IMMUNOLOGIC STUDIES OF HUMAN HIGH DENSITY LIPOPROTEINS*

By LOUIS DELALLA, LAWRENCE LEVINE, Sc.D., AND RAY K. BROWN, M.D.

(From the Division of Laboratories and Research, New York State Department of Health, Albany)

(Received for publication, March 27, 1957)

The possible role of lipoproteins in the pathogenesis of atherosclerosis has prompted the present investigation of their immunologic assay. The various flotation classes of low density lipoprotein are antigenically related and the cross-reactivity of different classes prohibits quantitative determination by serologic technics in whole serum (1). High density lipoproteins possess distinct serologic specificity and differ from low density lipoproteins in lipide and amino acid composition as well as N terminal amino acid residues (2, 2*a*, 3). During investigation of the applicability of immunologic assay for high density lipoproteins, it was found that these lipoproteins were altered serologically during purification or even on standing. The present immunologic study describes the changes that occur in high density lipoproteins during preparation and aging and the limitations inherent in their assay.

Materials and Methods

Preparation of Lipoproteins.—The high density lipoproteins were prepared from outdated ACD[1] plasma by ethanol fractionation (3), by ultracentrifugation, or a combination of the two methods. The antigen used in the production of antiserum 794 was prepared by ethanol fractionation. It was purified further by ultracentrifuging at solvent density 1.21 (1.63 M NaCl plus 1.78 M KBr in 1 liter of solution), removing the top portion with a tube slicing device, bringing its density to 1.10 with cold water, recentrifuging, and discarding the floating material. The remaining solution was adjusted to density 1.14 and the portion immobile in the ultracentrifuge was used. Several other antigens were prepared directly from freshly drawn blood by ultracentrifuging the serum at 1.24 density (prepared by adding an appropriate amount of a solution which was 2.97 M KBr and 2.62 M NaCl) for 48 hours, slicing off the material in the top of the tube, bringing its density to 1.063 with cold water, centrifuging for 24 hours, slicing off and discarding the top, again adjusting to density 1.24, and ultracentrifuging for an additional 48 hours. The top was sliced and that material used.

Production of Antisera.—Antisera were produced in rabbits by the intravenous injection of alum-precipitated lipoproteins. Sixteen injections, one every other day, were given. The doses were increased gradually from 0.5 to 7.5 mg. of protein. The rabbits were bled 6 days

* This work was supported in part by a grant from the Life Insurance Medical Research Fund.

[1] National Institutes of Health Formula B: 1.32 gm. sodium citrate, 0.44 gm. citric acid, and 1.47 gm. dextrose in 100 ml. of solution; 25 ml. of solution added to 500 ml. of blood.

after the last injection. The sera were cleared by centrifugation and complement (C′) was inactivated by heating for 30 minutes at 56°C.

Immunologic Methods.—Quantitative precipitin experiments were performed as described by Heidelberger and Kendall (4). The reaction mixtures were set up at room temperature and incubated at 0° to 2°C. for 2 or 3 days. The precipitates were collected in the cold, washed twice with ice-cold saline, and analyzed for nitrogen by the micro-Kjeldahl method.

Quantitative measurements of C′-fixation were made according to Mayer, Osler, Bier, and Heidelberger (5) except for a minor modification in the assay of hemolytic activity (6). Fixation was allowed to proceed for from 20 to 22 hours at 0° to 2°C. An aliquot of each mixture of C′ with antigen and antibody, as well as controls, C′ with antigen, C′ with antibody, and C′ alone, was titrated for residual hemolytic activity by the spectrophotometric method outlined by Mayer *et al.* (7). The number of $C'H_{50}$ fixed was calculated by subtracting the amount of residual hemolytic activity in the tube with antigen and antibody from the average of the values obtained in the antigen, antibody, and buffer controls. In all cases in which C′ fixation was used, a range of antigen concentrations sufficient to give a whole curve was employed.

Agar diffusion analyses were performed by the methods of Ouchterlony (8) and Oudin (9). Diffusion constants were obtained by the procedure of Becker and coworkers (10) with 13 μg. of antibody N per ml. in the solid phase. The reaction mixtures were incubated at 30° ± 0.005°C. in a water bath equipped with a Zilko thermoregulator. The migration of the bands was read to 0.01 cm. with a graduated travelling telescope.

Experimental Procedures and Results

Estimation of antigens by immunologic technics is dependent upon rigorous demonstration of immunochemical homogeneity of the system, identification of the antibody in the antigen-antibody reaction, and serologic similarity of the antigen in its purified state with the antigen in its native state.

The injection of eight rabbits with high density lipoproteins, a different preparation for each pair, produced precipitating antibody in seven. Immunochemical homogeneity was tested by the Ouchterlony and Oudin procedures (8, 9). Three to six bands developed when fresh human serum diffused into six of the antisera. Rabbit antiserum 794[2] gave only one band by single and double diffusion in agar and a single peak in C′-fixation experiments with whole serum as antigen. It did not react with crystalline human-serum albumin, gamma globulin, or crystalline heat-labile glycoprotein (11). The quantitative precipitin experiment described in Table I shows supernatant fluids consistent with immunologic purity. Antiserum 794 was used throughout the study.

In order to identify the antibody, the band formed during double diffusion on an agar plate was treated with Sudan black B. A positive reaction was observed but the stain intensity was less than that observed on bands formed between low density lipoproteins and their antisera (1). Identification of the antibody was demonstrated in another manner.

The immune precipitate formed in the region of antibody excess was analyzed for lipoprotein cholesterol. Thirty-seven μg. N of high density lipoprotein, ultracentrifugally prepared

[2] The lipoprotein preparation used for immunization was expended for chemical and immunologic studies and not analyzed for content of albumin, gamma globulin, or heat-labile glycoprotein.

and containing 113 μg. of cholesterol, was added to 5.0 ml. of antiserum at room temperature and incubated at 0° to 2°C. for 3 days. The precipitate was centrifuged in the cold, washed twice with ice-cold saline, and analyzed for cholesterol by the method of Abell, Levy, Brodie, and Kendall (12). The immune precipitate contained 73 per cent of the added cholesterol.

Other workers have separated this fraction into two fractions of cholesterol/N ratios of 1.5 and 2.3. If the immunologic non-reactive cholesterol is assumed to

TABLE I

Antibody N Precipitated from Antiserum 794 by Varying Quantities of Homologous Antigen

Lipoprotein N added	Total N precipitated* from 1.0 ml. serum	Antibody N precipitated from 1.0 ml. serum	Ratio antibody N to lipoprotein N in precipitate	Tests on supernatant fluids
mg.	*mg.*	*mg. (average)*		
0.002*	0.013‡	0.012*	6.0	Excess antibody
	0.016			
0.004*	0.024‡	0.024*	6.0	" "
	0.032			
0.008	0.059	0.053	6.6	" "
	0.063			
0.016	0.106	0.090	5.6	" "
	0.107			
0.024	0.133	0.108	4.5	No lipoprotein, no antibody
	0.131			
0.032	0.141	0.108	3.4	" " " "
	0.138			
0.048	0.139	0.090	1.9	Excess lipoprotein
	0.138			

* Values corrected by subtraction of serum blank (0.010 mg. N).

‡ These values are corrected to 1.0 ml. of antiserum. The experimental reaction mixtures contained 2.0 ml. antiserum.

arise from dissociation of high density lipoprotein, the ratio in this preparation would then fall within these limits.

Type-III pneumococcus and its homologous rabbit antiserum served to control the presence of cholesterol in precipitating rabbit antibody and the non-specific absorption or occlusion of high density lipoprotein in the immune precipitate. Less than 2 per cent of the added lipoprotein cholesterol was recovered in this precipitate. The limited amount of antiserum available prevented study of phospholipide in the specific precipitate.

Lack of similarity between purified high density lipoprotein and high density lipoprotein in freshly drawn serum was observed by three different technics.

(1) Threefold dilutions of these antigens, purified high density lipoproteins, and freshly drawn serum were allowed to diffuse into an agar mixture containing 13 μg. of antibody nitrogen per ml. The diffusion constants ($D_{20,\ H_2O}$) obtained were 1.64×10^{-7} cm.²/sec. and $3.25 \times$

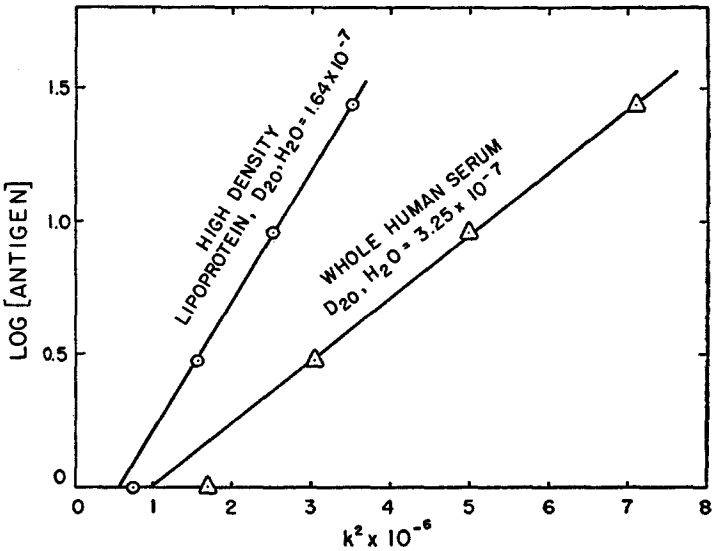

FIG. 1. Diffusion constant determination. Threefold serial dilutions of fresh human serum and purified lipoprotein in the liquid phase and 13.0 μg. of antibody N per ml. in the solid phase.

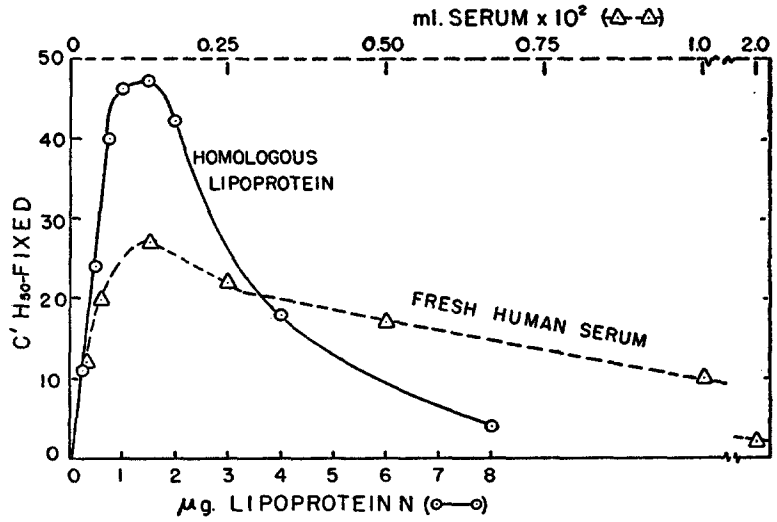

FIG. 2. Fixation of C′ by varying quantities of fresh human serum and purified high density lipoprotein with 2.2 μg. of anti-high density lipoprotein N at 0–2°C. for 20 to 22 hours.

10^{-7} cm.²/sec. respectively (Fig. 1). The different diffusion constants suggested a chemical or physical change in the antigen. (2) The two antigens and the antibody were allowed to diffuse into each other on an agar plate. A reaction of partial identity was observed. (3) Varying quantities of two antigens were reacted with 2.2 μg. antibody N in the presence of 100 $C'H_{50}$.

The data in Fig. 2 show that 48 $C'H_{50}$ were fixed at peak with purified lipoprotein, the homologous antigen, whereas 28 $C'H_{50}$ were fixed at peak with the serum.

Thus, during the course of purification, serologic specificity of the antigen had changed.

The purification procedures were studied to find which step caused this lipoprotein alteration. At various stages in the purification aliquots were analyzed by C′ fixation. Ultracentrifugation overnight, dialysis, and high salt concen-

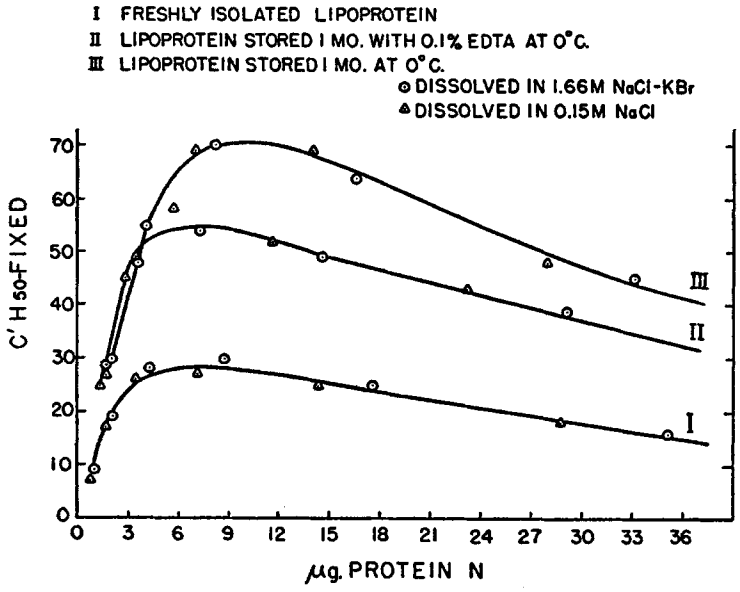

FIG. 3. Effect of aging on reactivity of isolated lipoprotein. Fixation of C′ by varying quantities of lipoprotein with 2.2 μg. of anti-high density lipoprotein N. Curve I, freshly isolated lipoprotein; Curve II, lipoprotein stored 1 month with 0.1 per cent EDTA at 0°C.; Curve III, lipoprotein stored 1 month at 0°C. ⊙ Lipoprotein dissolved in 1.66 M NaCl-KB_r. △ Lipoprotein dissolved in 0.15 M NaCl.

tration did not alter antigenic reactivity. These negative findings led to the study of the effect of aging on the immunologic reactivity of high density lipoproteins. It was possible to prepare a purified high density lipoprotein that fixed no more C′ than fresh serum, if prepared and analyzed promptly. Fig. 3 indicates that the serologic activity of a freshly prepared lipoprotein increased after 1 month at 0°C. This increase in reactivity occurred whether the freshly prepared lipoprotein was stored in concentrated or isotonic salt solution. When the lipoprotein was stored in isotonic saline containing 0.1 per cent disodium ethylenediaminetetraacetate (EDTA) at 0°C. for 1 month (13), the change in reactivity was less. The effect of aging on the reactivity of fresh serum was

also studied. Serum was stored aseptically at 0°C. and C′ fixation curves were obtained with aliquots from the freshly drawn serum, serum 1 month old, and serum 2 months old; peak fixation was 25, 36, and 46 $C'H_{50}$ respectively.

The serologically reactive portion of the lipoprotein was investigated with the thought that this might suggest the nature of the change on aging.

The lipoprotein was extracted with boiling alcohol-ether (3:1), a procedure that quantitatively removes the lipides (14). The extract was evaporated to dryness under nitrogen. Some of the protein moiety was denatured. This insoluble protein was removed by centrifugation and the remaining protein solution was used as the test antigen. Suspensions of lipide

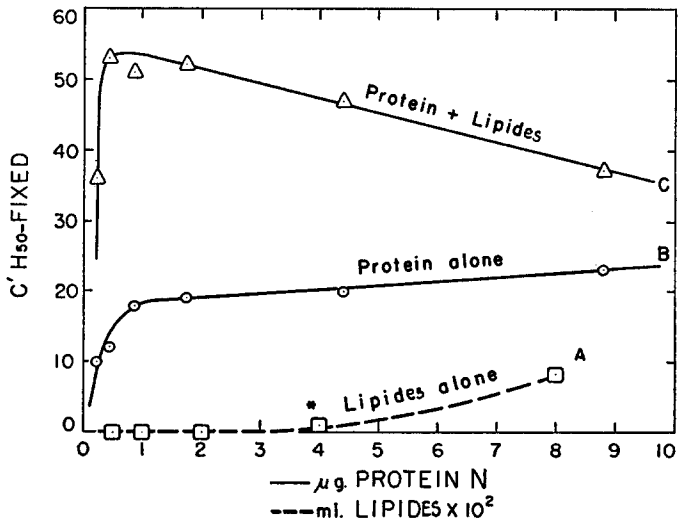

FIG. 4. Fixation of C′ by varying quantities of lipide and protein moieties from high density lipoprotein and protein to which 0.04 ml. of lipides were admixed with 2.2 μg. of anti-high density lipoprotein N.

in veronal buffer were made. They were reactive only when used in high concentration (curve *A*) while the protein exhibited activity over a wide range of concentrations (curve *B*) (Fig. 4). When a 25-fold dilution of lipide, a quantity unreactive alone, was used with increments of protein, the C′ fixed was markedly increased (curve *C*). These lipides extracted from lipoproteins did not enhance C′ fixation when added to the human serum albumin and gamma globulin–immune systems. When lipide suspension, lipide-free protein, and lipoprotein were tested against the antiserum by double diffusion, a line of identity was observed between the protein and lipoprotein. The lipide exhibited no precipitate band.

To determine whether the lipide effect was specific for lipides from high density lipoproteins, egg lecithin, and cholesterol, equivalent in amount to those in the extracted lipides, were added to the protein fraction. The data in Fig. 5 show that an increase in C′ fixation was observed.

Egg lecithin plus protein gave a variable increase in C′ fixation; cholesterol alone did not enhance reactivity. Triton WR-1339 in amounts up to 2.5 per cent was mixed with the protein

to determine whether further dispersion would affect the amount of C′ fixed. Although it is difficult to judge the extent to which the dispersion was affected, no change in C′ fixation occurred.

Whether the change during aging of lipoprotein was in the lipide or protein portion of the molecule was next investigated.

Lipides extracted from fresh and aged high density lipoproteins were mixed with protein from aged lipoproteins. In C′ fixation experiments, fresh lipides plus aged protein fixed 48 $C'H_{50}$ at peak; the aged lipides added to aged protein fixed 50 $C'H_{50}$. In similar experiments, fresh and old lipides were added to protein from fresh lipoproteins. Thirty-two $C'H_{50}$ were fixed at peak by the mixture of fresh lipides and fresh protein; 52 $C'H_{50}$ were fixed by aged

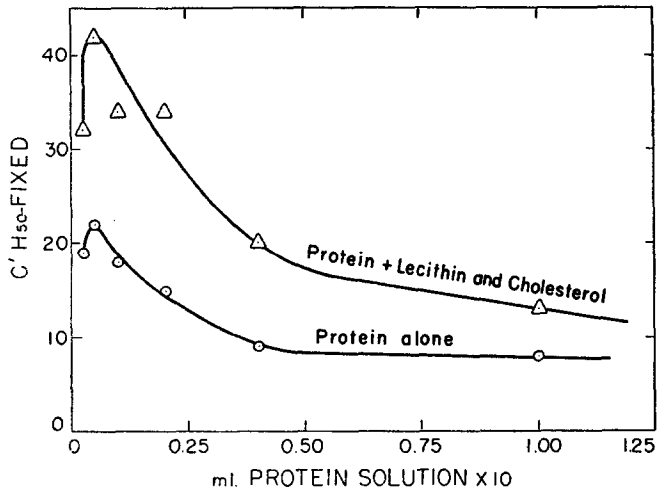

FIG. 5. Fixation of C′ by increments of the protein portion of high density lipoprotein with 2.2 μg. of antibody nitrogen. Each reaction mixture contained 50 μg. of egg lecithin and 25 μg. of cholesterol.

lipides mixed with fresh protein. Protein from aged lipoprotein fixed twice as much C′ at peak as did protein from freshly prepared lipoprotein.

The immunologic reactivity of both the protein and lipide portions of the lipoprotein appears to be altered by aging.

Since the protein portion of the high density lipoprotein reacted with this antibody, it was possible to determine whether any of the lipide-free protein existed in the serum.

A fresh serum, adjusted to density 1.24, was fractionated in an ultracentrifuge at 92,660 times gravity for 48 hours, and then the upper ⅙ was removed. Material in the lower portion of the tube was ultracentrifuged an additional 48 hours and then assayed by C′ fixation. The data (Fig. 6) show that the fixation of C′ by this fraction was decreased to the amount obtained with the protein fraction of the lipoprotein. Addition of lipide, itself inert, derived from the lipoprotein, enhanced activity.

If the protein portion of high density lipoprotein exists normally in serum, the bulk of this material should sediment from fresh serum at density 1.24. If the top fraction of the ultracentrifuged materials—the lipoprotein—is again ultracentrifuged at 1.24 density, the solution in the lower portion of the tube should contain little reactive material. As judged by C′ fixation, an equal amount of lipide poor protein was obtained from the bottom of the ultracentrifuged serum and the ultracentrifuged lipoprotein. Thus, it appears that the material sedimenting at density 1.24, which reacts with antiserum to high density lipoprotein, was derived during ultracentrifugation rather than occurring naturally. It was not possible to float any appreciable amount of reacting

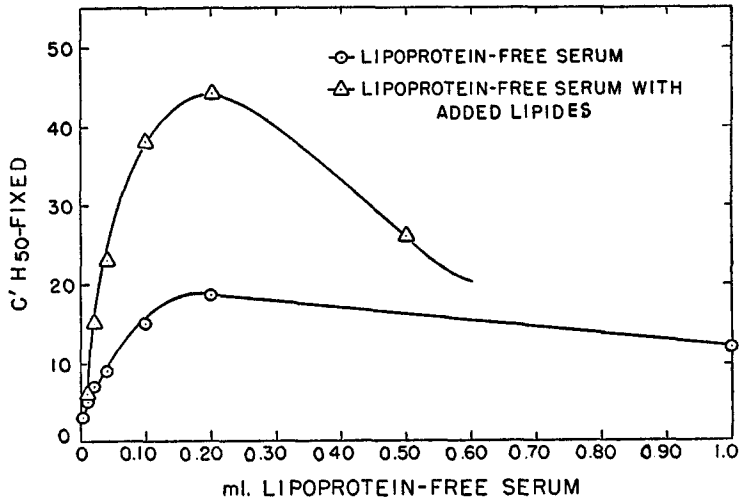

FIG. 6. Fixation of C′ by varying quantities of lipoprotein-free serum and lipoprotein-free serum plus lipides with 2.2 μg. of anti-high density lipoprotein N.

material from the lipoprotein-free serum by adding lipides and again ultracentrifuging.

It was previously shown that the lipoproteins of fraction IV+V (Cohn) from the serum of a patient with biliary cirrhosis[3] do not react with antibody to low density lipoproteins although almost all of the lipoprotein in the fraction has a density of less than 1.063 (15). Lipoproteins floated at density 1.063 from this serum react with antiserum 794 and fix 24 $C'H_{50}$ at maximum.

To determine whether the high phospholipide content of these lipoproteins caused this effect, lipides were extracted from these lipoproteins with Bloor's reagent and the protein part was mixed with lipides from normal lipoprotein. This mixture fixed 28 $C'H_{50}$. Lipides from the patient with biliary cirrhosis, added to protein from normal high density lipoprotein, fixed 38 $C'H_{50}$.

[3] We are indebted to Miss Ella M. Russ, Department of Medicine, New York Hospital, New York, for these fractions.

It is not possible to say from the data that all the lipoprotein in this preparation reacted with the antiserum to high density lipoprotein since the lipoprotein was not homogeneous—a difficulty also encountered with the low density lipoproteins. The losses of protein reactivity during extraction of lipide may be unequal and invalidate calculation of specific activity.

DISCUSSION

The antiserum used in this study was prepared against an aged lipoprotein in which alteration presumably had already occurred. Thus, fresh lipoproteins or any lipoprotein not having undergone such alteration would be expected to be less reactive with this antiserum. A manifestation of aging, then, would be an increased serologic reactivity approaching that of the homologous antigen. This is evident in Figs. 2 and 3 and in the increased reactivity of whole serum on standing.

The different diffusion constants obtained by immunodiffusion with fresh human serum and aged lipoprotein as diffusing antigens, 3.25×10^{-7} and 1.64×10^{-7} cm.^{2}/sec. respectively, suggest that the change in complement-fixation value was associated with aggregation or shape change of the lipoprotein.[4] Thus, the lipoprotein may have formed new determinant groups even before immunization. During aging, the same alteration may form these same determinant groups and increase reactivity to the antibody. This explanation, however, cannot be considered unique. Further clarification of the nature of the lipoproteins may provide alternative explanations.

The question of whether lipides are antigenic or direct some specificity to a molecule containing carbohydrate and/or protein is one that has long been debated (16). This study provides no clear answer. The increased serologic reactivity upon the addition of cholesterol and lecithin is usually explained by greater dispersion of the antigen. In this system, dispersion of the lipide-free protein to a state of aggregation similar to that in the homologous antigen might occur. Greater dispersion would probably lead to decreased serologic reactivity. It is possible, also, that the protein moiety is altered or denatured to a much greater extent than the lipoprotein used for immunization. This greater alteration may have obscured determinant groups that could be unmasked by dispersion with the lipides. The data in Fig. 5 show that egg lecithin and cholesterol increased the activity of delipidated protein. This would favor the general thesis that the state of molecular aggregation does indeed affect the serologic behavior.

While the observed data can be explained by a change in aggregation of the lipoprotein, the possibility of some antigenic specificity residing on the lipides must be considered. When fresh and old lipides were added to fresh protein, 32

[4] Similar serologic alterations in low density lipoproteins have been found during storage (17). These observations should be considered in the interpretation of immunologic experiments involving long periods of observation (18, 19).

and 52 $C'H_{50}$, respectively, were fixed at peak, suggesting a change in the lipide itself. Thus, it is possible that lipides react with the antibody but cannot form a lattice and fix C′. The admixed lipide and protein can form the lattice which fixes more C′ than does the protein alone. Egg lecithin might possess immunologic specificity similar to the lipides of lipoproteins since it enhanced the C′-fixing activity of the protein moiety (Fig. 5). It is pertinent to note that high concentrations of lipoprotein lipide do exhibit some ability to fix C′ (Fig. 4).

The effect of EDTA on the alteration of lipoprotein serologic reactivity with aging (Fig. 3) suggests that methods may yet be found to prevent this alteration. If this could be done, antiserum to native lipoprotein might be produced. However, the antigen must be pure enough to produce an antiserum in which the contaminating immune systems are known and are susceptible to absorption. We were not so fortunate as Aladjem *et al.* (19) in preparing rabbit anti–low density lipoprotein which is pure without absorption. As shown in Fig. 6, lipoprotein-free serum, used successfully to absorb antiserum to low density lipoproteins, would fail to preserve titer to high density lipoproteins since it contains reactive materials that are probably lipide-poor, high density lipoproteins. Exhaustive absorption of six of the antisera that were immunologically heterogeneous with lipoprotein-free serum resulted in loss of antibody to high density lipoproteins and to contaminants.

SUMMARY

High density serum lipoprotein underwent serologic and physicochemical alterations on aging during storage at 0°C. for 1 month, as judged by decrease of diffusion coefficient and increase of C′ fixation. Ultracentrifugation, dialysis, and high concentrations of sodium chloride did not cause these changes. A protein sedimenting at density 1.24 in the ultracentrifuge reacted with antiserum to high density lipoprotein. Probably it was the protein portion of α lipoprotein dissociated from the lipide during ultracentrifugation.

Although the antiserum to high density lipoprotein did not react with low density lipoprotein prepared from normal serum, it reacted with similarly prepared lipoproteins from the serum of a patient with biliary cirrhosis.

BIBLIOGRAPHY

1. Levine, L., Kauffman, D. L., and Brown, R. K., *J. Exp. Med.*, 1955, **102,** 105.
2. Brown, R. K., Davis, R., Clark, B. J., and Van Vunakis, H., *in* Proceedings of the Third International Conference on Biochemical Problems of Lipides, Brussels, 1956, in press.

2a. Avigan, J., Redfield, R., and Steinberg, D., *Biochim. et Biophysic. Acta*, 1956, **20,** 557.

3. Brown, R. K., DeLalla, L. S., and Kauffman, D. L., *Clin. Chem.*, 1955, **1,** 83.
4. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62,** 697.
5. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Immunol.*, 1948, **59,** 195.

6. Osler, A. G., Strauss, J. H., Mayer, M. M., *Am. J. Syph.*, 1952, **36,** 140.
7. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.*, 1946, **84,** 535.
8. Ouchterlony, Ö., *Acta Path. et Microbiol. Scand.*, 1953, **32,** 231.
9. Oudin, J., *Ann. Inst. Pasteur*, 1948, **75,** 30.
10. Becker, E. L., Munoz, J., Lapresle, C., and Le Beau, L. J., *J. Immunol.*, 1951, **67,** 501.
11. Peterkofsky, A., Levine, L., and Brown, R. K., *J. Immunol.*, 1956, **76,** 237.
12. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., *J. Biol. Chem.*, 1952, **195,** 357.
13. Ray, B. R., Davisson, E. O., and Crespi, H. L., *J. Physic. Chem.*, 1954, **58,** 841.
14. Sperry, W. M., *in* Methods of Biochemical Analysis, (D. Glick, editor), New York, Interscience Publishers, Inc., 1955, **2,** 83.
15. Russ, E. M., Raymunt, J., and Barr, D. P., *J. Clin. Inv.*, 1956, **35,** 133.
16. Landsteiner, K., The Specificity of Serological Reactions, Cambridge, Harvard University Press, revised edition, 1945, 111.
17. Spitzer, J. J., personal communication.
18. Aladjem, F., Lieberman, M., and Gofman, J. W., *J. Exp. Med.*, 1957, **105,** 49.
19. Aladjem, F., and Campbell, D. H., *Nature*, 1957, **179,** 203.