pH and Ionic Strength Dependent Aggregation of Serum Low-Density Lipoproteins*

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ABSTRACT: Normal serum low-density lipoproteins, density 1.02–1.06 g/cm³, undergo a reversible ionic strength pH-dependent aggregation as visualized in the analytical ultracentrifuge. Monodisperse low-density lipoprotein preparations of high salt concentration undergo a transition yielding multiple discrete components when the solution pH is decreased from neutrality to the range of 4.5–5.5. Below pH 4.5 visible precipitation of low-density lipoproteins occurs except in very dilute salt solutions. In the pH range of 4.5–5.5 the transition from a single to a multicomponent state is promoted by increasing ionic strength and occurs in solutions of KBr, NaCl, and NaBr, and this aggregation is further enhanced by increasing low-density lipoprotein concentration. Measurement of the buoyant density of the components indicates that the "monomer" has the same buoyant density as normal low-density lipoprotein, but the aggregates have slightly increased densities. Consistent with the decreased $\bar{V}$ of the aggregates, low-density lipoprotein shows enhanced aggregation under high hydrodynamic pressure occurring during centrifugation. These observations emphasize the need for control of pH and ionic strength in studying solutions of low-density lipoproteins.

The isolation and characterization of human serum lipoproteins by differential density flotation centrifugation is a standardized technique (DeLalla and Gofman, 1954). Serum lipoproteins have been well characterized and defined by their flotation and sedimentation characteristics in various salt solutions, and recent studies utilizing the high salt density flotation techniques have facilitated the characterization of low-density lipoprotein by analytical ultracentrifugation (Del Gatto *et al.*, 1959; Adams, 1966; Adams and Schumaker, 1969).

Under these conditions of high salt concentration, with the resultant high ionic strength, and in the presence of a low pH, normal low-density lipoprotein may be demonstrated to undergo a reversible transition from a single to a multiple-component macromolecular state which is presumed to be the result of a reversible aggregation. This phenomenon is the subject of the present report.

Materials and Methods

All reagents were of Analytical grade, and solutions were made either with deionized or glass-distilled water.

Nonbuffered salt solutions of various densities were prepared with solid KBr or other salts as desired and contained 100 mg of Na_2EDTA/l. Buffered salt solutions were prepared over a range of pH 3.0–10.0 by utilizing stock solutions of 0.025 M citric acid, KH_2PO_4, and K_2HPO_4. Each solution contained 100 mg of Na_2EDTA/l. These solutions were mixed to obtain the desired pH as measured with a pH meter, and then solid KBr was added to produce the appropriate density. Density measurements were performed in triplicate at 25° using 2-ml pycnometers. In calculating the ionic strength of the solutions, the minor contributions of the buffer ions and EDTA were neglected. The values for the viscosity of the salt solutions were obtained from the International Critical Tables (1926, 1929).

Dialysis was performed with commercially available membranes which were boiled in distilled water containing 100 mg of Na_2EDTA and 2.5 g of $NaHCO_3$ per l. These membranes were stored under refrigeration and rinsed thoroughly with distilled water prior to use.

For the preparation of lipoproteins, serum was obtained from the fasting blood of two normal subjects. Low-density lipoprotein (density 1.02–1.06 g/cm³) was isolated in a Spinco Model L-2 preparative ultracentrifuge by a method similar to that of DeLalla and Gofman (DeLalla and Gofman, 1954). Specifically, the density of the serum was adjusted to 1.02 g/cm³ by adding KBr, and Na_2EDTA was added to a concentration of 100 mg/l. The serum was then placed in 10-ml tubes and centrifuged at 50,000 rpm in the Ti-50 head for 24 hr at 5°. The top turbid layer was removed from each tube and discarded. The density of the lower fraction was readjusted to 1.06 g/cm³ and recentrifuged. The resulting top fraction was carefully removed with a syringe, and approximately 1-ml samples were transferred to dialysis membranes and dialyzed with appropriate solutions. Lipoprotein concentration was determined by the Lowry technique (Bailey, 1967), using a purified preparation of low-density lipoprotein as a standard. The concentration of the standard was determined by lyophilizing the solution and weighing the dried lipoprotein. All studies were performed on freshly prepared lipoproteins obtained from either of two normal subjects.

Analytical centrifugational data were obtained using a Spinco Model E analytical untracentrifuge with schlieren optics. Centrifugation was conducted at 42,040 rpm and 25° in an An-D rotor. Double-sector cells with epon–aluminum-filled centerpieces and fitted with standard or 1° positive

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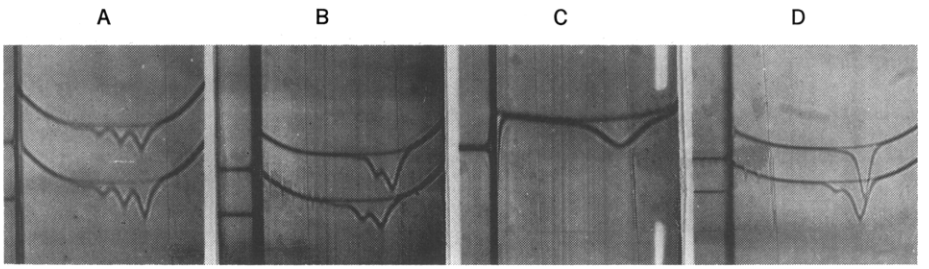

FIGURE 1: (A) Schlieren patterns of two solutions of normal low-density lipoprotein taken during analytical ultracentrifugation in KBr–Na_2EDTA solution of density 1.21 (μ = 2.6). Centrifugation at 42,000 rpm and photographs taken 14 min after reaching speed. Lipoprotein concentration wedged (upper) and standard (lower) cells are 2.9 and 3.4 mg per ml. Temperature 25°. Schlieren bar angle 65°. (B, C) Demonstration of reversible transition from single to multiple components. Condition of centrifugation as in part A with schlieren bar angle at 70°. Initial lipoprotein concentration 2.4 mg/ml. (B) Standard (lower) cell: initial LDL preparation at density 1.21 g/cm^3 (μ = 2.6), photographed at 11 min. (C) Same preparation dialyzed to density 1.06 g/cm^3 (μ = 0.78), photographed at 65 min. (B) Wedged (upper) cell: same preparation redialyzed to density 1.16 g/cm^3 (μ = 2.0), photographed at 11 min. (D) Demonstration of the effect of pH upon preparations of low-density lipoprotein. Centrifugational conditions as in part A with schlieren bar angle at 75°. Lipoprotein concentration 2.9 mg/ml. Photographed at 18 min. Wedged (upper) cell containing solution at pH 5.5 and standard (lower) cell at pH 4.9. Both solutions adjusted to density 1.16 (μ = 2.0) with KBr.

wedged windows were used. The cells were filled with 0.4 ml of sample and of reference solutions. All measurements were made from photographs of the schlieren patterns. Sedimentation coefficients for each run were calculated from the slopes of graphs of the logarithm of the distance from the center of rotation to the maximum ordinate of the lipoprotein peak *vs.* time.

Results

Following their isolation, solutions of low-density lipoprotein were dialyzed against KBr solutions of differing densities and containing Na_2EDTA. As indicated in Figure 1A and Table IA, during analytical flotation velocity ultracentrifugation, low-density lipoprotein solutions of low ionic strength ($\mu \leq 0.90$) were observed to have a single low-density lipoprotein component; however, in those solutions of high ionic strength ($\mu \geq 1.89$), several compoments were generally present.

The transition from single to multiple components was reversible as demonstrated in an experimental sequence where the density (and ionic strength) of an EDTA–KBr-containing solution of low-density lipoprotein was adjusted by dialysis in three stages as summarized in Table IB. The multiple components present in the initial solution at high ionic strength resolved into a single component upon reducing the ionic strength and again formed two components when the ionic strength was raised. Figure 1B,C shows the schlieren patterns observed with these solutions.

These initial observations were made with lipoproteins in salt solutions buffered only with low concentrations of Na_2EDTA. It was subsequently demonstrated that the pH of similar solutions varied within the range of 4.5–5.0.

In order to define the role of pH as a factor influencing the number of observed components in an isolated low-density lipoprotein preparation, EDTA-containing buffers of known pH were prepared over a pH range of 3.0–9.4. The ionic strengths of these buffered solutions were then adjusted by the addition of KBr, and isolated samples of low-density lipoprotein were dialyzed against four changes of these buffers in a 48-hr period. At the lower pH values visible precipitation of the lipoprotein occurred. The nonturbid solutions were studied by analytical ultracentrifugations, and the results are indicated in Figures 1D and 2. Upon inspection of Figure 2, which may be considered as a phase diagram, one may observe that at a given ionic strength, for example, 1.89, and a neutral pH, a single component is observed. When the pH is lowered to the range of about 5, multiple components become evident on ultracentrifugation, as shown in Figure 1D; however, this is a reversible process. Further acidification results in visible precipitation of the lipoprotein. By contrast, at a pH of 4.5 one may raise the ionic strength and induce a similar transition from one of several components. It thus is evident that the transition from a single to multiple components is influenced both by ionic strength and by pH.

In order to assess further whether this transition was specifically induced by KBr or was a general ionic strength

TABLE I: Ionic Strength Effect of KBr–Na_2EDTA Solutions upon Aggregation of Normal Low-Density Lipoprotein.

Subject	Soln Density	Ionic Strength	No. of Components
1	1.22	2.74	Multiple
	1.16	2.02	Multiple
	1.07	0.90	Single
	1.006	0.15	Single
2	1.21	2.62	Multiple
	1.21	2.62	Multiple
	1.15	1.89	Multiple
	1.06	0.78	Single
	1.006	0.15	Single
Reversibility of Ionic Strength Induced Low-Density Lipoprotein Aggregation			
Initial solution of low-density lipoprotein	1.21	2.62	Multiple
Dialyzed to	1.06	0.78	Single
Dialyzed to	1.16	2.02	Multiple

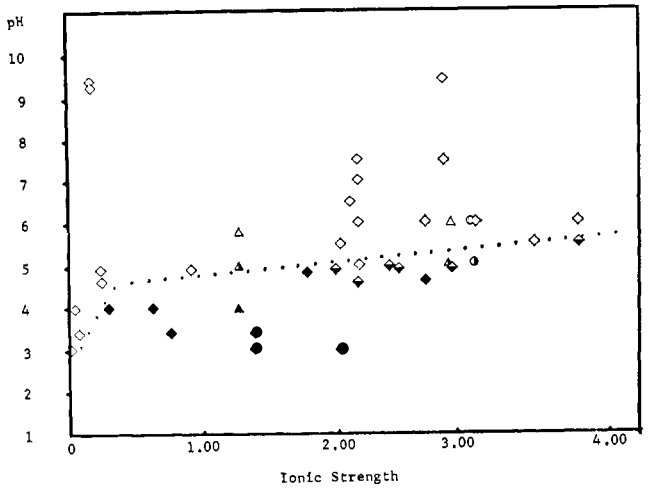

FIGURE 2: Modified phase diagram recording the effect of pH and ionic strength on number of low-density lipoprotein components observed in various salt solutions. (◇) KBr solutions, (△) NaBr solutions, and (○) NaCl solutions. Solutions represented by open symbols contained single low-density lipoprotein components and half-filled symbols represented solutions with multiple components. Solid symbols indicated solutions in which visible precipitation had occurred. (· · · ·) Approximate boundary between single and multicomponent low-density lipoprotein solutions.

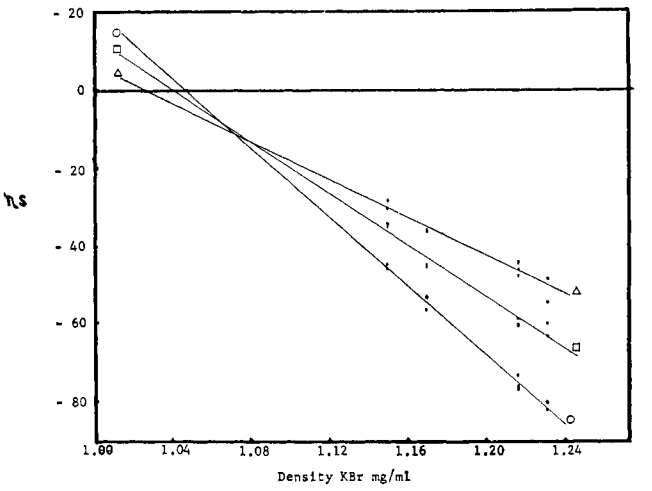

FIGURE 3: ηs *vs.* density plots of a triple component low-density lipoprotein (preparation 3) in KBr solutions. (○) Fastest component, (□) faster component, and (△) slower component. Each point represents a single analytical ultracentrifugational determination.

dependent phenomenon, samples of low-density lipoprotein were dialyzed against buffered solutions of NaCl and NaBr of known pH and ionic strength. The presence of single or multiple components was observed during analytical ultracentrifugation, and the results of these observations are incorporated in Figure 2. These results suggest that the transition from a single to multiple components is not ion specific.

In a further effort to clarify whether, in the transitional pH range (pH 4–5), the ionic strength or the density of the low-density lipoprotein solution was primary in determining the number of components observed, low-density lipoprotein was dissolved in solutions of density 1.15 g/cm^3 prepared alternately with KBr and KBr plus D_2O. In this manner isopycnic solutions of different ionic strength could be obtained. Specifically, aliquots of a solution of density 1.06 g/cm^3 (μ = 0.78), containing only a single low-density lipoprotein component, were adjusted to density 1.15 g/cm^3, first by adding KBr with a resultant μ = 1.89 and, second, by adding D_2O plus KBr with the resulting μ = 1.15. Upon analytical ultracentrifugation the former high ionic strength solution contained two low-density lipoprotein components; however, the latter solution of lesser ionic strength exhibited only a single component. Alternately, low-density lipoprotein multicomponent material in a KBr solution of density 1.21 g/cm^3 (μ = 2.63) was diluted with D_2O to a solution density of 1.15 g/cm^3 (μ = 1.07). Upon analytical centrifugation in one experiment the multicomponent system had resolved into a single component, though with many convection lines, while in a second experiment the multiple components were still present following dilution with D_2O. These results were interpreted as being consistent with the concept that the transition from a single to multiple components was a function of ionic strength rather than solvent density. The instance where the multicomponent system was maintained at μ = 1.07 may have resulted from stabilization of previously aggregated low-density lipoprotein by D_2O (Berns and Lee, 1968).

To ascertain further the characteristics of the aggregated lipoproteins, measurements of the buoyant density of the several components were undertaken. These studies were performed in Na_2EDTA containing KBr solutions of pH 4.5–5.[1] Sedimentation flotation measurements were made in solvents of different densities and the viscosity corrected, sedimentation coefficient, ηs, was plotted as a function of the solvent density, in a manner previously described (Fisher, 1970). From such plots the buoyant density of the various components may be determined as that density at which the line connecting the experimentally measured ηs values passes through zero. The plots of the data obtained from measurements of the three major components in one preparation (Figure 1A) are shown in Figure 3, and the data obtained from three separate preparations of low-density lipoprotein are tabulated in Table II.

In Figure 4 are shown the results of the measurement of the sedimentation coefficient as a function of lipoprotein concentration at a solvent density of 1.20 g/cm^3, for the three components of a single lipoprotein preparation.[1,2] The concentration dependence of s for the three components is minimal. The data from these studies are tabulated in Table II.

The influence of total low-density lipoprotein concentration upon the amounts of the several components observed was assessed using a solution of low-density lipoprotein

[1] These studies were performed before the role of pH in the transition between the single and multiple component states was appreciated, and there was undoubtedly some variation in the pH of these low-density lipoprotein solutions. In examining the data there is no evidence to suggest that the hydrodynamic properties of a given component, once it is formed, are influenced by variations of ±0.5 pH unit in the range of pH 4–5; therefore, it did not seem necessary to repeat these studies.

[2] s^0_{25}, ρ 1.20 g/cm^3 is the sedimentation coefficient at infinite dilution measured in a solvent of density 1.20 g/cm^3 at 25°. The value is corrected for the relative viscosity of the solvent (Fisher, 1970).

TABLE II: Physical Characteristics of Low-Density Lipoprotein Aggregates.

Prepn	Component	$s^0_{25^\circ}$, ρ 1.20 g/cm^3 ($\times 10^{13}$)	Hydrated Density (g/cm^3)	$\bar{V}^a$ (ml/g)	Mol Wt^b ($\times 10^{-6}$)
1	Slower	−43	1.036	0.965	2.8
	Faster	−53	1.045	0.957	4.3
2	Slower	−40	1.032	0.969	2.4
	Faster	−54	1.034	0.967	3.9
3	Slower	−42	1.030	0.971	2.6
	Faster	−54	1.042	0.960	4.2
	Fastest	−69	1.049	0.953	6.6

[a] $\bar{V}$ was determined by calculating the reciprocal of the hydrated density of each component which was evaluated from the ηs *vs.* density plots of the various lipoprotein preparations. [b] Molecular weight was calculated assuming spherical aggregates (Adams, 1966; Fisher, 1970).

at pH 5.05 with an ionic strength of 2.62 obtained by addition of KBr. The relative areas of the peaks from the slow and fast components were measured at three lipoprotein concentrations. The measurements demonstrated a relative increase in the concentration of the fast component with increasing lipoprotein concentration; however, this was a qualitative result, as the increasing relative concentration of the faster floating component could not be related to increased lipoprotein concentration in a precise manner.

Discussion

Recently, analytical ultracentrifugal analysis of serum lipoproteins is being performed increasingly with solvents of high salt density. Furthermore, it is recognized that decomposition of lipoproteins during their isolation is retarded by adding EDTA (Ray *et al.*, 1954). While many investigators routinely buffer their EDTA solutions to neutrality, this is not universally done, and the pH of a lipoprotein solution dialyzed against Na_2EDTA solutions can fall to values below pH 5.0. Under these conditions of acidic pH and high ionic strength, a monodisperse preparation of low-density lipoprotein undergoes a transition to a multicomponent state.

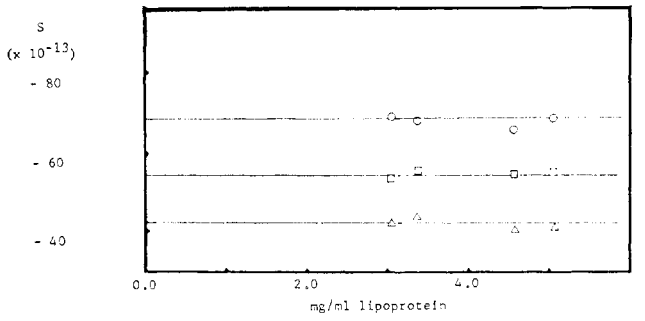

FIGURE 4: Concentration dependence of s for lipoproteins of a triple-component low-density lipoprotein aggregate (preparation 3) in KBr solution of density 1.20 g/cm^3. Symbols as in Figure 3.

The nature of these multiple low-density lipoprotein components will now be considered from the viewpoint of whether they represent the products of an aggregation phenomenon. In each of the low-density lipoprotein preparations studied a single component yielding a symmetrical schlieren peak was observed under conditions of neutral pH and either low or high ionic strength. Upon adjusting these parameters, however, multiple components appeared, and when carefully performed, the transition from single to multiple components was reversible.

The experimental finding of an increasing buoyant density with increasing particle size is consistent with a simple aggregation of identical particles associated with tighter packing of the subunits. The measured average buoyant density of the slow component (1.033 g/cm^3) agrees precisely with the buoyant density of monodisperse low-density lipoprotein isolated from the same subjects but studied under standard conditions of neutral pH and indicates that this lipoprotein has not undergone any appreciable change in its content of lipid or protein. This observation provides strong evidence against the hypothesis that under the experimental conditions low-density lipoprotein undergoes a rearrangement in composition with the formation of lipid-enriched components which are faster floating and a resulting slower floating protein-enriched species, which should, therefore, have an increased density. Actually, the measured densities of the faster floating species are greater rather than less than that of the slower floating component (Table II).

It has not proven feasible to isolate the unstable aggregates as these reassociate yielding a monodisperse product at low ionic strength; however, by adjusting the solution to a pH and ionic strength where visible precipitation occurs, the precipitate may be isolated by filtration. This objective was accomplished at pH 4.5 and ionic strength 1.26 where partial precipitation of low-density lipoprotein occurred. The precipitate was separated by passing the solution through a 0.8-μ filter (which removed 40% of the lipoprotein) and the lipoprotein in the filtrate as well as that in the original solution was analyzed for lipid content as previously described (Fisher, 1970). The analyses indicate a 76% lipid content of the lipoprotein in the original solution and a 79% content in the

filtrate, the difference being within the range of experimental error. These results demonstrate that the precipitated low-density lipoprotein was not selectively enriched in either lipid or protein, and are also consistent with the interpretation that under the conditions of these experiments low-density lipoprotein undergoes aggregation forming the larger components which have been observed.

Upon viewing Figure 1A it is evident that the several components separate from each other suggesting that they are discrete species. It thus seems reasonable to calculate approximate molecular weights for these major components, and these are recorded in Table II. The values are derived from sedimentation coefficients and buoyant density measurements of solutions containing multiple components; therefore, these measurements are subject to the errors inherent in sedimentation measurements of multicomponent solutions. Furthermore, the particles are assumed to be spheres in making these calculations, and this assumption it a reasonable approximation for unaggregated low-density lipoprotein (Oncley, 1963); in addition, the values calculated for the slower component are in approximate agreement with those reported recently for normal low-density lipoprotein with a molecular weight range of 2.3–2.5 (Adams and Schumaker, 1969). For the larger components the assumption of sphericity seems less reasonable. Alternately, if one assumes an axial ratio of 1:2 or 1:3 and utilizes the corresponding frictional ratios for ellipses derived by Perrin (Schachman, 1959a) to "correct" these estimated spherical molecular weights, the resulting values approach a molecular weight ratio of 1:2:3. It is perhaps not unreasonable to suggest that the observed components actually consist of monomers, dimers, and trimers.

As the partial specific volume of the larger components is less than for monomeric low-density lipoprotein, one would predict that there should be a demonstrable pressure dependent enhancement of aggregate formation under conditions of high gravitational force. This phenomenon has been encountered during ultracentrifugation of myosin (Josephs and Harrington, 1966). A partially aggregated preparation of low-density lipoprotein in KBr solution of density 1.20 g/cm^3, buffered to pH 5.1 was divided between two double-sectored analytical ultracentrifuge cells so that one cell contained twice the volume of solution present in the other cell. During ultracentrifugation the hydrodynamic pressure exerted on the protein in the bottom of the former cells was thus greater than in the latter cell. Figure 5 shows a photograph taken during such a centrifuge run and demonstrates that the slower floating monomeric component forms a larger peak in the partially filled cell. By contrast the cell with the larger volume of solution has a smaller mass of lipoprotein present as the slow-moving component and a proportionately greater amount of faster floating, aggregated lipoprotein of a lower partial specific volume. Measurements of the area of the major monomeric low-density lipoprotein peak revealed a decrease of approximately 25% in the concentration of this component present in the fully filled cell when compared with the half-filled cell.

The effects of pressure on associating protein systems has been ably discussed (Kegeles *et al.*, 1967; Ten Eyck and Kauzmann, 1967; Josephs and Harrington, 1967), and it is theorized that in such systems shifts in the equilibrium observed at high hydrostatic pressure result from differences

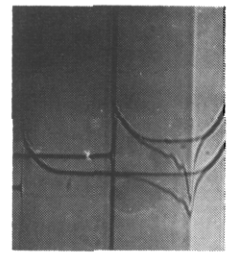

FIGURE 5: Analytical ultracentrifugation of low-density lipoprotein preparation in KBr solution of density 1.21 g/cm^3 buffered to pH 5.1. Lipoprotein concentration 12 mg/ml, centrifugation at 52,640 rpm and 25°. Photograph at 8 min after reaching speed. Standard cell (lower) contained 0.4 ml of solution and positive wedged window cell (upper) contained 0.2 ml of the same solution. Schlieren bar angle 75°.

in the partial specific volumes of the monomer and polymer so that the species with the lesser partial specific volume will be favored. For low-density lipoprotein the experimental observations are consistent with these predictions.

During analytical ultracentrifugation of lipoproteins convective disturbances are frequently observed, and the presence of such convective disturbances in the schlieren pattern is generally indicative of unstable interacting systems (Schachman, 1959b). For lipoproteins these may in part be explainable by the observed effects of pressure upon low-density lipoprotein during centrifugation.

The experimental observations thus indicate that when normal low-density lipoprotein is subjected to conditions of high ionic strength and mildly acid pH, the formation of larger, unstable, macromolecular aggregates occurs which may be observed during analytical ultracentrifugation. The observation that in the multicomponent state the relative concentration of the faster floating components increases with increasing lipoprotein concentration is consistent with this interpretation.

Though the aggregation is apparently enhanced by increasing hydrodynamic pressure, this effect is presumably of real significance only in the unstable region where the ionic strength and pH of the solution approach the transition boundary between the monodisperse and aggregated states (see Figure 2). Outside this transition zone the main determinants of aggregation clearly appear to be pH and ionic strength. These findings complement those of Oncley and coworkers (Oncley *et al.*, 1952), who showed that at a very low ionic strength, low-density lipoprotein has a minimum solubility at pH 5.4. The solubility of low-density lipoprotein is thus salt dependent with precipitation occurring at both high and low ionic strengths under acidic conditions.

In contrast to the unstable low-density lipoprotein aggregation phenomenon discussed in this report, the low-density lipoprotein fraction from certain patients with hyperlipemic diseases has been found to contain a number of discrete components which differ in their molecular weights and buoyant densities. These stable low-density lipoprotein components, which have been isolated and partially characterized, are quite different from the unstable aggregates which are the subject of the present report (Fisher, 1968, 1970). It is of interest that the isolated low-density lipoprotein components from the hyperlipemic patients will also form

unstable aggregates when subjected to low pH and high ionic strength.

Pragmatically, it is now routine practice in this laboratory to adjust all solutions used in the preparation of lipoprotein samples to pH 7.0 with 0.050 M potassium phosphate buffer containing 100 mg of EDTA/l. A concentrate of this buffer is added to all lipoprotein samples and dialysis solutions. In this manner problems of pH and ionic strength dependent aggregation have been minimized.

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