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Review

Molecular complexes in the isolation and characterization of plasma lipoproteins*

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The formation of insoluble complexes between sulfated polysaccharides and certain plasma lipoproteins was independently described by several investigators in 1955 (1, 2, 3). These complexes are formed at neutral pH and in the absence of organic solvents, and are readily dissociated by increasing ionic strength or by the addition of chelating agents which remove certain metal ions that appear to be necessary for the formation of the complex. Precipitation methods for the isolation and purification of plasma lipoproteins and turbidimetric procedures for their quantitative estimation based on this phenomenon have been developed. These methods satisfy many of the criteria proposed by Lindgren and Nichols (4) for the evaluation of lipoprotein isolation techniques. The mild conditions required for complex formation and dissociation, as well as the convenience and reproducibility of the methods, make them particularly attractive for the investigation of plasma lipoproteins. Comparable isolation procedures involving the formation of complexes between lipoproteins and the neutral polymer polyvinylpyrrolidone have also been described recently (5). In this review the formation of complexes between lipoproteins and macromolecules will be discussed and methods employing complex formation for the isolation and quantitation of plasma lipoproteins evaluated. Since the turbidimetric procedures are comparable in some ways to the older thymol (6) and phenol (7) turbidity tests for the estimation of γ-globulins and lipoproteins, a discussion of these is also included in this review. Fractionation procedures such as the Cohn cold-ethanol Methods 6 and 9 and Method 10, although frequently employed in lipoprotein studies, are not included since they have already been thoroughly discussed in the literature (4, 8 to 16).

Before discussing lipoprotein complex formation, it is appropriate to describe the kinds and properties of the lipoproteins which have been isolated from the plasma of human beings and other animal species in both normal and abnormal states. Plasma lipoprotein fractions represent classes of related, though not identical, molecules. Although these fractions are not homogeneous, their chemical and physical properties and metabolic interrelationships indicate that they do indeed represent distinct classes of related molecules rather than arbitrary fractions defined by the methods used in their isolation. This is most clearly demonstrated by the isolation of the same lipoprotein classes by different fractionation procedures from normal human plasma. However, distinct differences have been found between plasma lipoproteins isolated from normal humans and those isolated from human subjects in certain disease states. Also, the plasma lipoproteins isolated from other animal species are characteristic of the species and may differ considerably from species to species as well as from those in the human. In spite of this, the nomenclature employed to describe human lipoproteins has frequently been indiscriminately applied to animal lipoproteins simply because they correspond by one parameter. The methods which have been developed to separate and characterize human plasma lipoproteins are not generally applicable without modification to other lipoprotein systems. In the sections to follow an attempt is made to correlate the various nomenclature schemes which have resulted from the application of different fractionation procedures to the lipoproteins in normal human plasma. Some of the difficulties involved in applying a classification based on normal plasma to the lipoproteins found in certain disease states are then discussed. Finally, similarities

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and differences between the plasma lipoproteins of various animal species are enumerated to emphasize the problems involved in generalizing fractionation procedures from one species to another.

PLASMA LIPOPROTEIN FRACTIONS ISOLATED FROM NORMAL SUBJECTS

When the flotation rate in an ultracentrifugal field is employed as the defining parameter, the normal human plasma lipoproteins fall into three major categories: the chylomicrons, the low density lipoproteins, and the high density lipoproteins. For reasons to be discussed, the flotation rate, S_f, at density 1.063 has been used most frequently in the characterization of low density lipoproteins. Though perhaps somewhat arbitrary, the choice of this density as the dividing line between the low and high density lipoproteins is corroborated by other parameters of these two groups. Further subdivision of the low density lipoproteins into S_f 0–10 (average density 1.035) and S_f 10–400 (average density 0.98) fractions, though again appearing somewhat arbitrary, also can be justified by other considerations. In this review we shall classify the lipoproteins of normal human plasma as follows: (*a*) chylomicrons; (*b*) S_f 10–400 lipoproteins; (*c*) S_f 0–10 lipoproteins; and (*d*) high density lipoproteins.

Chylomicrons. Chylomicrons were originally defined as the fat particles in chyle or plasma which are visible by dark-field microscopy (17). A precise operational definition based on the isolation of pure chylomicrons has not yet been achieved because of the difficulty in attaining clear-cut separations from the upper S_f spectrum of the low density lipoproteins. Nevertheless, it is felt that true chylomicrons represent an entity distinct from very low density lipoproteins by virtue of their metabolic origin and fate (18). The term chylomicron, in this sense, refers to the vehicle for transport of exogenous triglyceride from the alimentary tract to the blood via the chyle. Chylomicrons are composed mainly of triglycerides, and contain only small amounts of cholesterol, phospholipid, and protein (13, 19 to 24). Part of the protein appears to be similar to, if not identical with, high density lipoprotein (25, 26). Upon entering the blood from the lymph, chylomicrons may acquire additional protein at the expense of high density lipoprotein in the plasma. It is of interest to note here that intravenously infused synthetic fat emulsions similarly associate with high density lipoproteins as demonstrated by ultracentrifugal flotation.[1] Chylomicrons have a density near 0.94 (12) and an S_f greater than 400 (4). Oncley has recently estimated the chylomicron flotation distribution as S_f 10,000 ± 5000 (27). Several flotation procedures at a density of 1.006 have been employed for the isolation of chylomicrons: 9500 × g for 10 minutes (28); 9300 × g for 30 minutes (18); 26,000 × g for 30 minutes (29); and 100,000 × g for 30 minutes (23, 25, 29, 30, 31). Some discrepancies in the literature concerning the metabolism of chylomicrons are probably attributable to differences in isolation technique. For example, Dole *et al.* (31) isolated postprandial chylomicrons by centrifugation at 100,000 × g for 30 minutes and concluded that dietary fats had little influence on their fatty acid composition. Bragdon and Karmen (29), on the other hand, found that chylomicrons isolated by centrifugation at 26,000 × g for 30 minutes did reflect the fatty acid composition of the dietary fat. The chylomicron fraction isolated at 100,000 × g probably contained appreciable amounts of S_f 10–400 lipoproteins.

Postprandial lactescence in the serum of normal individuals is caused by chylomicrons (17, 23); however, the assumption that all lactescence in the serum of hyperlipemic subjects is caused by these particles is probably not valid (18, 32). It has been reported that lymph chylomicrons migrate with the mobility of albumin in free electrophoresis (20, 32), whereas plasma chylomicrons have the electrophoretic mobility of α_2-globulins (19, 32, 33, 34). The turbid or lactescent region of the electrophoretic pattern of alimentary lipemic serum differs from that seen in essential, diabetic, or nephrotic hyperlipemic sera. In the former the turbidity is confined to the α_2 region, whereas in the latter it extends across the α_2 and β regions (32). While zone electrophoresis on paper has proved unsatisfactory for the study of chylomicrons because of adsorption, some interesting results have been obtained using starch blocks (34) or columns. Employing the latter, Carlson and Olhagen (35) observed the migration of chylomicrons, as detected by turbidity, from the serum of a patient with essential hyperlipemia as two distinct fractions, one an α- and the other a β-globulin. On the other hand, Kunkel and Trautman (34), employing the starch block technique, observed almost all of the visible lipemia in the α_2 region of normal postprandial serum. These results certainly suggest that more than one type of lipoprotein molecule can contribute to visible lipemia, but the significance of these variants remains to be elucidated. Another method which has been employed to isolate chylomicrons is flocculation with toluidine blue (19) or protamine (20); however, Laurell (33) has recently shown that other lipoproteins are precipitated together

[1] F. A. Kruger, D. G. Cornwell, G. J. Hamwi, and J. B. Brown, unpublished observations.

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with chylomicrons by protamine in the presence of fatty acid anions.

S_f 10–400 Lipoproteins. These lipoproteins are defined as molecules having flotation rates between 10 and 400 in a medium of density 1.063. They are generally isolated in the preparative ultracentrifuge by flotation at 100,000 × g for 18 to 24 hours in a medium of density 1.006 or 1.019 after preliminary removal of the chylomicron fraction. The fraction isolated under these conditions has been variously designated S_f 10–400 (18, 22, 36, 37, 38), S_f 12–400 (4, 39, 40, 41), S_f 20–400 (4, 41), and more recently, S_f 16–400 (42). Oncley *et al.* (37) originally suggested that this lipoprotein was concentrated in the S_f 10–100 region with a maximum near S_f 35, and had a mean density of 0.98. In a later paper Oncley (27) suggested that the fraction consisted of two subfractions: S_f 100 ± 60 with a density of 0.958, and S_f 30 ± 8 with a density of 0.990. Lipoproteins with a density near 1.02 and a flotation distribution approximated by S_f 10–17 (22, 38), S_f 5–15 (37), S_f 12–20 (4, 41), or S_f 10 ± 3 (27) are found in normal plasma at a very low concentration. This accounts for the somewhat arbitrary choice by different investigators of the S_f value separating the two main low density lipoprotein groups. It remains to be established whether the observation of lipoproteins in the S_f 10 region represents incomplete separation between these two groups, or actually represents part of a continuum embracing both groups.

The S_f 10–400 lipoprotein fraction contains 49% to 52% triglyceride, 18% to 19% phospholipid, 6% to 7% free cholesterol, 14% to 16% esterified cholesterol, and 7% to 10% protein (22, 37, 43). The cholesterol to phospholipid ratio varies from 0.78 to 0.90 (18, 22, 37, 38, 40). The fatty acid (44, 45), phospholipid (46, 47), and amino acid (48, 49, 50) compositions of the S_f 10–400 lipoproteins have been investigated. These lipoproteins have been reported to migrate as α_2-globulins by starch block electrophoresis (34); however, in view of the results which have been reported for chylomicrons, it would appear that this should be investigated further.

Both S_f 0–10 and S_f 10–400 lipoproteins are found in fraction I + III of cold-ethanol Method 10 (9, 12, 15, 16). Metabolically the S_f 10–400 lipoproteins represent the main vehicle for endogenous triglyceride transport (18, 36). Newly absorbed triglycerides are transported secondarily by them (36). They are synthesized in the liver (51, 52), and converted to S_f 0–10 lipoproteins (53) with the concomitant removal of triglyceride, presumably in the reticuloendothelial system (18, 36, 54, 55). Immunochemical studies indicate that the protein moieties of the S_f 10–400 and S_f 0–10 lipoproteins are closely related, if not identical (56, 57).

S_f 0–10 Lipoproteins. These low density lipoproteins, defined as having an ultracentrifugal flotation rate in the range from 0 to 10 in a medium of density 1.063, are usually isolated by centrifugation at 100,000 × g for 18 to 24 hours in a medium of this density (18, 22, 36, 37, 41 to 49, 53, 57, 58, 59). The S_f 10–400 lipoproteins are separated by a preliminary or final centrifugation at density 1.006 or 1.019. Oncley *et al.* (37) isolated and characterized three arbitrary subfractions within the S_f 0–10 spectrum and found a small increase in protein content with decreasing flotation rate. The S_f 0–10 lipoproteins are concentrated in the S_f 3–9 region (37), with a peak at S_f 6 ± 2 (27). These molecules have a mean density of 1.032 (27). Different investigators have used several S_f distributions to describe this lipoprotein fraction: S_f 0–10 (22, 38), S_f 3–9 (18, 36, 37, 57), S_f 0–12 (4, 39, 40, 41), S_f 0–20 (4, 41, 59), and S_f 0–16 (42).

These lipoproteins have the electrophoretic mobility of a β-globulin in moving boundary and zone electrophoresis (34, 37). They are found in fraction III-O of cold-ethanol Methods 6 and 9 (58), and fraction I + III of cold-ethanol Method 10 (9, 12, 15, 16) along with the S_f 10–400 lipoproteins. They differ markedly from the latter in chemical composition containing 9.0% to 12.5% triglyceride, 19% to 32% phospholipid, 7.5% to 9.5% free cholesterol, 37.0% to 39.4% esterified cholesterol, and 21.9% protein (22, 37, 43). The cholesterol to phospholipid ratio varies from 1.30 to 1.45 (18, 22, 37, 38, 40). The protein moiety has the same immunochemical specificity (56, 57) and amino acid composition (48, 49, 50) as that of the S_f 10–400 lipoprotein fraction.

The S_f 0–10 lipoproteins may arise either as the products of S_f 10–400 lipoprotein metabolism (53) or directly from the liver (18, 36, 51, 52). Although they constitute the largest lipoprotein group, their metabolic role, except as the end products of S_f 10–400 lipoprotein metabolism, remains obscure.

High Density Lipoproteins. The high density lipoproteins may be distinguished operationally from the low density lipoproteins by virtue of the fact that they sediment along with other plasma proteins at density 1.063. They may be isolated from the remaining plasma proteins by ultracentrifugal flotation at 100,000 × g for 22 hours in a medium of density 1.21 (4, 8 to 14, 22, 34, 41, 43 to 50, 59, 60). Some investigators (4, 12, 41, 59) have distinguished between two high density lipoprotein classes with mean densities of 1.09 and 1.14. Although these two fractions differ in chemical composition (61), they may not represent more than arbitrary fractions of a continuum. Oncley (27) has

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recently suggested that only a single high density lipoprotein class exists with a mean density of 1.12. We shall consider these lipoproteins as a single class in this review. These lipoproteins differ from the low density lipoproteins in their amino acid (48, 49, 50) and phospholipid (46, 47) composition as well as immunochemical specificity (57, 62, 63). They contain 4% to 8% triglyceride, 20% to 21% phospholipid, 2% to 3% free cholesterol, 13% to 15% esterified cholesterol, and 46% to 58% protein (22, 43, 61). Cholesterol to phospholipid ratios between 0.38 and 0.58 have been reported (18, 22, 38, 40, 60, 64). The high density lipoproteins exhibit the electrophoretic mobility of α_1-globulins (4, 8 to 14, 34), and are found in fraction IV + V + VI of cold-ethanol Method 10 (9, 12, 15, 16). They have also been separated from low density lipoproteins by paper chromatography (65).

As mentioned in the section on chylomicrons, the latter appear to contain a protein constituent similar in many ways to high density lipoprotein. It has been suggested that this lipoprotein may be liberated as such during the metabolism of chylomicrons in the reticuloendothelial system (36). High density lipoproteins have been shown to be synthesized in the liver (66). At least part of the protein of chylomicrons appears to be synthesized in the intestinal wall (23). The relation of this synthesis to the high density lipoproteins is not known.

A lipoprotein with a density greater than 1.21 has been isolated and partially characterized (22, 40, 61, 67). It contains only a small part of the total plasma phospholipid but is rich in lysolecithin (67). A more thorough investigation of this lipoprotein is necessary before the methods for its isolation and its significance in lipoprotein metabolism can be assessed.

The isolation and characterization of plasma lipoproteins is complicated by the rapid equilibration or exchange of lipid components including cholesterol (68, 69), phospholipid (70, 71, 72), and tocopherol (73) between the different lipoprotein fractions. Exchange between the triglyceride (36) or protein (53, 74) moieties of different lipoprotein fractions has not been demonstrated; however, a very rapid equilibration between the lipid-free protein moiety of the high density lipoprotein fraction and other lipoproteins has been reported (60).

PLASMA LIPOPROTEIN FRACTIONS ISOLATED FROM HUMAN SUBJECTS IN DISEASE STATES

Many disease states are accompanied by marked alterations in the plasma lipoproteins. These alterations may involve the elevation or diminution of normal lipoprotein fractions, changes in the S_f distribution within a lipoprotein fraction, or the appearance of atypical lipoproteins. Methods established for the fractionation of lipoproteins in normal human plasma can be applied in disease states where a normal fraction is elevated or depressed. This is illustrated in the investigation of plasma from hypercholesterolemic subjects. While the S_f 0–10 lipoprotein fraction is markedly elevated (4, 10, 18, 40, 59, 75, 76), it is characterized by normal cholesterol to phospholipid and fatty acid ester to cholesterol ratios (18, 76). The elevated S_f 0–10 fraction does not affect the isolation and characterization of other lipoprotein fractions (18, 76). The concentration of the high density lipoproteins is diminished in hyperlipemia and biliary cirrhosis (18, 38, 40, 76, 77). Fractionation at a density of 1.063 indicated that these lipoproteins had a low cholesterol to phospholipid ratio (18, 40). Baxter *et al.* (38), in a study of hyperlipemic sera from nephrotic individuals, found, however, that the cholesterol to phospholipid ratio of the high density lipoprotein fraction was normal when lipoproteins with a density greater than 1.21 were not included in the fraction.

It is more difficult to apply standard isolation procedures to plasma from hyperlipemic subjects. The S_f 10–400 lipoprotein fraction isolated from these is lactescent and contains molecules rich in triglyceride (18, 38), and with an elevated flotation rate (4, 53, 75). A large lipoprotein fraction is frequently isolated from lactescent sera by ultracentrifugal flotation at 9300 × g for 30 minutes (18). This procedure isolates postprandial chylomicrons from normal plasma; however, the composition of the "chylomicron fraction" from normal postprandial plasma is different than that of the "chylomicron fraction" from the plasma of hyperlipemic individuals (18). Jobst and Schettler (78) isolated chylomicrons by ultracentrifugal flotation at 22,000 × g for 60 minutes and found significant differences in composition between the "chylomicron fraction" from the serum of individuals with essential hyperlipemia as compared to alimentary chylomicrons. The "chylomicron fraction" from subjects with essential hyperlipemia contained less triglyceride and was generally similar in composition to S_f 10–400 lipoproteins (18, 78). Thus ultracentrifugal flotation may not distinguish between chylomicrons of alimentary origin and large low density lipoproteins with an enhanced flotation rate.

Elevated S_f 10–400 lipoprotein fractions isolated from hyperlipemic individuals do not represent a simple proportional elevation of lipoproteins normally present

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in this part of the S_f spectrum. They show a wide variation in chemical composition (18, 38, 40, 76). Cholesterol to phospholipid and fatty acid ester to cholesterol ratios may be increased or decreased from the normal range (18, 38, 76). In nephrosis the cholesterol to phospholipid ratio increases with increasing lactescence (38); this ratio is sometimes diminished in the corresponding lipoprotein fraction isolated from markedly lactescent sera from individuals with idiopathic hyperlipemia (18). An elevated S_f 10–20 lipoprotein, intermediate in composition and properties between the S_f 0–10 and S_f 10–400 lipoproteins of normal serum, has been found in some hyperlipemic sera (18, 38, 75, 76). Thus different hyperlipemias may involve elevations in specific regions of the S_f 10–400 lipoprotein spectrum, extension of the range beyond S_f 400 (75), or perhaps even the appearance of atypical lipoproteins not normally present in the low density lipoprotein spectrum.

S_f 0–10 lipoprotein fractions isolated from the sera of hyperlipemic subjects also differ from those isolated from normal sera. Cholesterol to phospholipid ratios are lower than normal, while fatty acid ester to cholesterol ratios are increased (18, 38, 76). These alterations in composition are most significant when the S_f 0–10 lipoprotein concentration is very low, as it is in markedly lipemic sera (18, 38, 76). These lipoproteins may represent a small residual fraction remaining after the disappearance of the major S_f 0–10 fraction, or they may represent atypical molecules synthesized in the hyperlipemic state.

The isolation and characterization of the lipoproteins from the sera of subjects with biliary cirrhosis present special difficulties. Whereas high density lipoproteins are greatly diminished or even absent (40, 77, 79), low density lipoproteins within the S_f 0–20 range are greatly increased (40, 75, 79, 80). However, these low density lipoproteins are atypical. Although they migrate electrophoretically as β-lipoproteins (77), their cholesterol to phospholipid ratio is more like that of the high density lipoproteins (77). Russ *et al.* (77) subjected the plasma of a biliary cirrhotic to cold-ethanol Method 10 and found β-lipoproteins in fraction I + III, IV + V, and VI. Normally, fraction VI contains only traces of lipid; in this case it represented the largest fraction. Of the three fractions, only I + III reacted immunochemically with antisera to normal low density lipoprotein. The unfractionated plasma, upon analytical ultracentrifugation, revealed three lipoprotein species: S_f 13 representing two-thirds of the total, and S_f 10 and S_f 7 equally representing the remainder.

It is thus evident that even within the human species the characterization of lipoprotein classes by a single parameter is inadequate and may lead to erroneous identification, especially in abnormal states. The relationships established between the various parameters of the lipoproteins in normal human plasma do not necessarily hold in abnormal conditions.

PLASMA LIPOPROTEIN FRACTIONS ISOLATED FROM OTHER ANIMAL SPECIES

Pedersen (81) first demonstrated the existence of the low density lipoproteins in human plasma by ultracentrifugation, which he designated by the term "X-protein," and also noted that a comparable protein was absent from other animal plasmas. This finding was substantiated by the use of cold-ethanol fractionation procedures (82). When applied to human plasma, a major portion of the lipoproteins appeared in fraction II + III; these were identified as β- or low density lipoproteins. By contrast, comparable fractions from animal plasmas contained very little lipoprotein (82). Most of the lipoproteins of animal sera appear in fractions IV-1 and IV-2, and are α-lipoproteins by electrophoresis (82). Both analytical and preparative ultracentrifugation have demonstrated that many animal species contain large amounts of high density and only small amounts of low density lipoproteins (40, 83). The correlation between low density, β, and fraction II + III lipoproteins on the one hand, and high density, α, and fraction IV lipoproteins on the other, which has been observed in human plasma, has not been established generally for the plasma of other animals. Only in the case of dog plasma have the lipoproteins been isolated and characterized by a variety of methods. Lipoprotein fractions obtained from human and dog plasma by cold-ethanol Method 10 (84), paper electrophoresis (85, 86), ultracentrifugal flotation (40), and analytical ultracentrifugation (83) are compared in Table 1. The agreement of the distribution of dog plasma lipids between the low density lipoproteins (fraction I + III and those with density 1.019–1.063) and the high density lipoproteins (fraction IV + V + VI and those with density >1.063) by procedures dependent upon solubility and ultracentrifugal properties would seem to indicate that the dog lipoprotein fractions correspond with the human. The paper electrophoretic data indicate a larger proportion of β-lipoprotein in the dog plasma than is indicated by the ultracentrifugal and solubility data. However, it may be noted that the dog serum cholesterol levels reported here are somewhat higher than usual (less than 150 mg/100 ml). In a paper electrophoretic study of dog serum by Fasoli *et al.* (87), the slow-mi-

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grating fractions accounted for only about 15% of the total cholesterol.

Although there appear to be two types of lipoproteins in dog plasma as demonstrated by three independent criteria, it is of interest to note that whereas the lipid compositions of comparable fractions isolated from human plasma differ widely, those isolated from the dog are quite similar at least with regard to the cholesterol to phospholipid ratio. Further chemical characterization with regard to other constituents such as triglyceride and the protein moiety is necessary before the significance of these fractions can be evaluated. This has been attempted in a study of N-terminal amino acids, which indicated that dog lipoproteins in the density 1.019–1.063 and density 1.063–1.20 fractions had different amino acid residues (25). Analytical ultracentrifugation (Table 1) demonstrates further differences between dog and human low density lipoproteins. In dog plasma these exhibit flotation rates, S_f 1–3 and $-S$ 23, which correspond to a minor human lipoprotein fraction, HDL_1, not separated from the major low density lipoproteins by ultracentrifugal flotation at density 1.063 (59). When dog lipoproteins are prestained with Sudan black B and separated by ultra-

TABLE 1. Isolation and Characterization of Human and Dog Plasma Lipoproteins

Method and Fraction	Human			Dog		
	Cholesterol	Phospholipid	Cholesterol / Phospholipid	Cholesterol	Phospholipid	Cholesterol / Phospholipid
		mg/100 ml			*mg/100 ml*	
Cohn Method 10 (84)						
Plasma	189	225	0.84	134	257	0.53
Fraction I + III*	123	98	1.25	10.2	23.7	0.61
Fraction IV + V + VI*	61	117	0.52	114	234	0.50
Paper Electrophoresis (85,† 86‡)						
Serum	245	259	0.94	243	455	0.53
α_2	27.5	36.0	0.77			
$\beta + \gamma$§	158	128	1.24	61	127	0.48
α_1	59.5	95.0	0.63	182	328	0.55
Ultracentrifugal Flotation (40)						
Serum	179	226	0.79	150	362	0.41
d <1.019	23	28	0.82	2	10	0.20
d 1.019–1.063	103	74	1.39	10	19	0.53
d >1.063	49	123	0.40	127	325	0.39

Analytical Ultracentrifugation (83)	Lipoprotein (Human)	Lipoprotein (Dog)
	mg/100 ml	*mg/100 ml*
Density 1.06		
S_f 20–60	20	0#
S_f 10–20	28	0
S_f 9	0	0
S_f 3–8	200	0
S_f 1–3	0	38
Density 1.21		
$-S$ 40–70	30	0#
$-S$ 35	0	0
$-S$ 30	200	0
$-S$ 23	14	77
$-S$ 6	0	0
$-S$ 3	140	400

* Fractions I + III and IV + V + VI are called fractions C + D and A in this study of dog serum (84).
† Paper electrophoresis of human serum (85).
‡ Paper electrophoresis of dog serum (86).
§ Fraction $\beta + \gamma$ signifies β-lipoproteins and lipoproteins trailing from the origin (85, 86).
# Different dogs were used in these ultracentrifugation experiments (83).

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centrifugal flotation in a density gradient,[2] a lipoprotein continuum is found instead of the separation into discrete low and high density fractions observed with human serum (88, 89).

As in the human, thyroid deficiency in dogs leads to alterations in the plasma lipoprotein pattern. These changes are quite different, however. In the human there is a marked increment in the S_f 0–10 lipoproteins, a small increment in the S_f 10–400 lipoproteins, and essentially no change in the high density lipoproteins (18). Milch *et al.* (90), studying I^{131}-thyroidectomized dogs, observed marked elevations in high density lipoproteins as well as in the S_f 0–12, S_f 12–20, and S_f 20–400 lipoproteins. In addition, they noted an almost twofold increase in the cholesterol to phospholipid ratio of the total serum lipids. This would indicate a change in the nature of at least some of the lipoproteins since, as indicated in Table 1, none of the lipoproteins isolated from normal dog plasma exhibit cholesterol to phospholipid ratios this high. The lipoproteins of thyroid-deficient dogs fed cholesterol have also been studied, and similar elevations in the various lipoprotein fractions found by cold-ethanol Method 10 (91) and electrophoresis (92). The cholesterol to phospholipid ratios of the total serum lipids (91, 92), as well as of both the high and low density fractions (91), were markedly elevated. An investigation of N-terminal amino acids in the lipoprotein fractions of hypercholesterolemic dogs, similar to the recent study by Shore and Shore (93) on hypercholesterolemic rabbits, would be highly significant.

In some respects the study of lipoprotein metabolism in animals like the dog is less complicated than it is in the human. For example, chylomicron metabolism can be followed in the dog with little chance of interference by low density lipoproteins in fractionation procedures (31, 94, 95). The guinea pig may offer certain advantages in the study of low density lipoproteins since there is a virtual absence of high density lipoproteins in guinea pig plasma (83). However, as has been pointed out, plasma lipoproteins differ from species to species in their physical and chemical properties, and perhaps also in their metabolic behavior. It should also always be kept in mind that lipoprotein fractionation procedures may require modification before they are applied to the plasma of a given species.

THE NATURE OF THE INTERACTIONS OF PLASMA LIPOPROTEINS WITH OTHER SUBSTANCES

The complexes formed by the interaction of plasma lipoproteins with certain sulfated polysaccharides bear at least a formal resemblance to those complexes isolated from biological systems which contain a protein bound to one of a variety of polyanions such as heteropolysaccharides, nucleic acids, or phosphoproteins. Polysaccharide anions such as hyaluronic acid in synovial fluid and chondroitin sulfate in cartilage form complexes with proteins in which electrostatic attractions between cationic groups on the protein and anionic (sulfate and carboxyl) groups probably play a major stabilizing role since the complexes are broken by the addition of alkali or increasing the ionic strength of the medium (96). It has been suggested that the anionic groups of mucopolysaccharides in connective tissue are in ion exchange equilibrium with metal ions and the cationic groups of the protein (97, 98). In a study of the various factors involved in the binding of protein to mucopolysaccharide in connective tissue, Loeven (99) concluded that although charge considerations are of primary importance, structural factors may alter the degree of binding. Partridge and Davis (100), studying chondroitin sulfate complexes in cartilage, arrived at similar conclusions, attributing the more specific aspects of the binding to hydrogen bonding and other short-range forces.

Nucleoproteins containing nucleic acid polyanions and either protamines or histones are undoubtedly stabilized mainly by electrostatic forces since the complexes are readily disrupted by increasing either the pH or the ionic strength of the medium (101, 102, 103). The situation is somewhat more complicated when the interaction between nucleic acids and other proteins is considered (101). High ionic strength alone does not suffice to disrupt these complexes. However, the components can be caused to separate by the use of protein denaturants such as urea, guanidine hydrochloride, or sodium dodecylsulfate. Conversely, the interaction between a given globular protein and nucleate polyanion to form a complex is generally weaker after denaturation of the protein than before denaturation. This is true even after the denaturing agent is removed, and also for heat denatured proteins. It therefore appears that certain structural features of the native protein in addition to net charge, which remains essentially unchanged with denaturation, contribute to the binding of globular proteins to nucleic acid polyanions. Recent studies on the formation of soluble and insoluble complexes between spermine and soluble ribonucleic acid (104) and spermine and synthetic polynucleotides (105) show that polynucleotide structure is also important in determining the type of complex formed.

Molecular complexes between proteins and phosphoprotein polyanions have not been studied as thor-

[2] D. G. Cornwell and F. A. Kruger, unpublished observations.

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oughly as the heteropolysaccharide and nucleic acid complexes; however, a phosphoprotein complex obtained during the fractionation of hen egg yolk has been partially characterized. Yolk, diluted with 10% sodium chloride, exhibits two sedimenting fractions in the ultracentrifuge (106). Although the major sedimenting fraction, isolated and dissolved in 10% sodium chloride, is homogeneous by sedimentation and electrophoresis, it has been shown to contain three proteins: lipovitellin (lipoprotein), phosvitin (phosphoprotein), and γ-livetin (globulin) (107). The γ-livetin component may be separated by sedimentation or electrophoresis in alkaline solution (pH 9) at low ionic strength ($\mu = 0.3$) or may be partially purified by precipitation from a 40% saturated ammonium sulfate solution (107, 108). The molecular complex is also disrupted in 0.4 M magnesium sulfate and phosvitin precipitated from this medium as a magnesium complex (109, 110). Dissociation in alkali or in magnesium sulfate both indicate that electrostatic bonding is important in stabilizing this complex system isolated from egg yolk. Its stability in 10% sodium chloride suggests that other factors assist in holding the molecular complex together. The structure of naturally occurring molecular complexes between phosphoproteins and lipoproteins in the whole yolk is more difficult to ascertain. The molecular complex containing lipovitellin, phosvitin, and γ-livetin may be an isolation artifact. Schjeide and Urist (111) have isolated granules or giant yolk complexes from egg yolk which contain only phosphoprotein (phosvitin) and dense and light lipoproteins (lipovitellin and lipovitellenin, respectively). Livetins were found only in the yolk fluid. Calcium ions are bound to the naturally occurring phosphoprotein, and the removal of these ions during isolation liberates anionic groups (112). These groups may then form electrostatic bonds with the cationic groups of a livetin and form the complex described by Joubert and Cook (107).

Other examples of complex formation between oppositely charged macromolecules include the association of protamine and heparin (113), the formation of a heparin-albumin precipitate below the isoelectric point of albumin (114, 115), the formation of an albumin-polylysine precipitate above the isoelectric point of albumin (116), and the coprecipitation of γ-globulins and low density lipoproteins in their interisoelectric region (117). Protein-protein and protein-synthetic polyelectrolyte interactions have been reviewed by Waugh (118) and Sela and Katchalski (119). Since these complexes are readily disrupted by altering the pH or increasing the ionic strength, it is difficult to establish the contribution of binding forces other than electrostatic forces. Furthermore, their formation is relatively nonspecific and does not appear to require unique structural relationships. Phytic acid, for example, precipitates most extracellular proteins in the region between pH 2 and pH 4 (120).

Although it was originally believed that heparin formed complexes with plasma proteins only in the pH region where the proteins were positively charged (121), evidence was obtained by Jaques (122), from considerations of solubility behavior, that complexes existed between heparin and proteins even under conditions where both macromolecular species carried a negative charge. A number of investigators have subsequently demonstrated that heparin forms molecular complexes with both albumin and low density lipoproteins in serum at hydrogen ion concentrations where the proteins carry a net negative charge (114, 115, 123 to 126). Complexes between fibrinogen and sulfated polysaccharides have been described in which protein and polyanion specificity at a pH above the isoelectric point would appear to indicate that polymer configuration and other secondary valence forces as well as electrostatic bonds contribute to the formation and stability of the complexes (127 to 130). Complex formation as determined by either electrophoretic behavior or precipitation is nevertheless very sensitive to changes in pH and ionic strength, generally decreasing with increases in either. However, Smith and Von Korff (129) have described the "salting-out" precipitation of fibrinogen-heparin complexes from concentrated sodium chloride solutions as well as the "salting-in" effect found at lower ionic strengths. Other evidence for nonelectrostatic interactions between proteins and polysaccharides has been obtained from studies on complex formation between glycogen and concanavalin-A (131, 132), between albumin and dextran (133), and between fibrinogen and dextran (134, 135). Glycogen which has been methylated no longer forms complexes with concanavalin-A (132). Cifonelli *et al.* (136) observed that whereas heparin precipitated concanavalin-A more efficiently than glycogen, no precipitation occurred with chondroitin sulfate or hyaluronic acid under similar conditions. These observations indicate that polymer structure and hydrogen bonding are important factors in complex formation. It is of interest to note here that Peterson and Sober (137) implicate nonionic bonding forces as contributing to departures from electrophoretic order in the elution of proteins from polyelectrolyte cellulosic absorbants. The neutral polymers polyvinylpyrrolidone[3] and poly-

[3] PVP. Technical Bulletin, Antara Chemicals, New York 14, N. Y.

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N-vinyl-5-methyl-2-oxazolidinone[4] have recently been shown to exhibit rather unique and specific complexing capacities for certain organic compounds as well as certain proteins. Interactions with the latter are discussed more completely later in this review.

The complexes formed by the interactions between polyanions and lipoproteins are comparable in many ways to the colloid complexes described by Bungenberg de Jong (138). We have already considered the interaction of polyanions with proteins below their isoelectric point; these result in the formation of dicomplex systems. In many instances, however, the presence of certain metal ions is essential for the formation of a complex. Bungenberg de Jong refers to these as tricomplex systems, amphion-cation-anion, in which the amphion may be a protein or other ampholyte such as egg lecithin, the cation a di- or trivalent metal ion, and the anion either a polyanion or a micro anion. Typical examples are: egg lecithin-La(III)-arabinate; isoelectric gelatin-Zn(II)-chondroitin sulfate; isoelectric gelatin-Cd(II)-thiocyanate. The role of metal ions in the formation of lipoprotein-polyanion complexes is discussed in the section on lipoprotein-polysaccharide complexes.

Albumin and the low density lipoproteins form complexes with simple anions above their isoelectric points. Albumin-anion interactions have been studied by a number of investigators and are discussed in reviews by Klotz (139) and Foster (140). The literature on low density lipoprotein-anion interactions is less extensive, but several important studies have appeared on the binding of free fatty acids (141, 142) and methyl orange (143). Goodman and Shafrir (142) showed that both S_f 10–400 and S_f 0–10 lipoprotein fractions bind a small number of fatty acid anions at strong binding sites, and a large number at weaker binding sites. While binding affinities are somewhat greater with the S_f 10–400 lipoproteins, they both bind the same number of fatty acid anions (142). These interactions may be compared with the binding of fatty acid anions by plasma albumin studied under the same conditions (144). Albumin binds more fatty acid anions at two somewhat stronger classes of binding sites than do the low density lipoproteins. However, more fatty acid anions are bound more strongly at the weaker binding sites of the low density lipoproteins than at the weakest class of binding sites of albumin. Rosenberg *et al.* (143) found that methyl orange binding corresponded rather closely in low density lipoprotein and albumin systems. These similarities in anion binding would appear to indicate that similar mechanisms are involved in anion interactions with albumin and the low density lipoproteins. Foster (140) has summarized the results of many studies on the binding by albumin of dyes and detergents and concluded that the tendency for water molecules to form a maximum number of hydrogen bonds with each other is a major factor in promoting protein-anion interactions. Aggregation of anions into micelles and the association of anions with proteins permit increased hydrogen bonding between solvent molecules, since hydrophobic groups constitute discontinuities in the surrounding hydrogen-bonded aqueous environment. These discontinuities may be minimized by the association of protein and anion hydrophobic groups (145). Foster (140) further suggests that albumin may assume a structural configuration in which hydrophobic groups are oriented toward the aqueous phase and are therefore available for nonionic bonding. Goodman and Shafrir (142) state that the large number of weaker binding sites on the lipoprotein molecule may result from hydrophobic interactions. The relative availability of hydrophobic groups may be characteristic of both albumin and low density lipoprotein molecules, and this structural feature influences the formation and specificity of protein-anion and protein-polyanion complexes.

The interaction of phenols with proteins has been attributed to the formation of hydrogen bonds between the phenolic hydroxyl groups and the peptide linkages in proteins (145 to 148). Mejbaum-Katzenellenbogen (149) studied the interaction of several proteins with naturally occurring phenolic polymers, the tannins, and found that insoluble complexes were formed at or below the isoelectric points of the proteins. These complexes did not dissociate at high ionic strength; however, they were dissociated by the addition of caffeine (150). Hydrogen bonding between phenols and caffeine may explain this phenomenon. The interaction of monomeric phenols, thymol and phenol, with proteins is somewhat more specific and will be discussed in the next section. It would appear, however, that considerations similar to those discussed above with reference to albumin and lipoprotein interactions with fatty acid and other anions may also apply to the interactions of proteins with phenols.

THE TURBIDIMETRIC ESTIMATION OF PLASMA LIPOPROTEINS WITH PHENOLS

In 1944, Maclagan (6) described a thymol turbidity test for the estimation of serum globulins in liver disease. He analyzed the precipitate formed on adding buffered aqueous thymol to human serum and found that it contained protein nitrogen, lipid phosphorus, and cholesterol as well as thymol. Cholesterol to phospholipid ratios

[4] Molecular Complexing with Devlex® Resins. Technical Bulletin, Dow Chemical Company, Midland, Mich.

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calculated from these data (average 1.43) indicate that the precipitate contained low density lipoproteins. Kunkel and Hoagland (151) found that thymol reacted with both γ-globulins and serum lipids. They also noted that the turbidity resulting from the γ-globulin did not occur at elevated ionic strengths, whereas that due to the serum lipids was unaffected by increased ionic strength. However, prior extraction of lipids from hepatitis serum with ether prevented the production of turbidity with γ-globulins. Reintroduction of serum lipid in the form of serum giving little thymol turbidity itself restored the turbidity (151, 152). It thus appears that the presence of serum lipoproteins is essential in the thymol turbidity reaction. Electrophoretic studies also indicate that γ-globulins are necessary in the production of thymol turbidity (151, 153). Albumin has an inhibitory effect on the production of turbidity (6, 154); however, this observation has been questioned by Albertsen *et al.* (155), who studied the effect of several purified protein fractions on turbidity. Thymol turbidity seems to depend on complex interactions between thymol, γ-globulins, low density lipoproteins, and perhaps albumin. Nevertheless, several investigators have used thymol turbidity (156) to estimate serum lipids and lipoproteins, especially the neutral fat and chylomicron concentrations (157 to 161). Walther (162) found that thymol turbidity was useful in following serum lipid alterations in a given individual, but it was difficult to correlate with the serum lipid levels of different individuals. The thymol test has been discussed in several reviews (163, 164).

Maclagan (6) investigated a number of phenolic compounds in his study of liver function tests. Kunkel *et al.* (7) subsequently used one of these, phenol, in a turbidimetric method for the estimation of serum lipids. Since the turbidity produced with γ-globulins is suppressed at elevated ionic strengths (7, 10, 151), the test was made more specific for serum lipids by the addition of 12% sodium chloride to the phenol reagent (7). This test was later modified by Polonovski *et al.* (165), who used 6% sodium chloride in their phenol reagent. These reagents have been used by a number of investigators and the turbidity produced appears to correlate to some degree with the total lipid and total cholesterol of serum (7, 165 to 181). Attempts have also been made, with some measure of success, to correlate the results of these methods with data obtained by paper electrophoretic procedures for the estimation of serum lipoproteins (167, 168, 177, 180, 182). It is possible that the phenol reagent which contains 6% sodium chloride is more sensitive to abnormal lipoproteins (165, 172); however, this observation should be investigated further with ultracentrifugal flotation procedures. Turbidity in a modified phenol test has been reported to be enhanced by the addition of heparin, and suppressed by the presence of free fatty acids (183). On the other hand, thymol turbidity appears to be suppressed by heparin (184).

The principal advantages of the thymol and phenol turbidity methods are simplicity, the small volume of serum required, and the availability of reagents. While temperature does affect the turbidity, the data can be readily corrected for this variable (185, 186). The major disadvantage of both phenol and thymol turbidity procedures is their incomplete specificity. They yield little information on the composition and concentration of the specific lipoprotein fractions altered in different disease states. Although they have been reported to yield results comparable to those obtained by methods employing sulfated polysaccharides (170, 178, 187), this correlation has been questioned (188). Furthermore, the turbidity obtained may be affected by the concentration of albumin (6, 154, 182), and fibrinogen and the products of fibrinolysis present in serum (189). Nevertheless, these procedures may have some application, within the limitations described, for following serum lipid levels in individuals during therapy.

PLASMA LIPOPROTEIN-SULFATED POLYSACCHARIDE COMPLEXES

The interaction of lipoproteins with sulfated polysaccharides may result in the formation of either soluble or insoluble complexes. Insoluble complexes have been studied by observing the development of turbidity or by the actual isolation and chemical analysis of the precipitate. Soluble complexes have been studied by moving boundary electrophoresis since the electrophoretic mobility of the lipoprotein-sulfated polysaccharide complex is greater than that of the free lipoprotein (190 to 193). Bernfeld *et al.* (190) found that the ascending and descending boundaries were both altered by the formation of a β-lipoprotein-sulfated pectic acid complex, whereas only the ascending boundary was altered with the formation of a β-lipoprotein-heparin complex (Table 2). These differences in electrophoretic behavior characterize complexes with little tendency to dissociate (irreversible complexes) and complexes with considerable tendency to dissociate (reversible complexes), respectively. Sugano (193) investigated soluble β-lipovitellin-heparin complexes and also found a tendency for complex dissociation in the descending limb during electrophoresis (Table 2). This tendency for dissociation may be expressed as the

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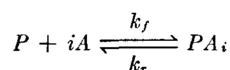
TABLE 2. Electrophoretic Mobilities Observed as Result of Interaction of Lipoproteins with Sulfated Polysaccharide

Buffer		Composition (mg/100 ml)		Mobilities × 10^5 (cm^2 sec^{-1} $volt^{-1}$)				Reference
				Ascending		Descending		
pH	N							
		β-Lipoprotein	Heparin					
8.6	0.1	980		3.0		3.0		(190)
8.6	0.1	980	80	4.5		3.1		
		β-Lipoprotein	Sulfated Pectic Acid					
8.6	0.1	980		3.0		3.0		(190)
8.6	0.1	980	80	4.9		4.2		
		β-Lipovitellin	Heparin	Fast	Slow	Fast	Slow	
9.8	0.1	800			5.7		5.3	
9.8	0.1		330	18.8		17.4		
9.8	0.1	800	330	18.3	7.8	16.2	5.5	
9.8	0.3	800			3.5		3.5	(193)
9.8	0.3	800	330	13.9	4.5	13.2	3.9	
7.8	0.25	800			1.25		1.2	
7.8	0.3*	800	330	13.6	3.35	13.0	2.05	

* Some precipitate formed. Electrophoretic analysis of the supernatant fraction.

reversible formation of a complex between the protein (P) and polyanion (A):



$$P + iA \underset{k_r}{\overset{k_f}{\rightleftharpoons}} PA_i$$

When the velocity constants for the forward (k_f) and reverse (k_r) reactions are both large, and the electrophoretic mobility of the polyanion is much greater than that of the protein, electrophoresis causes a depletion in the concentration of the former and dissociation of the complex (193, 194, 195). It is thus possible to distinguish between three types of molecular complex resulting from the interaction of lipoproteins and sulfated polysaccharides: (*a*) insoluble complexes; (*b*) reversible soluble complexes; (*c*) irreversible soluble complexes. The factors which affect the interaction of lipoproteins with sulfated polysaccharides include pH, ionic strength, and the presence of certain metal ions, as well as the nature of the polysaccharide and lipoprotein involved.

pH and Ionic Strength. Sulfated polysaccharides interact with many proteins on the acid side of their isoelectric points. Fibrinogen, albumin, and some lipoproteins, on the other hand, also form molecular complexes on the alkaline side of their isoelectric points. Serum, rather than plasma, is generally used in the investigation of lipoproteins in order to eliminate interference by fibrinogen-sulfated polysaccharide complexes. However, fibrinogen complexes are more soluble and more readily dissociated than lipoprotein complexes. Furthermore, certain sulfated polysaccharides selectively precipitate β-lipoproteins from plasma (191). Albumin forms soluble complexes with sulfated polysaccharides which do not interfere in precipitation methods for the isolation of lipoproteins (114, 115, 123 to 126).

Precipitation is a function of pH and the isoelectric point of the protein. The S_f 0–10 or β-lipoprotein fraction, isoelectric at pH 5.7 (196), forms a soluble complex with heparin in alkaline solutions and an insoluble complex at or below its isoelectric point (Table 3). The β-lipovitellin fraction of egg yolk, isoelectric at pH 5.9 (199), reacts in the same manner (Table 3). Since many other proteins precipitate in this pH range, reaction conditions must be adjusted for the selective precipitation of lipoproteins in the neutral to alkaline pH range. This is accomplished by the addition of specific metal ions to the reaction mixture, or by the utilization of sulfated polysaccharides which precipitate lipoproteins at an alkaline pH.

The specificity of some lipoprotein-sulfated polysaccharide interactions is a function of ionic strength. Thus heparin and calcium chloride flocculate the chylomicrons in lipemic serum but do not precipitate the β-lipoproteins unless the ionic strength is decreased (200, 201). Control of ionic strength is important in the selective precipitation of lipoproteins since other serum proteins may precipitate together with lipoprotein-sulfated polysaccharide-metal ion complexes at low ionic strengths (202). Insoluble lipoprotein-sulfated polysaccharide complexes dissolve in solutions of high ionic strength (37, 192, 201). This property

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TABLE 3. Effect of pH on the Formation of Lipoprotein-Sulfated Polysaccharide Molecular Complexes

Polysaccharide	Lipoprotein	pH	Molecular Complex	Reference
Heparin	β-Lipoprotein	8.6	Soluble-reversible	(190)
Heparin	β-Lipoprotein	5.0–5.7	Insoluble	(197)
Dextran sulfate*	β-Lipoprotein	8.6	Soluble-irreversible	(192)
Dextran sulfate*	β-Lipoprotein	5.0–5.7	Insoluble	(197)
Carrageenin-k	β-Lipoprotein	8.6	Soluble-irreversible	(192)
Carrageenin-k	β-Lipoprotein	6.8	Partially soluble	(198)
Carrageenin-k	β-Lipoprotein	4.6	Insoluble	(198)
Heparin	β-Lipovitellin	9.8	Soluble-reversible	(193)
Heparin	β-Lipovitellin	7.8	Partially soluble	(193)

* Low molecular dextran sulfate used as a synthetic anticoagulant.

of the molecular complex is important in many isolation procedures since the precipitate is isolated, dissolved in 5% to 12% sodium chloride, and reprecipitated by dilution with water or buffer (192, 201). The lipoprotein fraction may be purified by several reprecipitations.

Metal Ions. The formation of insoluble lipoprotein-sulfated polysaccharide complexes often depends upon the addition of metal ions to the reaction mixture. This effect has been investigated in detail by Burstein and his associates and the results of these studies are summarized in Table 4. Heparin and low molecular weight dextran sulfates both form soluble complexes with lipoproteins above pH 5.7 (192). Insoluble complexes are formed in the range from pH 6.5 to 8.5 by the addition of specific metal ions (206). The lipoprotein-sulfated polysaccharide-metal ion complexes are soluble above pH 9 (201, 206). Metal ions, pH, and ionic strength all contribute to the specificity of complex formation. For example, heparin forms a soluble complex with β-lipoproteins in the presence of Ca(II) or Mg(II), whereas chylomicrons in lipemic serum are flocculated under the same conditions (Table 4). An insoluble β-lipoprotein-heparin complex is formed with Ca(II) or Mg(II) if the ionic strength is lowered, but low molecular weight dextran sulfate precipitates β-lipoproteins in the presence of Ca(II) or Mg(II) without altering the ionic strength (Table 4). The γ-globulins are precipitated by heparin in the presence of Co(II), and Burstein and Prawerman (202) recently proposed a method for the turbidimetric estimation of γ-globulins in which β-lipoproteins are first precipitated in the presence of Ca(II), and then the γ-globulins are precipitated with Co(II).

The metal ions considered in Table 4 fall into two groups: (*a*) the alkali earths, Mg(II), Ca(II), Sr(II), and Ba(II), which interact primarily with carboxyl and sulfate groups, and (*b*) the transition elements, Mn(II), Co(II), and Ni(II), which are characterized by their strong tendency to form complexes with nitrogen containing ligands such as the amino and imidazole groups of proteins (214). The latter group of metal ions would be expected to form complexes with proteins and thereby contribute to the positive charge of the molecule. Thus the effect of these ions in promoting the formation of insoluble complexes with sulfated polysaccharides may be quite analogous to the effect of hydrogen ions. In this connection it

TABLE 4. Effect of Metal Ions on the Formation of Insoluble Sulfated Polysaccharide Molecular Complexes in Serum at pH 7.4*

Polysaccharide	Metal Ion	Composition of Insoluble Complex	Reference
Heparin	Ca(II), Mg(II)	β-Lipoprotein complex†	200, 201, 203, 204
Heparin	Ca(II), Mg(II)	Chylomicron complex	200, 201, 203, 204, 205
Heparin	Ni(II), Co(II), Mn(II)	β-Lipoprotein complex‡	200, 201, 203, 204, 206, 207, 208
Dextran sulfate	Ca(II), Mg(II)	β-Lipoprotein complex	200, 201, 203, 204, 207 to 212
Dextran sulfate	Ba(II), Sr(II)	Dextran sulfate-metal ion complex	204
Mepesulfate®,§	Ca(II)	S_f 0–10 and S_f 10–400 lipoprotein complex	213

* The reaction mixtures were buffered by serum; however, the exact pH was not measured in every experiment.
† An insoluble complex formed only at low ionic strength.
‡ Other serum proteins also precipitate at low ionic strength (200, 203).
§ Mepesulfate® is the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside obtained from Hoffmann-La Roche.

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should be noted (Table 4) that heparin in the presence of these ions precipitates other serum proteins as well as β-lipoproteins at low ionic strength.

Although the addition of metal ions may be necessary for the precipitation of β-lipoproteins by some sulfated polysaccharides, their presence does not appear necessary for precipitation by others. Bernfeld *et al.* (190, 191, 192) precipitated lipoproteins from serum with several sulfated polysaccharides without adding metal ions; however the Ca(II) already present in serum might have been sufficient. Florsheim and Gonzales (213) found that whereas it was necessary to add calcium ions to effect the precipitation of β-lipoproteins with Mepesulfate®, no addition of calcium ions was necessary with rice starch sulfate. Oncley *et al.* (37) precipitated low density lipoproteins from resin collected plasma with a high molecular weight dextran sulfate. Although the calcium ion concentration was probably very low in this case, analytical data on this point are not available. Insoluble lipoprotein-sulfated polysaccharide-metal ion complexes are readily dissociated by the addition of chelating agents such as oxalate or citrate (201, 206), by increasing the ionic strength (37, 192, 202, 204, 206), and by selectively precipitating the sulfated polysaccharide as a Ba(II), Sr(II), or protamine salt (192, 202, 204, 206). It is of interest to note here that Hoch and Chanutin (126) found that chelating agents did not affect the formation of soluble protein-heparin complexes as studied electrophoretically.

Structure of the Sulfated Polysaccharide. Bernfeld *et al.* (192) found that sulfate content and degree of polymerization are two major factors influencing complex formation. β-Lipoprotein is precipitated completely by sulfated corn amylopectin, which contains from 0.85 to 2.18 sulfate groups per hexose unit, while only partial precipitation is obtained with 0.22 to 0.57 sulfate groups per hexose unit (Table 5). Soluble complexes are not formed with corn amylopectins of low sulfate content. It thus appears that complexes, if formed at all with sulfated corn amylopectins, are of the insoluble variety. The effect of sulfate content was confirmed with sulfated hyaluronic acid and sulfated chondroitin sulfate, both of which form stronger complexes than the native polysaccharides (192). Precipitation is enhanced by increasing the concentration of polysaccharides with low sulfate content.

Insoluble complexes are formed with high molecular weight dextran sulfates, whereas soluble-irreversible complexes are formed with low molecular weight dextran sulfates with the same sulfate content per repeating unit (Table 5). Corn amylose sulfates varying in degree of polymerization from 80 to 800 demonstrated a moderate transition from soluble-irreversible to insoluble types. It is of interest to note that precipitation is not increased by increasing the concentration of low molecular weight polymer.

Bernfeld *et al.* (192) also found that the chemical composition of the polysaccharide influenced the nature of the molecular complex. Sulfated polysaccharides which contain carboxyl groups (sulfated pectic or polymannuronic acids, Table 5) form only soluble-irreversible complexes. The molecular complex is of the soluble-reversible type in the case of sulfated poly-

TABLE 5. EFFECT OF POLYSACCHARIDE STRUCTURE ON THE FORMATION OF MOLECULAR COMPLEXES WITH β-LIPOPROTEINS AT pH 8.6*

Polysaccharide	Sulfate Groups per Repeating Unit	Degree of Polymerization	Molecular Complex
Corn amylopectin sulfate	2.18	>2,000	Insoluble
Corn amylopectin sulfate	0.85	>2,000	Insoluble
Corn amylopectin sulfate	0.57	>2,000	Insoluble†
Corn amylopectin sulfate	0.22	>2,000	Insoluble†
Corn amylopectin	0	>2,000	None
Dextran Sulfate, from Strain			
NRRL-B512	1.44	240,000	Insoluble
NRRL-B512	1.58	440	Soluble-irreversible‡
NRRL-B1254	1.59	>6,000	Insoluble
NRRL-B1254	1.09	540	Soluble-Irreversible‡
Pectic acid sulfate	1.4		Soluble-Irreversible
Polymannuronic acid sulfate	1.15		Soluble-Irreversible
Hyaluronic acid sulfate	0.58–1.33		Soluble-Reversible
Chitin sulfate	0.68		Soluble-Reversible
Heparin	1.45–2.68		Soluble-Reversible

* Compiled from the investigation of Bernfeld *et al.* (192).
† Incomplete precipitation was obtained; however, no soluble complex was formed.
‡ A slight turbidity developed.

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saccharides containing N-acetyl and N-sulfatyl hexosamine residues. This is illustrated by sulfated hyaluronic acid, sulfated chitin, and heparin in Table 5.

The polysaccharides suitable for complex formation have no apparent specific requirement for degree of branching, branching points, configuration of glycosidic linkages, or hexose composition (192). However, this investigation was limited to a single pH, and metal ion interactions were not considered. Other relationships may become apparent when pH, ionic strength, and metal ion effects are considered along with polysaccharide structure.

Lipoprotein Specificity. Many of the experimental results obtained in the study of plasma lipoprotein-sulfated polysaccharide interactions are difficult to interpret because the criteria used to establish specificity were inadequate. In precipitation experiments specificity was usually demonstrated by paper electrophoresis (200 to 212). Paper electrophoresis may show the absence of β-lipoproteins from the supernatant solution. However, chylomicrons and S_f 10–400 lipoproteins may not be differentiated from one another by this technique. Groulade *et al.* (215) were able to correlate turbidity or precipitation with paper electrophoretic data by combining the β-lipoproteins and the less mobile lipoproteins as one fraction. This indicates that precipitation involves both S_f 0–10 and S_f 10–400 lipoproteins, though the correlation was less apparent with lipemic sera (215). Moving boundary electrophoresis indicates that serum lipoprotein-sulfated polysaccharide interactions involve the β-lipoprotein or S_f 0–10 lipoprotein fraction (190, 191, 192, 216). Starch gel and agar electrophoretic data are difficult to interpret since molecular size affects protein mobility in a starch gel (217), and agar, a sulfated polysaccharide, interacts with the low density lipoproteins (218).

Analytical and preparative ultracentrifugation have been used by several investigators to determine lipoprotein specificity in sulfated polysaccharide interactions. Oncley *et al.* (37) fractionated the lipoproteins from a high molecular weight dextran sulfate complex by ultracentrifugation in a density gradient. Analytical ultracentrifugation was then used to measure the S_f distribution in the lipoprotein fractions. These fractions contained S_f 3–9, S_f 5–15, and S_f 10–100 lipoproteins; however, the quantitative recovery of the different low density lipoproteins from serum was not measured. Florsheim and Gonzales (213) compared serum and the lipoproteins obtained as an insoluble rice starch sulfate complex by analytical ultracentrifugation. The precipitate contained the S_f 0–12 lipoproteins and most of the S_f 12–400 lipoproteins. Boyle and Moore (198) fractionated serum by preparative ultracentrifugation into the lipoprotein-free infranate ($d > 1.21$), high density lipoproteins (d 1.063–1.21), S_f 0–16 lipoproteins (d 1.006–1.063), and $S_f > 17$ lipoproteins ($d < 1.006$). Only the S_f 0–16 lipoprotein fraction formed an insoluble complex with the carrageenin used in their study. Sulfated corn amylopectin precipitated S_f 2–12 lipoproteins from serum, according to a study by Bernfeld *et al.* (192). In an earlier study Bernfeld *et al.* (216) found the turbidity with sulfated corn amylopectin to be specific for the β-lipoprotein fraction as isolated by zone electrophoresis on a starch block.

An insoluble complex has been obtained by the flocculation of lipemic serum with heparin and Ca(II) ions, and attributed to the chylomicron fraction (201, 203, 204, 207, 208). Since the chylomicron fraction is often poorly defined, and since lactescent serum also contains increased amounts of S_f 10–400 lipoproteins, ultracentrifugation studies are necessary to confirm this important observation. Burstein and Samaille (205) prepared antibodies against this chylomicron fraction and found them to flocculate chylomicrons, whereas they only reacted weakly with β-lipoproteins. However, the chylomicrons employed in the production of antibodies were subject to centrifugation during the isolation procedure from the heparin complex and may therefore have been separated from any S_f 10–400 lipoproteins coprecipitated with them. Burstein and Samaille (201) also found that the protein content of the lipoprotein-heparin-Ca(II) ion precipitates was altered by varying the ionic strength of the reaction mixture. The fraction isolated at low ionic strength contained more protein than the fraction isolated directly from serum. To what extent this difference indicates contamination by nonlipoprotein protein or selectivity between precipitation conditions for the chylomicrons, S_f 10–400 lipoproteins, and S_f 3–9 lipoproteins remains to be investigated.

Electrophoretic and ultracentrifugal techniques indicate that the sulfated polysaccharide interactions with respect to human serum are specific for chylomicrons and low density lipoproteins. This is further substantiated by the more sensitive immunochemical techniques. Burstein and Oudin (219) prepared antibodies against the lipoproteins precipitated from serum as a dextran-Ca(II) complex, and demonstrated the immunochemical homogeneity of the lipoprotein fraction. Antibodies to this fraction have been used to estimate the β-lipoprotein content of serum (220, 221). The antibodies are probably not strictly specific for β-lipoproteins alone, since S_f 0–10 lipoproteins and S_f 10–400 lipoproteins do not differ immunochemically (57). As discussed earlier, there is some question concerning the electrophoretic behavior of S_f 10–400 lipo-

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proteins; at least some portions of this fraction do not migrate as β-lipoproteins. Briner *et al.* (57) isolated low density lipoproteins by precipitation with high molecular weight dextran sulfate, fractionated the precipitated lipoproteins in a saline density gradient into S_f 0–10 and S_f 10–400 lipoproteins, and prepared antibodies against the two fractions. These two fractions cross-reacted completely, and therefore cannot be distinguished by immunochemical means.

Although the composition of different lipoprotein fractions isolated from normal sera by other procedures has been studied in detail, chemical analysis has not been used extensively in the characterization of lipoprotein-sulfated polysaccharide precipitates. This is due in part to the fact that the precipitates often contain mixtures of S_f 0–10 and S_f 10–400 lipoproteins, and the composition of the precipitate may vary, depending on the relative concentration of these two lipoproteins in the serum. The total lipid content of sulfated amylopectin precipitates (190) and the range in protein, sterol, phosphatide, and glycerol content for a low molecular weight dextran sulfate precipitate (206) have been reported. Polonovski *et al.* (211) studied the total lipid, cholesterol, and phospholipid composition of a dextran sulfate precipitate and the corresponding supernatant solution. Cholesterol to phospholipid ratios calculated from this study indicate that the precipitate contained low density lipoproteins and the supernatant contained the high density lipoprotein fraction. Castaigne and Amselem (212) measured the total cholesterol, esterified cholesterol, and free cholesterol in similar fractions and found the appropriate esterified to total cholesterol ratios for low and high density lipoproteins in the precipitate and supernatant, respectively. Oncley *et al.* (37) isolated S_f 10–100 and S_f 3–9 lipoprotein fractions from a high molecular weight dextran sulfate precipitate by ultracentrifugal flotation and showed that the composition of these fractions was identical with that of lipoprotein fractions isolated directly by ultracentrifugation. The cholesterol distribution between lipoprotein-sulfated polysaccharide precipitate and supernatant solution has been measured in several studies (178, 206, 208, 209, 222), and is similar to the cholesterol distribution between low and high density lipoproteins as determined by ultracentrifugal, electrophoretic, and cold-ethanol fractionation methods. However, Beaumont and Beaumont (223) found an appreciable quantity of vitamin A in lipoprotein-dextran sulfate-Ca(II) precipitates, whereas Krinsky *et al.* (224) found only traces of vitamin A in lipoprotein-dextran sulfate precipitates further purified by ultracentrifugal flotation.

Sulfated polysaccharide precipitation methods have been used to investigate the plasma lipoprotein distribution in disease states associated with hypercholesterolemia and hyperlipemia (170, 172, 173, 178, 180, 181, 198, 208, 209, 211, 212, 216, 222, 225, 226, 227). These studies are difficult to evaluate since the methods are usually standardized with sera from normal subjects, or lipoprotein fractions isolated from normal subjects. Since the S_f distribution and composition of lipoprotein fractions show wide variation in many diseases, direct extrapolation from normal to pathological sera may be misleading. The cholesterol distribution between precipitate and supernatant observed in hyperlipemic sera is generally similar to that obtained by other methods, although in some cases the cholesterol content of the supernatant (high density lipoprotein) is greater than would be expected from other studies (178, 209, 211, 212, 222). It is possible that all portions of the low density lipoprotein spectrum are not precipitated under the conditions employed. It is also important to be certain that the concentration of precipitating polyanion is adequate for quantitative precipitation.

The investigation of plasma lipoproteins in different animal species by sulfated polysaccharide precipitation methods presents special problems. Castaigne and Amselem (212) used dextran sulfate to investigate the cholesterol distribution in a number of animal species. Their results were similar to those obtained by other methods; however, the characterization of animal lipoproteins is as yet too incomplete for a significant correlation between methods. Burstein and Samaille (228) studied lipoprotein-dextran sulfate complexes in several animal species by paper electrophoresis and were able to demonstrate precipitation of β-lipoproteins. However, Florsheim and Gonzales (213) obtained variable precipitation of different animal β-lipoproteins with rice starch sulfate, a polyanion which precipitates human low density lipoproteins quantitatively. These workers found that Mepesulfate® precipitated many animal β-lipoproteins in a quantitative manner. The use of sulfated polysaccharides will undoubtedly contribute to the characterization of animal serum lipoproteins, but it is most important that they be initially employed in conjunction with electrophoretic and ultracentrifugal techniques.

Concentration. The solubility of lipoprotein-sulfated polysaccharide complexes is influenced by the relative concentration of lipoprotein and sulfated polysaccharide. Soluble complexes are formed if either lipoprotein or sulfated polysaccharide is present in large excess. Oncley *et al.* (37) suggested that a serum aliquot be titrated to maximum turbidity in order to establish the optimal sulfated polysaccharide concentration for

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precipitation. Bernfeld *et al.* (216), Boyle and Moore (198), and Antoniades *et al.* (226) have published experimental results relating precipitation or turbidity to sulfated polysaccharide concentration. The region of maximum precipitation is broad, and a single polyanion concentration is adequate for most studies. Nevertheless, this region should be determined particularly in the evaluation of new sulfated polysaccharides or in the isolation of lipoproteins from markedly hypercholesterolemic or hyperlipemic sera.

Isolation of Plasma Low Density Lipoproteins. Several lipoprotein isolation procedures involving the use of sulfated polysaccharides have been reported and three of these are outlined in Charts I, II, and III. In two procedures (Charts I and II) the lipoproteins are purified by reprecipitation of the sulfated polysaccharide complex and the low density lipoproteins are isolated as a single fraction. In the third procedure (Chart III) density gradient ultracentrifugation is employed to further purify the lipoproteins and at the same time separate them into S_f 0–10 and S_f 10–400 fractions. The sulfated polysaccharide is removed either as an insoluble barium salt (Chart I), or an insoluble protamine complex (Chart II), or is concentrated in the infranatant solution by ultracentrifugation (Chart III). These methods are amenable to modification, and certain steps such as reprecipitation and ultracentrifugation may be employed in the same procedure. In studies where the rapid isolation of low density lipoproteins is required and purity is not critical, a

CHART I

ISOLATION OF β-LIPOPROTEINS WITH SULFATED AMYLOPECTIN (192)

Serum 60 ml

a. Add 9 ml 1% sulfated amylopectin
b. Centrifuge at 15,000 × g

Precipitate	Supernatant
β-Lipoprotein-sulfated amylopectin complex	Discard

a. Dissolve in 3 ml 12% NaCl
b. Reprecipitate by diluting with 57 ml 0.02 M Na_2HPO_4
c. Centrifuge at 15,000 × g

Precipitate	Supernatant
β-Lipoprotein-sulfated amylopectin complex	Discard

a. Dissolve in 3 ml of 12% NaCl
b. Add 0.75 ml 0.1 M barium acetate
c. Centrifuge

Precipitate	Supernatant
Amylopectin sulfate-Ba (II) complex	β-Lipoproteins

a. Pass through Amberlite® IR 120* column charged with NaCl to remove excess Ba (II)†

β-Lipoprotein solution‡

* Amberlite® IR 120 obtained from Rohm and Haas.

† Lipoprotein stability is improved if excess Ba (II) is precipitated by the addition of 1 ml 0.1 M sodium sulfate rather than passage through an ion-exchange column. (P. Bernfeld, private communication.)

‡ The β-lipoprotein solution is stabilized by the addition of ethylenediaminetetraacetic acid, 2 mg/10 ml. or pyrophosphate, 0.01 M final concentration.

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CHART II

ISOLATION OF β-LIPOPROTEINS WITH DEXTRAN SULFATE (201)

Serum 100 ml

a. Add 2 ml 10% dextran sulfate* and 10 ml 1 M $CaCl_2$
b. Centrifuge at 5450 × g for 10 minutes†

Precipitate	Supernatant
β-Lipoprotein-dextran sulfate complex	Discard

a. Dissolve in 10 ml 5% NaCl
b. Reprecipitate by diluting with 90 ml distilled water and 10 ml 1 M $CaCl_2$
c. Centrifuge at 5450 × g for 10 minutes†

Precipitate	Supernatant
β-Lipoprotein-dextran sulfate complex	Discard

a. Dissolve in 5 ml of 0.1 M sodium oxalate
b. Add 2 g NaCl and 2.5 ml 2% protamine sulfate‡
c. Centrifuge

Precipitate	Supernatant
Dextran sulfate-protamine complex	β-Lipoprotein solution

a. Dialyze against 0.15 M NaCl

β-Lipoprotein solution

* Dextrarine® obtained from Equilibre Biologique.
† M. Burstein, private communication.
‡ High ionic strength prevents formation of a protamine-lipoprotein complex.

single precipitation step may be adequate for the isolation of a low density lipoprotein fraction (229, 230). The purified lipoprotein fractions should be analyzed for the presence of residual sulfated polysaccharides. This may be accomplished by metachromatic staining with toluidine blue (231) or the thiazine dye Azure A (232), or by measuring the antithromboplastic activity of the fraction (232). Ribonuclease inhibition has been used as a sensitive assay for heparin (233). Jaques and Bell (234) have recently discussed the methods used for the determination of heparin.

It should perhaps be re-emphasized that isolation procedures developed for use with normal human serum may require modification before they are applied to the sera of hyperlipemic individuals or of other animal species. For example, the insoluble complexes obtained from hyperlipemic sera may float rather than sediment upon centrifugation (201, 203, 205, 206, 208, 211). In adapting these methods to other than normal human sera, preliminary studies including electrophoretic, ultracentrifugal, and chemical analyses of both precipitate and supernatant fractions should be carried out. It may be necessary to alter certain of the parameters which affect specificity and quantitative precipitation such as pH, ionic strength, metal ions, and concentration of precipitant.

Quantitative Estimation of Plasma Lipoproteins. Several procedures involving the formation of insoluble sulfated polysaccharide complexes have been proposed for the estimation of plasma lipoproteins. In some of these the cholesterol content of precipitate and supernatant fractions is determined (178, 203, 207, 208, 209, 212). In others the turbidity produced is measured as an estimate of the amount of insoluble lipoprotein complex formed (170, 178, 188, 191, 203, 206, 210, 215, 216, 225, 226). Both methods have limitations. The cholesterol content of the precipitate and the turbidity give an estimate of the total low density lipo-

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proteins. No information is obtained concerning the relative amounts of the major low density lipoprotein fractions. This defect could perhaps be partially remedied by determining the lipid phosphorus content of the precipitated complex in addition to the cholesterol. Since the cholesterol to phospholipid ratios of the S_f 0–10 and S_f 10–400 subfractions differ considerably (1.30–1.45 and 0.78–0.90, respectively), some idea of the relative contribution of each to the total might be obtained. It should be kept in mind, however, that the cholesterol to phospholipid ratios of the S_f 0–10 and S_f 10–400 lipoproteins are frequently altered in abnormal states. Turbidimetric methods are also subject to error dependent upon the relative contribution of S_f 0–10 and S_f 10–400 lipoproteins since the turbidity increment is probably different for the two fractions. Lactescent sera exhibit an inherent turbidity which interferes with evaluation of the turbidity due to complex formation. Furthermore, turbidity produced by abnormal lipoproteins may not be comparable to that

CHART III

Isolation of Low Density Lipoprotein Fractions with Dextran Sulfate (37, 57)

Serum

a. Add dextran sulfate*
(Titrate aliquot to maximum turbidity)
b. Stir 2 hours at 2°
c. Centrifuge 30 minutes 1000 × g at 2°

Supernatant
Discard

Precipitate
Low density lipoprotein-dextran sulfate complex

a. Dissolve in 5 ml 2 M NaCl† per 200 ml original serum
b. Prepare density gradient tube

Gradient Tube A
13.5 ml lusteroid tube
a. Place 6 ml lipoprotein solution in tube
b. Fill tube by layering 0.15 M NaCl† above lipoprotein

Centrifuge§ 20 hours → 100,000 × g, 2° to 5°

Gradient Tube A‡
a. Top layer: turbid S_f 10–400 lipoproteins
b. Intermediate layer: S_f 5–15 lipoproteins
c. Middle layer: orange S_f 0–10 lipoproteins
d. Infranate: protein contaminants and dextran sulfate

Gradient Tube B
13.5 ml lusteroid tube
a. Dialyze S_f 0–10 lipoproteins against 20 volumes 2 M NaCl† for 18 hours
b. Place 6 ml lipoprotein solution in tube
c. Fill tube by layering 0.15 M NaCl† above lipoproteins

Centrifuge§ 20 hours → 100,000 × g, 2° to 5°

Gradient Tube B‡
a. Top layer: discard
b. Middle layer: orange S_f 0–10 lipoproteins
c. Bottom layer: discard
Dialyze S_f 0–10 lipoproteins against 0.15 M NaCl†

Centrifugation C
13.5 ml lusteroid tube
a. Dialyze S_f 10–400 lipoproteins against 20 volumes 0.15 M NaCl† for 18 hours
b. Fill tube

Centrifuge§ 20 hours → 100,000 × g, 2° to 5°

Centrifugation C‡
a. Top layer: turbid S_f 10–400 lipoproteins#
b. Infranate: small amount S_f 10–20 lipoproteins

* High molecular weight dextran sulfate (37).
† All salt solutions contain 0.1 g per liter of the disodium salt of ethylenediaminetetraacetic acid adjusted to pH 7.0 ± 0.2 with 1 N NaOH.
‡ Separate layers with tube cutter.
§ Spinco Model L ultracentrifuge.
# Chylomicrons and lipoproteins with a high S_f may be separated by centrifugation for a shorter time in a lower centrifugal field.

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produced by a like amount of normal lipoprotein. Finally, it should be pointed out that purified lipoproteins have not proved satisfactory as standards for the turbidimetric analysis of serum lipoproteins, largely because a number of substances present in serum enhance turbidity (192).

Molecular Complexes in Plaque Formation. The demonstration of synthetic lipoprotein-sulfated polysaccharide complexes led Gerö *et al.* (235) to suggest that lipid deposition in the aorta resulted from the formation of a plasma lipoprotein-mucopolysaccharide complex at the site of plaque formation. Atherosclerosis is accompanied by an elevation in aortic mucopolysaccharides (235, 236, 237), and there may be a focal increase in metachromatic material in the area of plaque formation (238). Farquhar *et al.* (239) have suggested that atheroma and plasma fatty acids are in a dynamic equilibrium; however, the net deposition of lipid or preformed plasma lipoprotein in the aorta has not been demonstrated. Also, lipid synthesis has been shown to occur in arterial tissue (240). The relative contribution of plasma lipid and synthesis of lipid, *in situ*, to the lipid in the atherosclerotic lesion, as well as the nature of the binding of the lipid therein, remains obscure (241).

THE INTERACTION OF LIPOPROTEINS WITH NEUTRAL POLYMERS

Polyvinylpyrrolidone (PVP) is a neutral polymer which has been used as a plasma extender.[5] Although PVP preparations are available in several molecular weight ranges, the one studied most contains between 150 and 170 monomer units and has a mean molecular weight of about 40,000 (242). PVP as obtained from the manufacturer migrates in an electric field and exhibits two types of ionizable groups (pK 4.3 and 10) on titration with alkali (243). However, it has been shown that these groups are associated with a low molecular weight inpurity and PVP which has been subjected to exhaustive dialysis is essentially nonionogenic throughout the pH range 2.5 to 10 (244). Also, ultraviolet absorption indicates that the structure of the polymer remains essentially unaltered over a wide pH range (245, 246). PVP forms molecular complexes with a number of compounds (242, 244, 245, 247, 248). Higuchi and Kuramoto (247) have suggested that complex formation is effected by hydrogen bonding. Another neutral polymer, poly-N-vinyl-5-methyl-2-oxazolidinone (Devlex®), has binding properties similar to PVP, and this has been attributed to the formation of resonance complexes.[6]

The literature on the interactions between PVP and serum proteins appears to be contradictory. May *et al.* (242), Ardry (249), and Hirsch and Cattaneo (250) found no evidence of interaction by electrophoretic techniques. However, Grabar (251) concluded from the results of hemagglutination studies that PVP did interact with serum proteins. Furthermore, Ramos (252) reported that PVP altered the electrophoretic mobilities of albumin and γ-globulins. Banerjee (253) studied the interaction between PVP and fibrinogen by measuring viscosity effects and found that high molecular weight PVP formed complexes with fibrinogen, whereas low molecular weight PVP did not appear to do so. It is therefore possible that some of the apparent discrepancies are due to differences in the PVP preparations used by the several investigators.

In 1957 Burstein (5) reported that PVP formed an insoluble complex with the β-lipoproteins. Burstein and co-workers (5, 254, 255, 256) have studied this interaction in detail. Precipitation with PVP is a function of pH, ionic strength, and PVP concentration. Only chylomicrons complex above pH 9.5, whereas β-lipoproteins also form precipitates in the intermediate pH range, and both lipoproteins and γ-globulins precipitate when the pH is lowered to 4 (5, 254, 255). The formation of insoluble complexes with β-lipoproteins and γ-globulins is inhibited in hypertonic sodium chloride solutions (5, 254, 255). Hypertonic salt solutions do not inhibit PVP interaction with chylomicrons or the flocculation of chylomicron-PVP complexes (254, 255). Only chylomicrons are flocculated when the final PVP concentration is 5%, both chylomicrons and β-lipoproteins are precipitated when the concentration is raised to 8%, and other globulins are also precipitated in 12% PVP (5, 254, 256). The lipovitellenin or rising lipoprotein fraction of egg yolk may be flocculated as a Devlex-130® complex.[7]

PVP has been employed in a turbidimetric method for the estimation of chylomicrons and lipoproteins in serum (256). Burstein and Prawerman (255) recently developed a method for the isolation of low density lipoproteins from hyperlipemic sera which involves varying ionic strength and PVP concentration for the selective formation of insoluble lipoprotein-PVP complexes (Chart IV). The lipoprotein fractions are further purified as heparin complexes, and the chylomicrons isolated by a final ultracentrifugal flota-

[5] See footnote 3.

[6] See footnote 4.

[7] D. A. Wolfe and D. G. Cornwell, unpublished observations.

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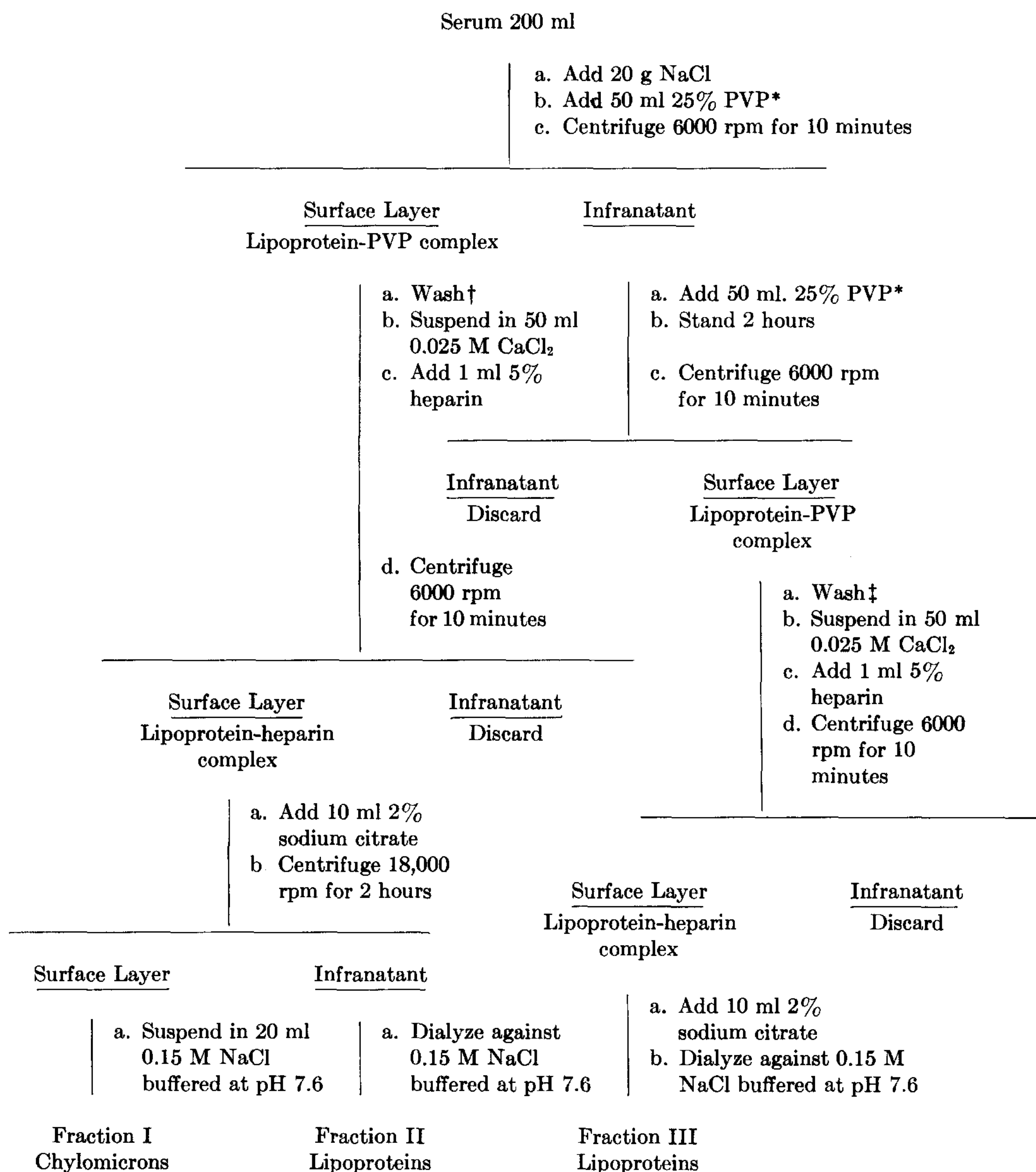
CHART IV

ISOLATION OF LOW DENSITY LIPOPROTEINS FROM HYPERLIPEMIC SERUM WITH PVP (255)



* Subtosan retard® obtained from Specia.
† Wash 3 times by suspending in 50 ml of a solution containing 10% NaCl and 5% PVP.
‡ Wash 3 times by suspending in 50 ml of a solution containing 10% NaCl and 8% PVP.

tion step. The composition of the three fractions obtained is summarized in Table 6. The lactescence of the three fractions (comparing aliquots with equal lipid content) decreases from fraction I to fraction III. Fraction III is more opalescent than the β-lipoprotein fraction obtained from normal serum. It would appear from this study that considerable specificity can be attained within the low density lipoprotein spectrum with precipitation techniques employing neutral polymers. However, much work remains to be done in defining conditions and characterizing the fractions obtained.

In summary, the fractionation, estimation, and characterization of lipoproteins by complex formation with a variety of high molecular weight substances is an active area of research. However, although the formation of insoluble molecular complexes has proved valuable in the initial concentration and purification of certain

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TABLE 6. Composition of Lipoprotein Fractions Isolated from Lipemic Serum by Precipitation with PVP

Lipoprotein Fraction	Percentage Composition (Range)*		
	Protein	Cholesterol	Phospholipid
I	1.3–2.0	4.4–6.4	7.4–8.8
II	3.8–5.2	7.2–10.1	9.5–11.0
III	6.3–7.0	11.0–12.6	14.0–15.4
β-Lipoproteins†	16–20	26–33	14–20.5

* Calculated from the data of Burstein and Prawerman (255).
† Lipoproteins isolated from the serum of a normal subject.

lipoprotein classes, our knowledge and methodology have not yet progressed sufficiently to employ these methods to the exclusion of others for the preparation of homogeneous lipoprotein fractions. Ultracentrifugal procedures remain the method of choice for the isolation and estimation of specific lipoproteins.

ADDENDUM

Several important studies have appeared since the completion of this review. Bernfeld *et al.* (257) have employed sulfated amylopectin in a nephelometric procedure for the estimation of relative serum lipoprotein concentrations in normal as well as disease states. They describe a method for the standardization of sulfated amylopectin preparations based on the turbidity produced in the formation of sulfated amylopectin-Ba(II) complexes. Ledvina *et al.* (258), using heparin in a turbidimetric procedure, have published a study on lipoprotein concentrations in thyroid disorders. Amenta and Waters (259) have isolated a mucopolysaccharide from human aortas and used this sulfated polysaccharide to precipitate lipoprotein fractions obtained from the serum of hypercholesterolemic rabbits. Amenta and Waters (260) have also investigated the formation of insoluble molecular complexes between rabbit lipoprotein fractions and gelatin.

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