Composition of the Protein from Dog Lymph Chylomicrons and Dog Serum High Density Lipoprotein*

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ABSTRACT: The protein moiety of dog serum high density lipoprotein and the proteins isolated from dog lymph chylomicrons by various methods of extraction were on the basis of a quantitative amino acid analysis found to be different in composition. The four chylomicron protein fractions studied—the Veronal buffer soluble, the urea-soluble, the material insoluble in either media, and the unfractionated chylomicron protein—also were found to vary in their quantitative amino acid composition. These results indicate that either the serum high density lipoprotein and any one of the chylomicron proteins are different proteins or that the chylomicron protein under consideration contains other proteins as well as high density lipoprotein.

Conclusive evidence has been offered that the low and high density lipoproteins of plasma are not interconvertible (Avigan *et al.*, 1956; Shore, 1957; Gitlin *et al.*, 1958). Similarities, however, have been observed between the protein composition of the plasma high density lipoprotein and the lymph chylomicrons. These similarities were found in their electrophoretic mobilities, N-terminal amino acids, and the fingerprinting of their peptides (Rodbell and Fredrickson, 1959). This paper describes the results of a quantitative amino acid analysis of dog high density lipoprotein and the proteins isolated from dog lymph chylomicrons by various methods of extraction.

Experimental Procedure

Collection of Lymph Chylomicrons. Lymph chylomicrons were obtained by cannulation of the thoracic duct of male mongrel dogs. The animals were anesthetized with sodium nembutal and fed heavy cream *via* a stomach tube before cannulation of the duct. The feeding of cream was repeated at approximately hourly intervals during the 6–7 hr of lymph collection. The yield of lymph from each animal varied from 120 to 360 ml. The lymph was allowed to clot at room temperature. The clot was removed by straining through cheesecloth; the lymph was then layered under an equal volume of 0.9% sodium chloride and centrifuged in a Beckman–Spinco Type 30 rotor at 22,500 rpm (44,100*g*) for 68 min. The solid buttery layer at the top of the tube and the lactescent portion of the liquid immediately beneath it were removed by aspiration and mixed together with saline. This mixture was emulsified by extrusion through a 20-gauge needle. The chylomicrons thus obtained were washed twice with saline and finally reemulsified with saline by repeated extrusion through a 24-gauge needle. To check the possibility of contamination of the chylomicrons by other lipoproteins or proteins, analyses were made at 26° in a Beckman–Spinco analytical ultracentrifuge at densities of 1.063 and 1.21 g/ml. No other lipoproteins or proteins were detected.

Delipidation of Chylomicrons. Removal of lipid was accomplished by the method of Rodbell and Fredrickson (1959). One volume of the reemulsified chylomicrons was added dropwise to 12 volumes of chloroform–methanol (2:1) at room temperature. After standing for 2 hr the protein which flocculated at the interface between the aqueous and organic layers was packed by centrifugation and reextracted with 10 volumes of ethanol–acetone (1:1). This was followed by two washes with acetone and two washes with diethyl ether. The protein residues thus obtained were air dried and stored in a desiccator over Drierite.

Isolation of High Density Lipoprotein from Dog Serum. Blood was collected from male mongrel dogs by cannulation of the femoral artery. The blood was allowed to clot at room temperature and the serum separated by centrifugation. The low density lipoproteins were removed by precipitation with sulfated rice starch (Bernfeld *et al.*, 1957). Following removal of the low density lipoproteins, the serum was adjusted with crystalline sodium bromide to a density of 1.21 g/ml and centrifuged in a Beckman–Spinco Type 30 rotor at 20,000 rpm (34,850*g*) at 4° for 69 hr. The high density lipoprotein which floated to the top of the tube under these conditions was removed with a tube slicer. To remove any low density lipoproteins which might still be present, the mixture was refloated by layering

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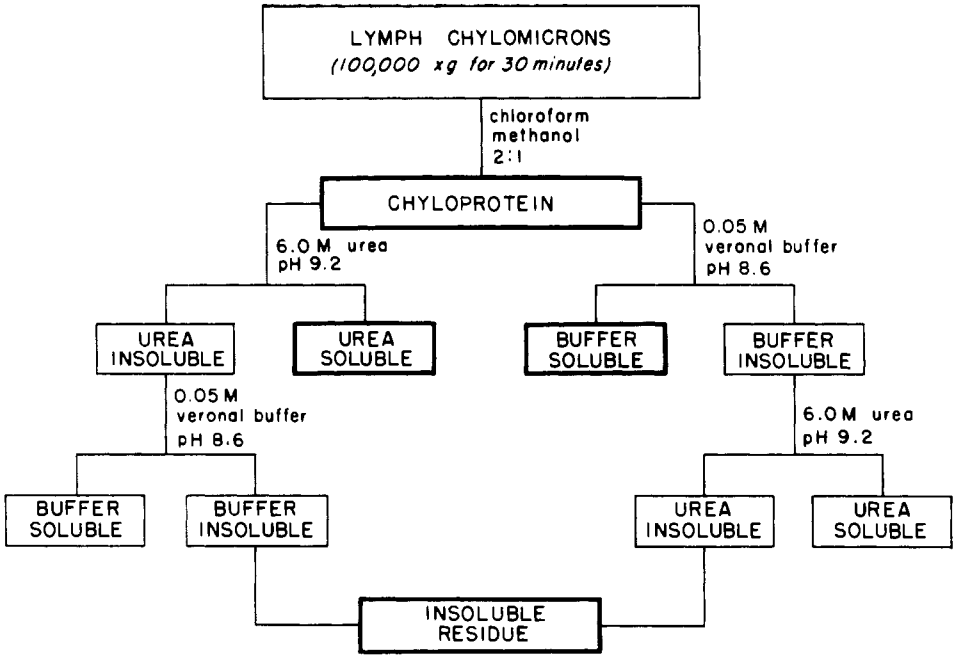

FIGURE 1: Isolation procedures for chylomicron proteins.

beneath an equal volume of sodium chloride (density 1.05 g/ml) and centrifuged in a Beckman–Spinco Type 40 rotor at 37,500 rpm (92,660*g*) at 4° for 40 hr. The low density lipoproteins, if present, floated to the top of the tube and were removed.

Homogeneity of the high density fraction was determined by moving boundary electrophoresis and analytical ultracentrifugation. No other lipoproteins or proteins were found to be present. The high density lipoprotein was delipidated by employing the same method used in the delipidation of the chylomicrons. To ensure the completeness of delipidation, both the chylomicron and high density protein residues were examined for lipid phosphorus (Lowry *et al.*, 1954). None was detected.

Differentiation of Chylomicron Proteins by Their Solubility Behavior. Separate aliquots of the lipid-free protein were extracted with 5-ml portions of 6.0 M urea (pH 9.2) and 0.05 M Veronal buffer (pH 8.6), respectively. The extractions were repeated until the amount of protein that could be extracted was negligible as determined by the Kabat and Mayer (1961) modification of the Folin and Ciocalteu protein method. The residue remaining after urea extraction was reextracted with buffer, and the residue remaining after buffer extraction was reextracted with urea. These remaining insoluble materials were combined and suspended in 0.05 M sodium chloride. The three samples, *i.e.*, the buffer-soluble, the urea-soluble, and the insoluble chyloproteins, were dialyzed against two changes of 0.05 M sodium chloride for 48 hr and then lyophilized (Figure 1).

Aliquots of the various air-dried proteins were hydrolyzed with constant boiling 6 N HCl (200 times weight of protein) for 22 hr at 110 ● 0.5°. Samples of the hydrolysate containing the equivalent of approximately 2 mg of protein, based on the factor 6.25 times the Kjeldahl nitrogen content (Lang, 1958), were analyzed quantitatively for amino acid constituents on a Beckman–Spinco Model 120 amino acid analyzer. After corrections were applied for decomposition of serine (0.90), threonine (0.95), and tyrosine (0.95) during hydrolysis (Crestfield *et al.*, 1963), it was found that about 92% of the hydrolyzed protein was recovered in the amino acids listed. Cysteine, cystine, and tryptophan were not determined. All values were converted from micromoles of amino acid per milligram of protein to moles of amino acid/100,000 g of protein.

Results

In order to demonstrate that the amino acid analyzer is capable of determining the identity of a protein and, therefore, is able to distinguish between two different proteins, two hydrolysates were prepared from the same protein, *i.e.*, a highly purified human serum β-lipoprotein which had been found to be homogeneous and free from any other lipoprotein or protein. Application of Student's *t* test to the data obtained from duplicate analyses of these two hydrolysates[1] showed that at a

[1] It is assumed that each pair of hydrolysates is normally distributed with means μ_1 and μ_2 and equal variances. This assumption appears to be justified by comparing the standard deviations. The hypothesis to be tested by the *t* test is $\mu_1 = \mu_2$ and the test statistic is

$$t = \frac{2(\bar{X}_1 - \bar{X}_2)}{\sqrt{(X_{11} - X_{12})^2 + (X_{21} - X_{22})^2}}$$

where $f = n_1 + n_2 - 2 = 2$.

TABLE I: Amino Acid Composition of Protein from Human Serum β-Lipoprotein.[a]

Amino Acid	Human Serum β-Lipoprotein Hydrolysate I[b]	Human Serum β-Lipoprotein Hydrolysate II[b]	Value of t[c]
Alanine	48.59 ± 1.17	48.49 ± 0.38	0.11
Arginine	26.69 ± 0.98	27.18 ± 1.42	0.40
Aspartic acid	78.84 ± 0.95	77.54 ± 0.27	1.86
Glutamic acid	88.88 ± 1.17	88.99 ± 0.26	0.13
Glycine	38.92 ± 0.02	38.37 ± 0.25	3.14
Histidine	18.83 ± 1.16	19.69 ± 0.13	0.98
Isoleucine	44.56 ± 0.48	44.59 ± 0.29	0.08
Leucine	87.98 ± 1.18	87.67 ± 0.13	0.37
Lysine	61.44 ± 1.70	64.93 ± 0.21	2.88
Methionine	9.30 ± 0.71	9.80 ± 0.14	0.84
Phenylalanine	39.13 ± 0.61	40.03 ± 0.82	1.24
Proline	32.06 ± 1.65	32.79 ± 0.48	0.60
Serine[d]	69.42 ± 0.40	67.48 ± 0.45	4.62
Threonine[d]	52.29 ± 0.45	50.93 ± 0.18	3.94
Tyrosine[d]	28.82 ± 2.85	26.13 ± 0.00	1.33
Valine	42.23 ± 1.61	42.84 ± 0.81	0.48

[a] Moles of amino acid/100,000 g of protein. [b] Mean values and standard deviations from duplicate analyses of two identically prepared 22-hr hydrolysates from the same protein (human serum β-lipoprotein). [c] The t value obtained on application of Student's t test to these data; $t_{0.95} = 4.303$. [d] Corrected for decomposition during 22-hr hydrolysis (serine, 0.90, threonine, 0.95, and tyrosine, 0.95).

95% level of confidence only one of the sixteen amino acids measured was significantly different (Table I).

The variously extracted proteins from the lymph chylomicrons—the buffer-soluble, urea-soluble, and the insoluble as well as the unfractionated chyloprotein and the serum high density lipoprotein—were analyzed in duplicate on the amino acid analyzer. The results were calculated in moles of amino acid/100,000 g of protein (Table II).

The high density protein and the Veronal buffer soluble chyloprotein are, according to Rodbell and Fredrickson (1959), identical. A comparison of their amino acid composition is shown in Figure 2. To facilitate comparison, a semilog plot is made of the amount in moles of each amino acid in decreasing order of concentration. This simplified method of comparison is similar to that suggested by Cook and Martin (1962). Application of the t test to duplicate analyses of hydrolysates of these two proteins showed that at a 95% level of confidence fifteen out of the sixteen amino acids determined were present in significantly different quantities. Only valine showed no significant difference in amount in the two proteins.

The four protein fractions from the chylomicrons were then compared with each other in a similar fashion (Figure 3). None appears to be identical with any other, but with the exception of the insoluble residue their patterns follow a similar trend.

Discussion

These results have been compared with those of Allerton *et al.* (1962) who examined the amino acid

TABLE II: Amino Acid Composition of the Protein from Soluble and Insoluble Dog Lymph Chylomicron Protein and Dog Serum High Density Lipoprotein.[a]

Amino Acid	Buffer-Soluble Chyloprotein[b]	Urea-Soluble Chyloprotein[b]	Insoluble Residue[b]	Unfractionated Chyloprotein[b]	Serum HDL Protein[b]
Alanine	72.33 ± 0.50	69.80 ± 0.64	61.92 ± 0.35	66.41 ± 0.07	84.26 ± 0.71
Arginine	33.30 ± 0.07	31.92 ± 0.00	36.98 ± 0.85	33.96 ± 0.35	50.84 ± 0.14
Aspartic acid	62.61 ± 0.07	62.32 ± 0.28	39.58 ± 0.14	64.78 ± 0.21	67.15 ± 0.14
Glutamic acid	102.97 ± 0.71	98.02 ± 0.35	75.00 ± 0.57	96.42 ± 0.50	148.43 ± 0.42
Glycine	45.38 ± 0.28	50.19 ± 1.13	64.58 ± 0.64	49.30 ± 0.14	38.02 ± 0.35
Histidine	18.39 ± 0.21	18.84 ± 0.64	17.80 ± 0.64	18.82 ± 0.28	6.82 ± 0.14
Isoleucine	15.44 ± 0.21	18.68 ± 0.21	25.51 ± 0.35	19.19 ± 0.07	8.06 ± 0.14
Leucine	82.84 ± 0.07	80.47 ± 0.00	77.77 ± 0.21	77.84 ± 0.35	117.12 ± 0.14
Lysine	69.28 ± 0.00	63.86 ± 1.27	52.64 ± 0.78	60.79 ± 0.50	55.72 ± 0.42
Methionine	4.94 ± 0.07	3.79 ± 0.07	4.48 ± 0.01	7.59 ± 0.07	2.13 ± 0.00
Phenylalanine	35.68 ± 0.50	30.80 ± 0.42	33.91 ± 0.14	34.74 ± 0.99	15.51 ± 0.28
Proline	46.40 ± 0.64	51.60 ± 1.20	58.74 ± 2.76	48.54 ± 0.42	30.10 ± 0.64
Serine[c]	55.20 ± 0.07	63.65 ± 1.06	90.70 ± 0.14	65.40 ± 0.00	50.58 ± 0.28
Threonine[c]	45.59 ± 1.06	50.63 ± 0.50	60.37 ± 0.14	47.77 ± 0.21	25.30 ± 0.21
Tyrosine[c]	28.46 ± 0.35	29.14 ± 0.35	29.07 ± 0.01	28.90 ± 0.35	21.59 ± 0.28
Valine	56.55 ± 1.49	58.68 ± 2.12	67.08 ± 0.07	58.88 ± 0.21	49.45 ± 0.42

[a] Moles of amino acid/100,000 g of protein. [b] Mean values and standard deviations from duplicate analyses of a 22-hr hydrolysate. [c] Corrected for decomposition during 22-hr hydrolysis (serine, 0.90, threonine, 0.95, and tyrosine, 0.95).

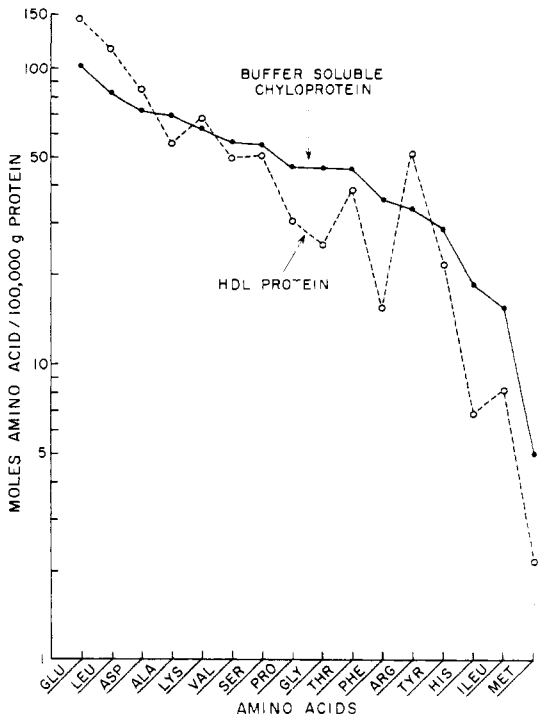

FIGURE 2: Semilog plot of the concentrations of sixteen amino acids found in buffer-soluble chyloprotein and serum high density lipoprotein from the dog.

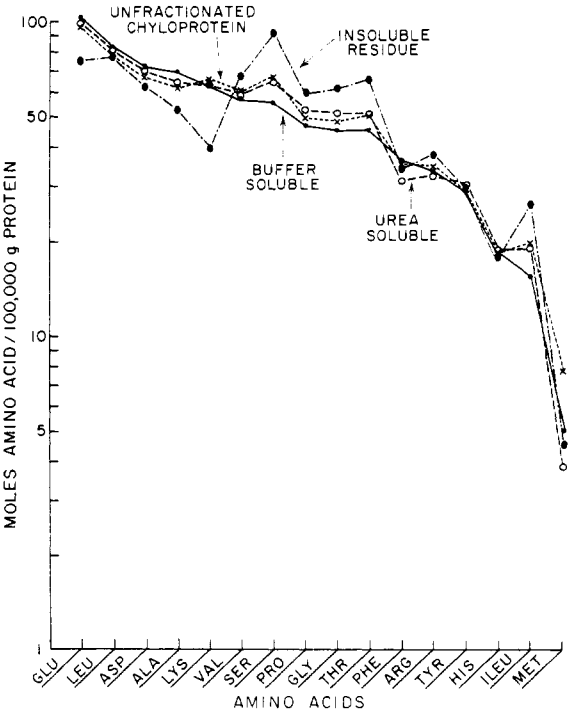

FIGURE 3: Semilog plot of the concentrations of sixteen amino acids found in buffer-soluble, urea-soluble, unfractionated chyloprotein, and the residue insoluble in either urea or Veronal buffer.

composition of dog plasma albumin. The differences detected between the chylomicron proteins themselves and between the chylomicron proteins and the high density protein could not have been due to contamination by dog plasma albumin.

Rodbell and Fredrickson contended on the basis of end group analyses, electrophoretic mobilities, and fingerprinting techniques that the protein from plasma high density lipoprotein and the Veronal buffer soluble protein from lymph chylomicrons are identical in the dog. We have isolated these proteins by methods similar to those used by Rodbell and Fredrickson. Electrophoretic and ultracentrifugal analysis indicated that our products were essentially the same as theirs. However, our present study would indicate that on the basis of amino acid composition the two proteins are not the same. At least two explanations for this dissimilarity can be advanced. Either the high density protein and buffer-soluble chyloprotein are two completely different proteins, or the buffer-soluble chyloprotein contains not only the high density protein but another protein as well. Such a mixture could account for a nonidentical amino acid composition This latter possibility was considered by Lindgren and Nichols (1960) in proposing a hypothetical model for lipoproteins of the low density classes (to include chylomicrons): "In the low density serum lipoproteins, the properties of the protein moiety predominate over that of the lipid moiety indicating exposure of a significant portion of this protein moiety. On the other hand, considering both the chemical composition and the molecular weights of the major classes of low density lipoproteins, only a portion of the lipoprotein surface could be covered with protein, thus requiring substantial exposure of the lipid moiety. Further, consideration of the molecular weights of the protein moiety of the lipoproteins of the S_f 20–10^5 class would require the presence of increasing numbers of substructural protein units (50,000–300,000 in molecular weight) in order to yield the proper total protein content. Thus, a lipoprotein model might consist of a lipid core partially surrounded by substructural protein or lipoprotein units." If some of the substructural units which surround the lipid core of the chylomicrons are high density lipoproteins, then any similarities between the protein moieties of the high density lipoprotein and chylomicrons would be understandable.

Since in the present study none of the proteins derived from chylomicrons were identical with the protein of serum high density lipoprotein, speculation suggests that at least two forms of protein might be associated with chylomicrons. One form could be a combination of protein and lipid identical with high density lipoprotein of serum; another form could include a protein or proteins not associated with lipids. The further separation of these protein components was not attempted in these experiments because of the limited amount of material available.

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The Specific Cleavage of Ribonucleic Acid from Reticulocyte Ribosomal Subunits*

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ABSTRACT: Ribonucleic acid (RNA) isolated from each of the two subunits of reticulocyte ribosomes has been digested with pancreatic ribonuclease. The conditions were adjusted so that the early stages of digestion could be observed by separation of the products by polyacrylamide gel electrophoresis. The course of digestion is highly specific suggesting that certain regions are exceptionally prone to enzymatic attack. The patterns of degradation of the two types of RNA are entirely different implying that these regions are distributed at characteristic intervals in each case. The first products of digestion are relatively large, having molecular weights in the range of 250,000–400,000. As the digestion proceeds, the higher molecular weight species are degraded into smaller fragments in the range of 20,000–250,000 molecular weight.

The results indicate that the number of vulnerable regions is of the order of 5 for the 30S component and 3 for the 19S component. It is suggested that these regions are attacked preferentially because the RNA chain is folded in a specific manner which leaves them uniquely exposed.

Excellent resolution of high molecular weight ribonucleic acid (RNA) species in the range of 2–14 S has been obtained using polyacrylamide gel electrophoresis (Richards *et al.*, 1965; McPhie *et al.*, 1966). This technique has been used in the present study to analyze the early stages in the enzymatic digestion of ribosomal RNA from rabbit reticulocyte subunits.

Previous studies have shown that specific intermediates may be formed upon mild nuclease digestion of total ascites ribosomal RNA (*e.g.*, Huppert and Pelmont, 1962). Studies of the RNA from the two subunits, individually, have so far been confined to the examination of the products formed upon the complete digestion with specific nucleases. Aronson (1963), and Sanger *et al.* (1965), have shown that the complete enzymatic digests contain different relative amounts of various oligonucleotides, and therefore the nucleotide sequences are different.

Likewise, the results to be described below indicate that the molecular weights and numbers of products formed in the early stages of digestion of RNA from the two subunits differ. It is inferred that the regions most susceptible to enzymatic attack are distributed in a

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