Dissociation of low density lipoprotein–antibody precipitates at alkaline pH

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ABSTRACT The effect of alkaline pH on the dissociation of immunoprecipitates of low density lipoproteins (LDL) of the S_f 0–10 class was studied by immunological and ultracentrifugal methods. The precipitates prepared at the equivalence point were dissolved and centrifuged in sodium chloride solutions of density 1.063 and pH's between 10.25 and 11.5.

Analytical centrifugation of the top fraction, which floated at density 1.063, after dialysis against 0.9% sodium chloride of pH 7.4 revealed the presence of LDL and of soluble LDL–antibody complex. The amount of soluble complex was greater for the preparations obtained at lower pH than those obtained at higher pH and was undetectable at pH 11.5. The yield of immunoglobulin from the bottom fractions was maximal when the pH of the centrifugation medium was 11.0. Below pH 11.0, the greatly reduced yield of immunoglobulin was due partly to incomplete dissociation and partly to aggregation of soluble complex, while above pH 11.0 the decreased yield was possibly due to alkaline denaturation of the globulin. The immunoglobulin separated at pH 11.0 and dialyzed to pH 7.4 was reprecipitatable by LDL, and the reactivity did not seem to be appreciably influenced by the alkaline treatment.

KEY WORDS low density lipoproteins · anti-low density lipoproteins · immunoprecipitates · Solubilization · alkaline · analytical centrifugation · immunoglobulin · soluble complex

ALTHOUGH IMMUNOCHEMICAL METHODS have been widely used in studies of human plasma lipoproteins, no attempt has yet been made to isolate the specific antibodies. Kleinschmidt and Boyer reported that egg albumin–anti-egg albumin precipitates dissolved completely at a pH of 11.0 or greater (1). Little dissociation was observed at pH 11.5, but at pH 11.7 approximately 17% was dissociated as shown by electrophoresis; dissociation was complete at pH 12.3. Although it is well known that alkaline conditions facilitate the denaturation of antibodies, these authors observed little or no change in the reactivity of immunoglobulin with its antigen after exposure of the globulin to a pH of 11.7 or lower. More recently, Edelhoch, Lippoldt, and Steiner (2,3) reported that no irreversible changes occurred in either rabbit or bovine γ-globulin below pH 11.0, as indicated by no loss in solubility, unchanged optical rotation, and unaltered fluorescence intensity of antibody-1, dimethylaminonaphthalene-5, sulfonyl chloride conjugate.

In the present study, the effect of alkaline pH on the dissociation of LDL–anti-LDL precipitates was investigated, and a method for the purification of anti-LDL immunoglobulin was established.

MATERIALS AND METHODS

Human plasma LDL of the S_f 0–10 class was isolated from pooled plasma in the presence of 0.05% EDTA according to the method of Gillies, Lindgren, and Cason (4) and Gofman et al. (5) as previously described (6). A typical LDL preparation used in this study contained 80.6% lipid and 19.4% protein on a dry weight basis as determined by gravimetric methods (7).

Antiserum against LDL was prepared essentially according to the intraveneous injection method of Briner, Riddle, and Cornwell (8). Prior to injections, the LDL was dialyzed against 0.9% sodium chloride solution (the pH of which had been adjusted to 7.4 by the addition of dilute sodium hydroxide) for 24 hr, with four changes of the external solution to remove the EDTA. 12 rabbits received injections of LDL containing 0.5 mg of protein on 3 alternate days; then the amount of protein injected was increased to 0.7 mg for three more injections. 1 wk after the last injection, the rabbits were bled.

Abbreviation: LDL, low density lipoprotein.

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Double diffusion in agar was carried out by the method of Ouchterlony (9) in 1% Difco Special Agar (Noble) purified by the method of Grabar (10). The rabbit serum produced a precipitation line with human LDL, but not with human serum albumin, γ-globulin, or high density lipoproteins.

The antibody precipitates of LDL were prepared at the equivalence point at pH 7.4 in the following manner. A solution, containing 3.94 mg of protein, of LDL in 0.9% sodium chloride of pH 7.4 was added to 5 ml of antiserum in 12-ml graduated centrifuge tubes. Nitrogen gas was passed into the tubes, and the tubes were sealed with rubber stoppers. The solutions were then mixed, left at room temperature for 1 hr, and held at 4°C for 24 hr. The resultant precipitates were washed with 4 ml of 0.9% sodium chloride of pH 7.4 for the first and second washings, and 6 ml for the third.

To study the effect of alkaline pH on the solubilization of immunoprecipitates of LDL, we dispersed the precipitates prepared as described in 3 ml of 0.9% sodium chloride of pH 7.0, and diluted 0.2 ml of the suspension to 1.0 ml with 0.9% sodium chloride containing appropriate buffers; the concentration of buffers was 0.02 M. The mixtures were left for 10 min at room temperature under nitrogen, then centrifuged; the protein content in the supernatant fraction was determined by the method of Lowry, Rosebrough, Farr, and Randall (11).

For the study of the dissociation of insoluble LDL–antibody complexes at alkaline pH, the precipitates prepared as above were suspended in 2.3 ml of 0.9% sodium chloride, and 0.216 g of sodium chloride was added. The mixtures were then brought to the various desired pH's with 0.1 N sodium hydroxide and to a volume of 2.5 ml with 0.9% sodium chloride of corresponding pH's. The density of the medium so obtained was 1.063. Final volumes of 3 ml were reached by the addition of sodium chloride solutions of density 1.063 and pH's corresponding to each medium. The solutions were subjected to centrifugation for 20 hr at 108,900 *g* in Spinco cellulose tubes 303369. A total of approximately 0.8 ml of the yellowish top fraction, 1.7 ml of the transparent middle fraction, and 0.5 ml of the bottom fraction were collected from the tubes and dialyzed against 0.9% sodium chloride of pH 7.4 at 4°C for 24 hr with three changes of the external solution; the total volume of the external solution used was 2400 ml. After dialysis, we confirmed that the pH of the solution in the dialysis bag was 7.4. The volumes of the dialyzed top, middle, and bottom fractions were increased to 2, 2, and 1 ml, respectively, with the 0.9% sodium chloride. All the salt solutions used contained 0.01% EDTA.

Ultracentrifugal analyses of the top, middle, and bottom fractions were carried out at 20°C in 0.9% sodium chloride of pH 7.4 in a Spinco model E ultracentrifuge at 52,640 rpm with an acceleration time of 5 min 20 sec. The protein content of these fractions was determined by the method of Lowry et al. (11), with a crystalline bovine plasma albumin as standard.

RESULTS

The solubility of LDL immunoprecipitates increased appreciably when the pH of the suspension media (0.9% sodium chloride–0.02 M buffer) was increased above 9.5 (Fig. 1), and was complete at pH 10.5. When the pH was raised by the addition of dilute sodium hydroxide, the solubilization curve was essentially similar to that obtained by the use of buffers.

The effect of alkaline conditions on the immunoprecipitates was investigated as follows. The top, middle, and bottom fractions obtained by preparative centrifugation of the solubilized LDL–anti-LDL precipitates at pH 11.0 in a sodium chloride medium of density 1.063 were subsequently dialyzed in 0.9% sodium chloride of pH 7.4. Ultracentrifugal analysis of the dialyzed top fraction showed a major peak with an apparent sedimentation coefficient of 6.0 and a minor peak with an apparent sedimentation coefficient of 10.3 (Fig. 2, picture 3). The sedimentation coefficient of the main peak closely corresponded to the value 5.9 obtained for untreated LDL. Double diffusion in agar of the top fraction showed a precipitation line with the antiserum, but no reaction with the antigen, indicating that this fraction consisted essentially of LDL. Neither antibody nor antigen was detected in appreciable amounts in the middle fraction. The bottom fraction, which showed a single peak with an apparent sedimentation coefficient of 6.5 by ultracentrifugal analysis, gave a precipitation line with the antigen, but not with the antiserum. The substance in the bottom fraction, therefore, appears to be antibody. We found that the LDL and the antibody isolated after centrifugation and subsequent dialysis contained 44.4 and 43.7%, respectively, of the total protein originally present in the immunoprecipitates (Table 1). During centrifugation, a small pellet was formed at the bottom of the centrifuge tube and was found to contain 1.6% of the total protein. In addition, 6.1% of the total protein was recovered from the precipitate formed during dialysis of the bottom fraction.

When the pH of the medium used for solubilization of the immunoprecipitates was varied from 10.25 to 11.5, the apparent sedimentation coefficient of the bottom (antibody) fraction did not change, but the amount of antibody recovered from the bottom fraction was greatly influenced by the pH value of the medium, the maximum recovery being obtained at pH 11.0 (Table 2). We found that the lower yield of antibody at pH 10.25 and 10.5 was not due to the presence of increased amounts of anti-

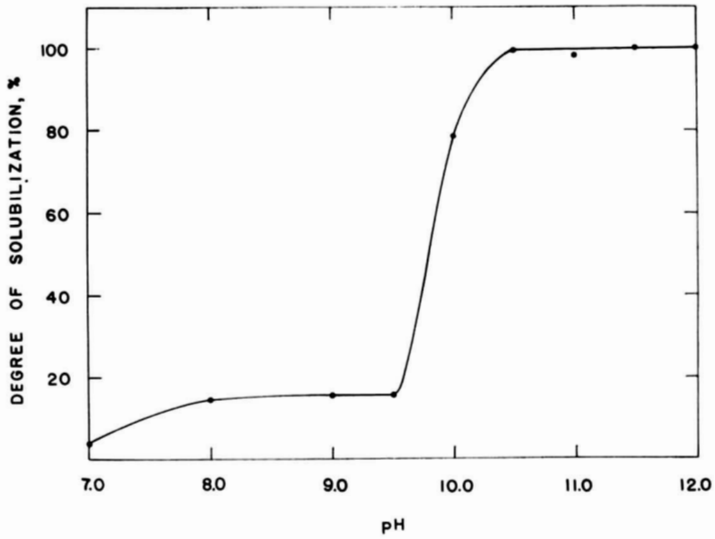

FIG. 1. Solubilization of immunoprecipitate of LDL at alkaline pH. The precipitates were suspended in 0.9% sodium chloride containing 0.02 M buffers, and the degree of solubilization was determined as described in Materials and Methods. The buffer solutions used were potassium dihydrogen phosphate–dipotassium hydrogen phosphate for pH 7.0, tris (hydroxymethyl) aminomethane–hydrochloric acid for pH 8.0 and 9.0, sodium bicarbonate–sodium carbonate for pH 9.5 and 10.0, and dipotassium hydrogen phosphate–sodium hydroxide for the pH's ranging from 10.5 to 11.5.

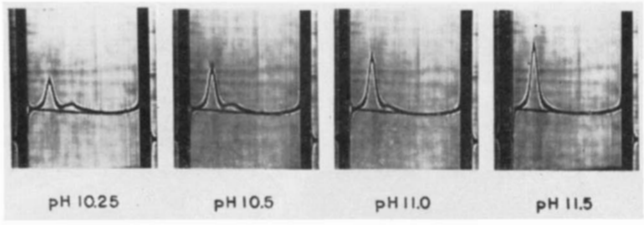

FIG. 2. Sedimentation patterns of top fractions isolated by centrifugation at d = 1.063 of LDL immunoprecipitates solubilized at various pH's, followed by dialysis against 0.9% sodium chloride solution of pH 7.4. Pictures were taken at a bar angle of 55°, 40 min after a speed of 52,640 rpm had been obtained (acceleration time, 5 min 20 sec).

body in the middle fraction. Although the recovery of protein from the top fraction was influenced by the pH of the medium in a manner similar to that observed for the bottom fraction, the extent of decrease in the recovery at lower pH values was considerably less pronounced for the top fraction.

Analytical centrifugation of the top fractions, obtained at different pH values and dialyzed against 0.9% sodium chloride solution of pH 7.4, revealed that the area of the slow-moving LDL peak progressively increased as the pH increased from 10.25 to 11.5, while the area of the fast-moving peak decreased with increasing pH (Fig. 2 and Table 2); the latter peak, which had an apparent sedimentation coefficient between 12.1 and 10.3, disappeared completely at pH 11.5. Since the material responsible for this fast-moving peak floated at a medium density of 1.063 and possessed an apparent sedimentation coefficient much larger than those of either antigen or antibody, it appears to be a soluble complex in the "antigen excess" region. The apparent sedimentation coefficients of the slow-moving components in the top fractions obtained at pH values 10.25 to 11.5 changed from 6.2 to 5.8. Although we suspected that this difference was due to the effect of alkaline treatment on LDL, it appears not to be so. When LDL alone was treated at alkaline pH's ranging from 10.25 to 11.5 in the same manner as for immunoprecipitates of LDL, the apparent sedimentation coefficient remained the same as that obtained for untreated LDL. The increase in the sedimentation coefficient of the slow-moving LDL in the top fractions ob-

TABLE 1 PROTEIN RECOVERY IN VARIOUS FRACTIONS OBTAINED BY CENTRIFUGATION OF LDL IMMUNOPRECIPITATE DISSOLVED AT pH 11.0*

Fraction	Protein Recovery	
	mg	%
Whole	7.66	100.0
Top	3.40	44.4
Middle	0.05	0.7
Bottom	3.35	43.7
Pellet	0.12	1.6
Precipitate	0.47	6.1
Total recovery from fractions	7.39	96.5

* The immunoprecipitate prepared by the addition of LDL containing 3.94 mg of protein to 5 ml of antiserum was solubilized and centrifuged in sodium chloride solution of density 1.063, pH 11.0. The top, middle, and bottom fractions were dialyzed against 0.9% sodium chloride of pH 7.4. "Whole fraction" refers to the immunoprecipitate dissolved at pH 11.0; "pellet" to the material aggregated at the bottom of the tube after centrifugation; and "precipitate" to the material precipitated during dialysis of the bottom fraction.

TABLE 2 EFFECT OF pH ON DEGREE OF DISSOCIATION OF LDL IMMUNOPRECIPITATE*

pH	Protein Recovery		Observed Sedimentation Rate			Relative Area†	
			Top Fraction			Top Fraction	
	Top Fraction	Bottom Fraction	Slow-Moving Peak	Fast-Moving Peak	Bottom Fraction	Slow-Moving Peak	Fast-Moving Peak
	mg						
10.25	2.97	0.37	6.2	12.1	6.7	48.2	28.3
10.5	3.06	1.92	6.1	11.5	6.5	61.2	21.6
11.0	3.25	3.55	6.0	10.3	6.5	83.2	11.6
11.5	3.32	2.93	5.8	—	6.5	100.0	0

* The procedure for obtaining the top and bottom fractions from immunoprecipitates solubilized at various pH's is described in Table 1.
† Relative area of slow- and fast-moving peaks, measured with a planimeter on tracings of 10 X (linear) enlargements of the photographic plates, are given to indicate the approximate proportions of LDL and soluble complex, respectively, in the top fractions obtained at various pH's. The peak area of the slow-moving material (LDL) in the top fraction obtained at pH 11.5 was taken as 100.

tained by the treatment of immunoprecipitates at lower alkaline pH's might have been caused mainly by a combination of two factors. The Johnston-Ogston effect, which is known to exist in two-component systems, might have slightly increased the sedimentation value, with an increase in the concentration of fast-moving soluble complex. Secondly, the decrease in the concentration of LDL, which is evident from a comparison of the relative areas of the slow-moving peak (Table 2), might also be a factor in increasing the sedimentation coefficient.

DISCUSSION

The present study reveals that the recovery of immunoglobulin from immunoprecipitates of LDL, after alkaline treatment and subsequent centrifugation in sodium chloride media of density 1.063, is greatly influenced by the pH of the medium. The highest yield of immunoglobulin was obtained at pH 11.0; however, a small amount of soluble complex present in the lipoprotein (top) fraction indicated incomplete dissociation of the precipitate. Although the precipitate dissociated completely at pH 11.5, the lower yield of soluble immunoglobulin indicated some aggregation of immunoglobulin at this pH.

The yields of immunoglobulin separated at pH 10.25 and 10.5 were approximately 10 and 50%, respectively, of that obtained at pH 11.0. The extraordinarily low yield of antibody (bottom fraction, Table 2) at these lower pH's was apparently caused by the incomplete dissociation of the immunoprecipitate, as indicated by the presence of greater proportions of soluble complex (fast-moving peak) in the lipoprotein fraction. We also noted that after preparative centrifugation of lipoprotein–antibody preparations in sodium chloride medium of pH 10.25, density 1.063, approximately 43% of the total protein originally present in the immunoprecipitate was recovered from the pellets formed at the bottom of the centrifuge tubes because of incomplete solubilization of the precipitate and aggregation of the soluble complex.

Although the exact nature of association between antigen and antibody is not known, we suspected that alkaline conditions may facilitate the conversion of an immunoprecipitate to the soluble complex, which can further be dissociated mainly into antigen and antibody upon centrifugation at solvent density 1.063. Concerning the state of immunoprecipitates prepared at the equivalence point and solubilized in 0.9% sodium chloride media of pH 11.0 and 11.5, we have obtained results that were dependent on the antiserum used. In about half the cases, ultracentrifugal analysis of the solubilized precipitates showed heterogeneous sedimentation boundaries indicative of the presence of a series of large soluble complexes varying greatly in molecular weight; but in the rest of the cases these soluble complexes were absent and analysis showed the composite patterns of the lipoprotein and antibody fractions separated by the subsequent centrifugation. The ultracentrifugal patterns of the isolated lipoprotein and antibody fractions were identical in all cases, however, which indicates that the soluble complex can easily be dissociated by centrifugation at a density (1.063) at which free antigen floats and antibody sediments. It is interesting to note that the immunoprecipitate of egg albumin, dissolved at pH 11.5, existed almost entirely as soluble complex, while egg albumin and immunoglobulin when mixed at the same pH remained dissociated as separate components (1). The components of soluble complexes at alkaline pH appear to be weakly and delicately associated. Minor differences in the binding property or surface configuration of antibody may greatly influence the degree of interaction at alkaline pH, but may not influence the amount of immunoprecipitates formed at neutral pH.

We observed that the immunoglobulin separated at pH 11.0 and dialyzed at pH 7.4 can be reprecipitated by the addition of LDL: 3.72 mg of the immunoglobulin and LDL containing 3.94 mg of protein produced 6.78 mg of immunoprecipitate, the value being comparable to the amount of protein recovered from the precipitate

formed by the addition of the same amount of LDL to antiserum (Table 1).

Kleinschmidt and Boyer observed (1) that the exposure of immunoglobulin to pH 11.2 and 11.7 for a period of 24 hr caused very little change in the reactivity of the globulin. The lower yield of immunoglobulin obtained at pH 11.5 in the present study was in agreement with the result of Edelhoch et al. (2), whose experiment indicated that a critical extent of ionization seems to occur above pH 11.0.

Immunochemical properties of plasma lipoproteins have previously been investigated by immunodiffusion and immunoelectrophoresis with the use of unfractionated antisera. To obtain more information about surface configuration and antigenicity of plasma lipoproteins, we are currently studying the interaction of purified immunoglobulins with lipoproteins having structures enzymatically or otherwise modified.

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