo* and analyzed by the carboxyl ester method (11). (*b*) Preparations of αP (20–70 mg) dissolved in 4–10 ml 1.0 N acetic acid were chromatographed on a 2 × 200 cm column of Sephadex G-200 as previously described (3). A 0.2–0.5 ml aliquot of each 10 ml fraction was analyzed by the colorimetric ninhydrin reagent after alkaline hydrolysis.

Electrophoresis of αP and fractions of αP in 7.5% acrylamide gel was done in two systems: pH 8.9, 5.0 M urea; and pH 4.0, 5.0 M urea. The techniques of Ornstein and Davis (12) were used throughout, except that urea was added to the gel to a final concentration of 5 moles/liter.

Immunodiffusion of αLP, αP and fractions of αP was done as described previously (3). Antisera were purchased from Behringwerke and Hyland Laboratories, and their specificity was verified as in the previous study.

Phospholipid, total cholesterol, and carboxyl ester content of both ethanol-acetone (1:1) and chloroform-methanol (2:1) extracts of lyophilized αP preparations were determined according to Fiske and Subbarow (13), Abell, Levy, Brodie, and Kendall (14), and Rosenthal, Pfluke, and Callerami (11), respectively.

For measurement of the amino acid composition of αP and fractions thereof, duplicate samples of 1–2 mg of protein were hydrolyzed in 6.0 N HCl at 110°C for 24, 48, or 72 hr in sealed evacuated tubes. The hydrolysates were taken to dryness on a rotary evaporator at 40°C, and analyzed on a Beckman-Spinco 120 C instrument with the "4 hr protein hydrolyzate system" (15). Except for $\frac{1}{2}$-cystine, methionine, and tryptophan, values for each residue were calculated by extrapolation of 24, 48, and 72 hr hydrolysate data to zero time, or by averaging the two highest among the 24-, 48-, and 72-hr values as indicated in Table I. $\frac{1}{2}$-cystine and methionine were determined as cysteic acid and methionine sulfone on duplicate 24 hr 6.0 N HCl hydrolysates of performic acid–oxidized protein; a 94% recovery of cysteic acid was assumed (16). Calculation of the protein's content of $\frac{1}{2}$-cystine and methionine was based on the ratio of cysteic acid or of methionine sulfone

to leucine recovered in the same analysis of oxidized protein, and on the known content of leucine in the unoxidized protein. Tryptophan, destroyed by acid hydrolysis, was determined by the method of Spies and Chambers (17). The composition of the protein was expressed as moles of each residue per 1000 moles of amino acid.

RESULTS

Delipidation of αLP on LH-20 column. From 270 ml of normal human plasma, we obtained 80–100 ml of αLP solution. After documentation of purity by electrophoresis on cellulose acetate and by immunodiffusion against antisera to various human plasma proteins, a 25 ml batch of this solution was freed of salt by dialyzing against 12.0 liters H_2O overnight and then concentrated 4 to 5-fold by dialysis against 20% polyvinylpyrrolidone (grade 40). To 5 ml of this turbid concentrate was added dropwise, with stirring, a mixture of 1 ml acetic acid with 4 ml sec-butanol. The αLP solution became transparent during this addition. This solution (containing about 40–70 mg lipoprotein-protein) was then subjected to gel filtration on LH-20, with the result shown in Fig. 1. 90% of the protein of αLP was recovered in tubes 23–27 (cumulative volume 230–270 ml) unaccompanied by any detectable lipid; all the lipid was recovered in tubes 33–42. Tubes 23–27 were pooled,
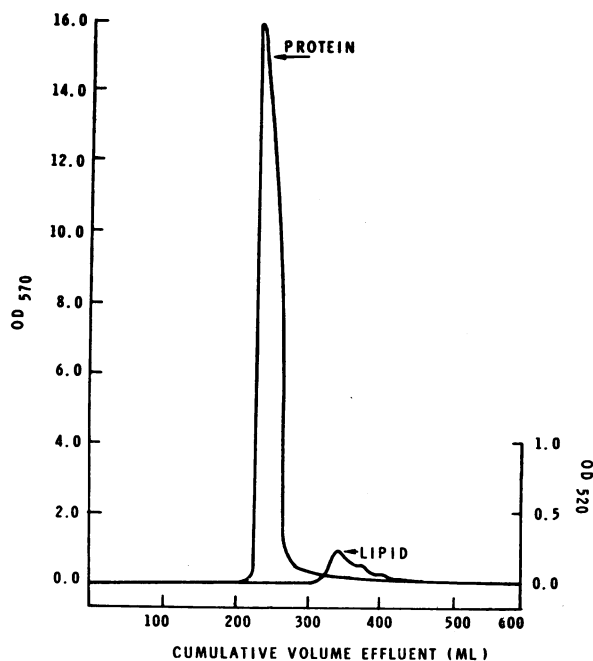

FIGURE 1 Gel filtration of αLP containing 50 mg protein on a 3 × 100 cm column of Sephadex LH-20 in a medium of 2 butanol : acetic acid : H_2O 4 : 1 : 5 (v/v/v). Left-hand ordinate scale represents optical density of colorimetric ninhydrin analysis; right-hand ordinate shows optical density of carboxyl ester analysis.
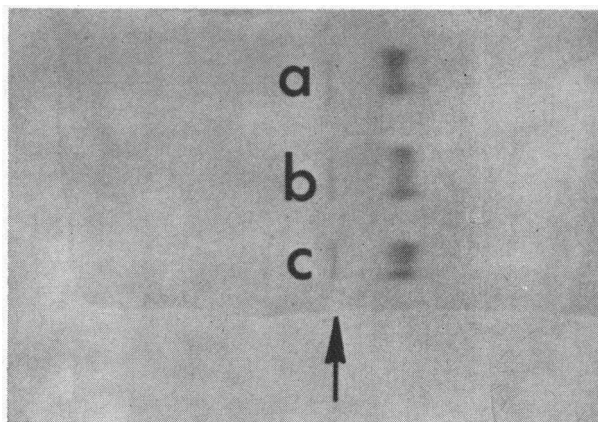

FIGURE 2 Electropherogram on cellulose acetate in barbital buffer (pH 8.6) containing 2.0 M urea of (*a*) unlyophilized αP (LH-20), (*b*) lyophilized αP (LH-20), and (*c*) αP (E/A). Arrow indicates line of sample application.

and concentrated from a volume of 50 ml to 8 ml by a stream of N_2 at 40°C. This concentrated solution contained about 7–11 mg protein/ml. In order to characterize the protein in the solution and to determine the optimal means of recovering the protein from it, the following experiments were done.

Characterization of αP prepared by LH-20. Preparatory to immunodiffusion or electrophoresis in various systems, 1 ml aliquots of this concentrated solution were dialyzed against 1.0 liter of each of these solutions: 0.05 M Na_2HPO_4 for immunodiffusion and for electrophoresis at pH 8.6–8.8 on cellulose acetate or in acrylamide gel; 1.0 N acetic acid for electrophoresis at pH 3.9 on cellulose acetate or at pH 4.0 in acrylamide gel. These preparations will be referred to as "unlyophilized αP (LH-20)." A 5 ml aliquot was dialyzed against 1.0 liter of 1.0 N acetic acid and then lyophilized to yield 30–50 mg of white fluffy powder readily soluble in aqueous buffers from pH 3 to pH 11. Samples of this material (termed "lyophilized αP [LH-20]"), dissolved in the appropriate buffer, were compared in some tests with unlyophilized αP (LH-20), in order to determine the possible effect of lyophilization on the electrophoretic and immunodiffusion patterns; the lyophilized preparation was also used in determining the lipid content and quantitative amino acid composition of αP prepared by the LH-20 technique. Finally, in some experiments, from the same batch of αLP used to prepare αP (LH-20), a sample of αP (termed αP [E/A]) was prepared by the previously described method (3) of lyophilizing αLP, extracting the lipid with ethanol-acetone, dissolving the residual αP in 1.0 N acetic acid, and relyophilizing. This was done in order to compare the properties of αP prepared by two different techniques of delipidation. In addition, several preparations of fractions I, II, and III
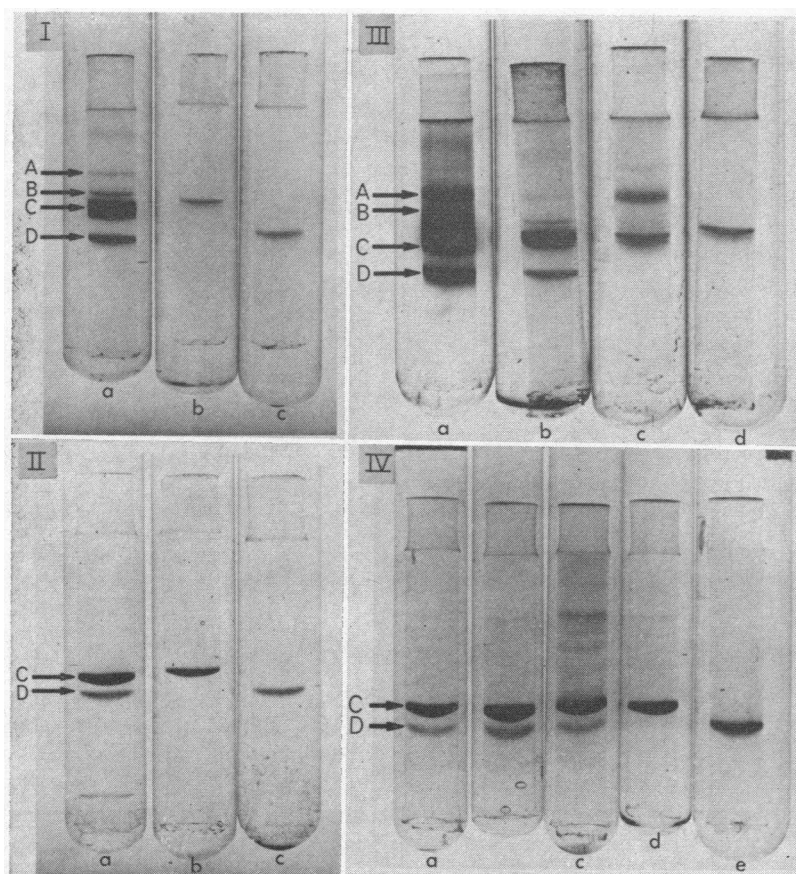
FIGURE 3 Panel I. Disc electrophoresis in 5.0 M urea, pH 8.8 buffer for 1 hr, (a) unlyophilized αP (LH-20); (b) unlyophilized fraction II; (c) unlyophilized fraction III. Panel II. Disc electrophoresis in 5.0 M urea, pH 4 buffer for 1 hr, (a) unlyophilized αP (LH-20); (b) unlyophilized fraction II; (c) unlyophilized fraction III. Panel III. Disc electrophoresis in 5.0 M urea, pH 8.8 buffer for 1.5 hr, (a) lyophilized αP (LH-20); (b) unlyophilized αP (LH-20); (c) unlyophilized fraction I; (d) unlyophilized fraction II. Panel IV. Disc electrophoresis in 5.0 M urea, pH 4 buffer for 1.5 hr, (a) unlyophilized αP (LH-20); (b) lyophilized αP (LH-20); (c) αP (E/A); (d) lyophilized fraction II; (e) lyophilized fraction III.

derived from αP (E/A) in our previous study (3) by gel filtration on Sephadex G-200 were reexamined in the present investigation by acrylamide-gel electrophoresis in 5.0 M urea, pH 8.9 buffer.

αP prepared by the LH-20 technique contained no detectable phospholipid, cholesterol, or triglyceride (less than 0.01 mg of each lipid species per mg of αP). On cellulose acetate membranes at pH 8.6 or pH 3.9, unlyophilized αP (LH-20), lyophilized αP (LH-20), αP (E/A), and αLP itself all moved as single bands with identical mobility (Fig. 2). All four preparations gave a single line of precipitation on double diffusion against anti-αLP; the three preparations of αP showed a reaction of identity, while spur formation was present between this line of reaction and the line of reaction be-

tween αLP and its antiserum. The three types of αP showed similar but not identical patterns on acrylamide-gel electrophoresis (Fig. 3). At pH 8.9, unlyophilized αP (LH-20) exhibited two major bands, labeled C and D, D being the more rapid. Two fainter bands, A and B, followed C, and occasionally a faint component slower than A could be seen (Fig. 3, I-a,[1] 3, III-b). The effect of lyophilizing αP (LH-20) was to increase the prominence of the A and B bands (Fig. 3, III-a). αP (E/A) resembled lyophilized αP (LH-20) but additional bands slower than A were more prominent. At pH 4, unlyophilized αP (LH-20) showed only two visible bands, the slower being labeled C and the faster D because subsequent experiments identified them as corresponding to

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[1] Electropherogram a in panel I of Fig. 3.
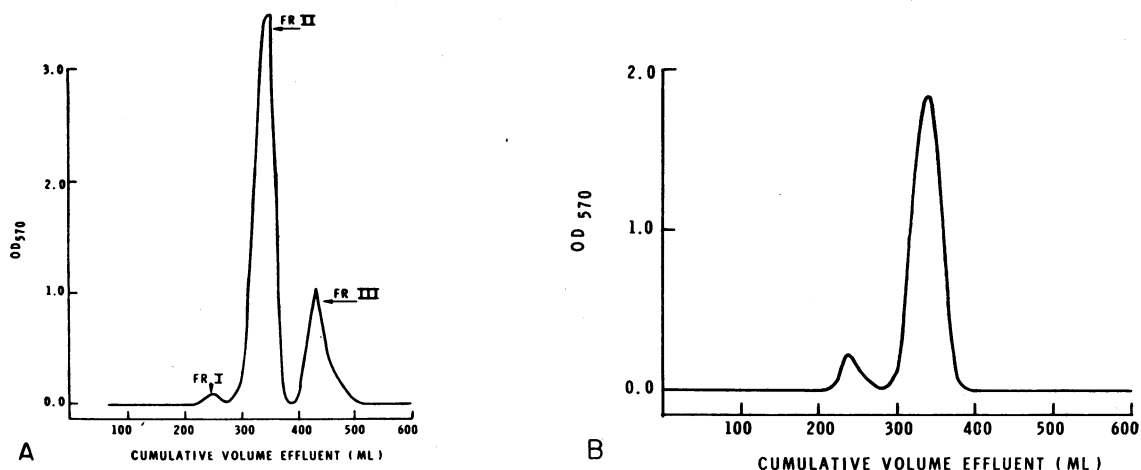
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FIGURE 4 A. Gel filtration of 40 mg unlyophilized αP (LH-20) on 2 × 200 cm column of Sephadex G-200 in 1.0 N acetic acid. Ordinate shows ninhydrin value (OD_{570}) per ml of hydrolyzed effluent. Abscissa shows cumulative volume of effluent, collected in 10-ml fractions. B. Gel filtration under same conditions of 20 mg lyophilized fraction II.

the C and D bands in the alkaline electropherograms. Lyophilized αP (LH-20) showed a series of faint bands preceding C (Fig. 3, IV-a); this series was more prominent in αP (E/A) (Fig. 3, IV-c).

Separation of αP (LH-20) into three fractions by gel filtration on G-200. 40 mg of unlyophilized αP (LH-20) in 4 ml 1.0 N acetic acid was placed on the Sephadex G-200 column and eluted with the same solvent. Ninhydrin analysis of the effluent (Fig. 4 A) revealed three peaks: a minor fraction I, K_d value 0.1; and major fractions II and III with K_d values 0.4 and 0.7. Each peak was pooled and concentrated 10-fold by evaporation under nitrogen at 40°C. 1 ml aliquots of these three concentrates were dialyzed against 5.0 M urea buffer of pH 8.8 and 4.0, preparatory to acrylamide-gel electrophoresis. The electropherograms at both pH's showed that fraction II contained only band C, and that fraction III contained only band D (Fig. 3, I-b and c, and 3, II-b and c). Fraction I consisted of a mixture of C with varying amounts of A and B (Fig. 3, III-c). The remainder of these three fractions were then lyophilized. Yields from 100 mg αP averaged 2 mg fraction I, 68 mg fraction II, and 22 mg fraction III. Lyophilization led to the appearance of a series of bands (including A and B) slower and fainter than band C in both the pH 8.8 and pH 4 disc electropherograms of fraction II; it did not alter the appearance of fraction III (Fig. 3, IV-d and e). On immunodiffusion fraction III reacted with anti-αLP, showing a line of identity with lyophilized αP (LH-20); fraction II was unreactive.[a] When *unlyophilized* fraction

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[a] These observations were confirmed with preparations of fractions II and III from five different blood donors and with three different lots of rabbit antiserum to human αLP: Behringwerke lots 927/c67, 1228b/g67, and 9713/c67.

II was passed through the G-200 column in 1.0 N acetic acid a second time, it emerged with unchanged K_d of 0.4; but when *lyophilized* fraction II was passed through the G-200 column a second time, the elution profile revealed about 15% fraction I and 85% fraction II (Fig. 4 B). Fractions II and III showed major differences in their content of the following amino acids: histidine, arginine, ½-cystine, isoleucine, and tryptophan (Table I). Fractions I and II, on the other hand, did not differ significantly in amino acid composition.

We had previously (3) prepared αP (E/A) by extraction of lyophilized αLP and separated this αP on G-200 into fractions I, II, and III with K_d values of approximately 0.1, 0.4, and 0.6. For comparative purposes samples of these three earlier fractions were now reexamined in the present acrylamide-gel systems. Fraction III consisted of a doublet in the region of the D band with one minor component slower than A. Fraction II consisted mainly of C band, with A and B visible; fraction I was a mixture of C, A and several bands slower than A.

DISCUSSION

Gel filtration of αLP in 2 butanol: acetic acid: H_2O (4:1:5) appears to be an effective way of preparing totally (> 99%) delipidated αP. Unlyophilized αP prepared by this method presents a gel electrophoretic pattern in 5.0 M urea, pH 8.8 buffer of two major bands C and D, with two minor slower bands A and B. This is generally similar to that of the αP of Shore and Shore (7), delipidated with ethanol-ether and examined in 8.0 M urea at pH 8.8. Lyophilization of the present αP preparation increases the prominence of bands migrating

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more slowly than C in both acid and alkaline electropherograms. On gel filtration of αP (LH-20) on G-200 in 1.0 N acetic acid, these minor bands of slow mobility are recovered in the minor, highly aggregated fraction I. Lyophilization of fraction II containing only C band (but not of fraction III containing only D band) leads to appearance of A and B by disc electrophoresis and fraction I by gel filtration. Thus bands A and B appear to be aggregates of band C. This is supported by the closely similar amino acid compositions of fractions I and II. Numerous slow bands, including A and B, are also prominent in αP which has been prepared by the previous method of extracting lyophilized αLP and then lyophilizing the resulting lipid-free protein. This latter type of αP also contains a more prominent high molecular weight component, K_d 0.1, by gel filtration on G-200, than does the present unlyophilized αP (LH-20) (3). Thus it seems probable that exposure of αP in the solid phase to ethanol-acetone, as well as lyophilization, leads to extensive aggregation of the "C component" of αP, leading to a more complicated pattern on analysis by gel filtration or by acrylamide-gel electrophoresis.

αP (LH-20) thus contains two major components, C and D, by acrylamide-gel electrophoresis. The C band is recovered in pure form as fraction II (K_d 0.4) after gel filtration on G-200, and the D band as fraction III (K_d 0.7). These two components differ in their amino acid composition, showing that C is not merely a more aggregated form of the same protein present in D. The absence of ½-cystine and of isoleucine in C (fraction II), while they constitute about 13 and 19 moles/1000 residues in D (fraction III), indicates that if D is a single protein species it is not present to a detectable extent in C. Reaction of D but not of C with the antiserum to αLP is additional evidence for the same conclusion. Conversely, the absence of tryptophan in D, and its presence in C, show that if C is a single protein species, it is not present to a detectable extent in D. The simplest hypothesis consistent with all the data is that αP is made up of two nonidentical subunits, corresponding to the C and D bands. The work of others (4, 5, 7) indicates that these two nonidentical subunits have closely similar molecular weights, although estimates of this value have ranged from 15,000 to 31,000. The separability of C and D components by gel filtration despite this similarity in molecular weight suggests that after delipidation C has either a greater tendency to aggregate with itself in 1.0 N acetic acid, or a larger radius, or both, than does D.

The present information does not rule out the possibility that more than two subunits could be present. Thus, if the isoleucine-containing K_d 0.6 fraction III (band D) contained two peptides, one of which lacked isoleucine, then the absence of isoleucine in K_d 0.4 fraction II (band C) would no longer prove that fractions II and III do not have a subunit peptide in common. Conversely, if fraction II (band C) contained two proteins, one of which lacked tryptophan, then the absence of tryptophan in fraction III (band D) would not prove that C and D do not contain a subunit in common. For the present, however, there is no actual evidence for more than two components in αP or for heterogeneity of components C and D.

The C and D bands in the alkaline gel electropherogram of the present αP resemble the two major components in Shores' αP, analyzed in a similar electrophoretic system (7). Presumably the present C (fraction II) is identical to the Shores' slow component R-thr, while D (fraction III) is identical with their fast component (R-gln). This conclusion is supported by the general similarity in amino acid composition of fraction II and R-thr, and of fraction III and R-gln (Table I). Thus fraction II in comparison to fraction III contains a substantially greater proportion of histidine, arginine, leucine, and tryptophan, and a considerably lesser proportion of lysine, threonine, ½-cystine, isoleucine, tyrosine, and phenylalanine. Similar relationships were reported by Shore and Shore (8) for R-thr compared to R-gln. Nevertheless the two sets of amino acid data do not agree completely. All three preparations of fraction III contain small but definite proportions of histidine and arginine, reported to be absent in R-gln. Lesser discrepancies concern lysine and aspartic acid in fraction II vs. R-thr, and aspartic acid, threonine, and serine in fraction III vs. R-gln. The explanation for these inconsistencies between the two sets of data is not presently apparent.

What is the relation of the present C and D components to fractions I, II and III of αP described in our earlier report (3)? Acrylamide-gel electrophoresis indicates that D is the major component in fraction III, and C the major component in fractions I and II but that some cross-contamination of the two nonidentical subunits was present in these earlier preparations. The cleaner separation of the two types of subunit by Sephadex G-200 in the present than in the earlier study may be related to the lesser proportion of highly aggregated material in αP (LH-20) than in αP (E/A).

The present technique has the advantage of a complete separation of C and D. No fraction representing an aggregate of both subunits is obtained, as in previously reported methods (3, 7). Components C and D are now consistently isolated from αP in a weight ratio close to 3:1. The amino acid compositions of αP, fraction II, and fraction III (Table I), when expressed as grams of each residue/100,000 g of protein, are similarly consistent with the presence of these two subunits in αP in a weight ratio of 3:1. If both peptides have a molecular

weight close to 15,000 (7, 8), then the molar ratio of C and D in αP will also be 3:1.

Perhaps each αLP particle likewise contains peptides C and D in a molar ratio of 3:1, together with various lipids. Alternatively αLP may not be homogeneous with regard to peptide composition. As an extreme example, human serum might contain two types of αLP (with closely similar electrophoretic mobilities and densities), one based on peptide C and the other on D. Determination of the ratios of C to D in appropriate subfractions of αLP will clarify this problem.

ACKNOWLEDGMENTS

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