THE ANTIGENIC SIMILARITY OF HUMAN LOW DENSITY LIPOPROTEINS

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The present studies were undertaken to explore the immunologic relationships between the ultracentrifugally separable low density plasma lipoproteins.

Several immunologic studies on β-lipoprotein fractions have been described (1–5). Gitlin (2) has ascribed the numerous antigen-antibody reactions in part to heterogeneity of the β-lipoproteins. Korngold and Lipari (3), using the agar diffusion technic of Ouchterlony (6), observed several antigen-antibody systems in the reaction between fraction III and antiserum to fraction III. Only one of the bands gave a positive test with specific lipid stains and only one band developed in the reaction between β-lipoprotein and its antiserum. They suggested that Gitlin's results may have been due to contaminating proteins. Kunkel (1) demonstrated the antigenicity of the protein portions of β-lipoproteins by removing 90 per cent of the lipid and obtaining precipitin reactions with this modified lipoprotein. Middleton (4) in a study of the reaction between rabbit anti-β-lipoprotein and steapsin-hydrolyzed chylomicrons suggested that the protein portions of these substances are similar.

The results of the present study indicate that the various low density lipoprotein fractions tested are antigenically similar.

Materials and Methods

The lipoproteins of human plasma may be divided into two main groups: (*a*) the α-lipoproteins of density 1.075 and 1.14; (*b*) the β-lipoproteins which include the lipoprotein of density 1.050 as well as the β-lipoprotein of Oncley, Gurd, and Melin (7) and the large group of lipoproteins described by Lindgren, Elliott, and Gofman (8) which have densities of 1.020 or less and are usually designated in terms of flotation rates, as $S_{f_{1.063}}$ 10 or greater.

Lipoprotein Purification.—A dense β-lipoprotein, lipoprotein A, was prepared by ethanol and ammonium sulfate fractionation and ultracentrifugation as described in an earlier study (9) in which they were referred to as α_2-lipoproteins. Lipoprotein B was similarly prepared except that material from the middle portion of the tube, centrifuged at density 1.063, was used as the starting material. Although Oncley and Gurd (10) have suggested that this lipoprotein is found only in aged plasmas, it also has occasionally been observed in this laboratory in ultracentrifuge flotation of lipoproteins from freshly drawn sera, and material of a similar S_f has been purified from such sera. In order to determine whether the high salt

concentrations used in flotation might have caused the appearance of this lipoprotein, one lot was prepared using 0.15 M NaCl in D_2O to adjust the density of the medium. It was found that similar amounts of dense β-lipoprotein were observed in samples prepared in high salt medium and in deuterium oxide solution. Lipoprotein C was prepared from freshly drawn and outdated human plasma by bringing its density to 1.063 with an appropriate amount of a solution, 2.62 M NaCl and 3.03 M KBr, centrifuging for 16 hours at 105,000 *G* in celluloid tubes, removing the top fraction with a tube-slicing device, bringing its density

TABLE I

Physical and Chemical Characteristics of Low Density Lipoproteins Studied

Lipoprotein	Source	Method of preparation	$-S_{1.21} \times 10^{13}$	$-S_{1.063} \times 10^{13}$	D_{20}, H_2O $\times 10^7$	Ratio cholesterol to N by weight
A	Fr. III-0	Sed. D = 1.063 AMS Fr. Flot. D = 1.20	28	*	0.93	2.9
B	Fr. III-0	Immob. D = 1.063 AMS Fr. Flot. D = 1.20	28	*	1.60	6.5
C	Plasma	Flot. D = 1.063 Sed. D = 1.050 Flot. D = 1.10	32	6	—	8.4
D	Fr. III-0	Flot. D = 1.063 Immob. D = 1.040	33	5	0.98	8.4
E	Plasma	Flot. D = 1.020 Sed. D = 1.006	65	19	0.52	3.2
F	Plasma	Flot. D = 1.006	160	70	0.35	11.2

Note:—Sed. = sedimentation; AMS Fr. = ammonium sulfate fractionation; Flot. = flotation; Immob. = immobility; $-S_{1.063} = S_f$ of Gofman.

* The density of these lipoproteins is so similar to the density of the medium that accurate *S* values are difficult to obtain.

to 1.050 with water, centrifuging as before, slicing, and discarding the top fraction. The remainder was brought to a density of 1.10 with NaCl-KBr solution and again centrifuged. The top fraction constituted lipoprotein C. A β-lipoprotein, lipoprotein D, was prepared from fraction III-O (11) as described previously (9). It was separated at a density of 1.040 by similar centrifugation. The middle portion of the tube was used. Lipoprotein E, of the S_f 10–20 lipoprotein group, was separated from plasma by flotation at density 1.020 and subsequent sedimentation at density 1.006 in the preparative ultracentrifuge. Lipoprotein F, a lipoprotein of S_f greater than 20, was obtained from plasma by flotation at density 1.006 by the same methods. All samples were dialyzed against 0.15 M NaCl before use.

Physical and chemical studies of the low density lipoproteins used showed that significant differences existed among them (Table I).

Production of Antisera.—Antisera were produced in rabbits by intravenous injections of alum-precipitated lipoproteins A and D. Sixteen injections, one every other day, were given. The doses were increased gradually from 0.5 mg. to 7.5 mg. of the protein. The rabbits were exsanguinated 6 days after the last injection. The sera were cleared by centrifugation and heated for 30 minutes at 56°C. to inactivate the complement, C′.

Immunologic Methods.—Quantitative precipitin experiments were performed as described by Heidelberger and Kendall (12). The reaction mixtures were set up at room temperature and incubated at 0–2°C. for 24 hours. The precipitates were collected in the cold, washed twice with ice-cold saline, and analyzed for nitrogen by the microKjeldahl method.

Quantitative C′ fixation measurements were made by the method described by Mayer, Osler, Bier, and Heidelberger (13) except for a minor modification in the assay of hemolytic activity (14). Quantities of C′ from 100 to 110 $C'H_{50}$ were used and fixation was allowed to proceed for from 20 to 24 hours at 0–2°C. An aliquot of each mixture of C′ with antibody and antigen, as well as the controls, C′ with antigen, C′ with antibody, and C′ alone, was titrated for residual hemolytic activity by means of the spectrophotometric method outlined by Mayer *et al.* (15).[1] The number of $C'H_{50}$ fixed was calculated by subtraction of 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.

Agar-diffusion analyses were performed by the methods of Ouchterlony (6) and Oudin (16). Diffusion constants were obtained by the procedure described by Becker and coworkers (17) using 20 μg. of antibody N per ml. in the solid phase; the reaction mixtures were incubated at 30°C. ± 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

It was recognized that the antigenic relationship of the low density lipoproteins could not be determined unless a single antigen-antibody system was being analyzed. In the reaction between whole serum and anti-A lipoprotein, 5 bands, which moved with time, were observed by the single diffusion method. One of these immune reactions was found to be the human serum albumin system[2] and another the heat-labile α_2-glycoprotein system (18). The presence of the antiglycoprotein was not surprising since the heat-labile α_2-glycoprotein is derived from the same fraction, III-O, as lipoprotein A. Similarly, in the reaction between whole serum and anti-D lipoprotein, 4 bands developed, one of which was due to the heat-labile α_2-glycoprotein.

Since interpretation of the results would be difficult with antisera containing multiple antibodies, the following absorption experiments were done:—

The human plasma used for absorption of the anti-A lipoprotein was freed of low density lipoproteins by bringing it to a density of 1.063 with a solution of 2.62 M NaCl and 3.03

[1] We are indebted to Dr. Manfred Mayer and Dr. Herbert Rapp of the Department of Microbiology, Johns Hopkins University School of Hygiene and Public Health, Baltimore, for the antiserum to boiled sheep stromata used as hemolytic antisera.

[2] The impurities present in the lipoprotein fraction that give rise to immunochemical heterogeneity probably are a result of occlusion. One possible cause of this may be convection currents arising in dilute solutions during ultracentrifugation. Lipoprotein D was recentrifuged at density 1.063; in the top fraction and adjacent layer, 0.3 per cent and 1.3 per cent, respectively, of the protein was found to be human serum albumin.

M KBr, centrifuging it for 16 hours at 105,000 *G*, and discarding the top fraction. The remaining material was brought to a density of 1.12 with the NaCl-KBr solution and centrifuged similarly. The top fraction was discarded. For absorption of the anti-D lipoprotein, the procedure was the same except that the density was raised to 1.21 before centrifugation. Both preparations were dialyzed against 0.15 M NaCl before use.

One volume of lipoprotein-free plasma was added to each ten volumes of the antiserum. The mixture was kept at 0–2°C. for 20 hours and centrifuged at 22,000 *G* in the cold. The process was repeated and the antiserum examined by single diffusion performed with equal volumes of agar and undiluted serum in the solid phase.

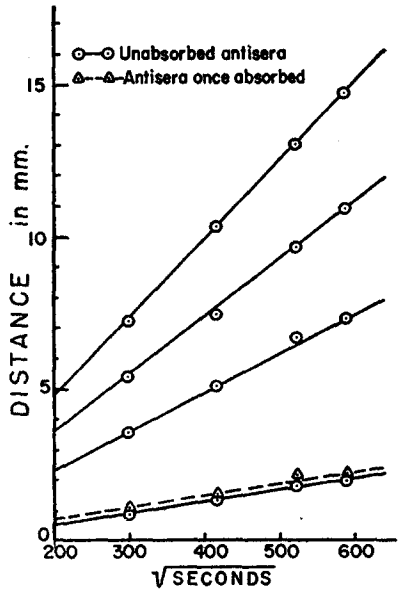

FIG. 1. Single diffusion of undiluted human plasma into unabsorbed and once absorbed rabbit anti-D lipoprotein agar mixture, 1:1 by volume.

The removal of three antigen-antibody reactions, as determined by single diffusion, was complete after one absorption of the anti-D lipoprotein (Fig. 1). Two absorptions with lipoprotein-depleted plasma were required to remove the last traces of antibodies to the heat-labile glycoprotein and two unidentified proteins from anti-A lipoprotein. It should be noted that four bands were detected with whole plasma as the diffusing reactant, whereas only two bands developed with D lipoprotein overlaying the agar-serum mixture. This was probably due to a higher concentration of one or two contaminants in the whole plasma. These experiments were repeated with various dilutions of the antiserum with essentially the same results. With high dilutions of antiserum some bands disappeared.

When the absorbed antisera were overlaid with plasma freed of low density

lipoproteins, no antigen-antibody reactions were observed. C′ fixation was also performed on this lipoprotein-free plasma and anti-A lipoprotein with negative results. The limit of detection by this method is about 0.2 μg. N/ml. of the lipoprotein-free plasma, which corresponds to about 0.002 per cent of the plasma proteins. Thus, no lipid-poor plasma protein reactive to anti-low-density lipoproteins was found. Two high density lipoproteins, an α_1-lipoprotein (9), and a lipoprotein of density 1.075 isolated from whole plasma gave no reaction with the absorbed anti-A lipoprotein when observed by single diffusion. Lipoproteins present in fraction IV + V[3] from a patient with biliary cirrhosis also gave no reaction. It is possible that a lipid-poor protein reactive to lipoprotein antibody is present in plasma and that, during the absorption procedure, antibody cross-reactive to this hypothetical protein was removed. Similarly, antibody cross-reactive to the high density lipoproteins may have been absorbed. These possibilities are under investigation.

Since the immune systems, A lipoprotein–anti-A lipoprotein and D lipoprotein–anti-D lipoprotein, appeared to be single antigen-antibody reactions, the reaction was studied by the quantitative precipitin technic. In this manner, the cross-reactions could be studied. Also, another criterion of immunochemical homogeneity, supernatant fluid analyses, could be fulfilled. Accordingly, quantitative precipitation tests were set up with constant amounts of anti-A lipoprotein and varying quantities of A, B, D, and E lipoproteins. The four different classes of lipoproteins reacted with the anti-A lipoprotein, although a quantitative difference was apparent (Table II). Supernatant fluid analyses of these reaction mixtures for excess antigen and antibody showed no evidence of overlapping.

Precipitin analyses with D lipoprotein and anti-A lipoprotein were performed at 0°C. and 37°C. Unlike many cross-reacting systems (19), this antigen-antibody reaction displayed no marked temperature coefficient.

Although the data shown in Table II demonstrate cross-reactions between lipoproteins of different flotation classes, objection might be raised as to the validity of estimating antibody nitrogen by subtracting the antigen nitrogen added. The contribution of N from the contaminating proteins has been ignored, and it has been assumed that all of the N added in antibody excess has been precipitated. This possible error may be magnified when a relatively large amount of antigen N is subtracted from a comparatively small quantity of precipitable N, as in these studies. Moreover, the total precipitable N does not lend itself to unequivocal interpretation, since the percentage of nitrogen in different classes of lipoproteins varies considerably.

Although these objections exist, the quantitative precipitin technic indi-

[3] Obtained from Dr. David M. Barr and Ella M. Russ, New York Hospital, New York City.

cates that the cross-reaction is not due to A lipoprotein contaminating B, D, and E lipoproteins, since increasing increments of heterologous antigen

TABLE II

Antibody N Precipitated from A-Lipoprotein Antisera by Varying Amounts of A, B, D, and E Lipoproteins at 0–2°C.

Lipoprotein added		Total N precipitated from 2.0 ml. serum	Antibody N precipitated from 2.0 ml. serum	Ratio antibody N to lipoprotein N in precipitate	Tests on supernatant fluids
	mg. N	*mg.*	*mg.*		
A	0.048	0.160	0.112	2.3	Excess antibody
	0.096	0.232	0.136	1.4	" "
	0.182	0.396	0.214	1.2	" "
	0.268	0.528	0.260	0.97	" "
	0.360	0.605	0.245	0.68	" "
	0.450	0.684	0.234	0.52	No lipoprotein, no antibody
B	0.220	0.614	0.394	1.8	Excess antibody
	0.440	0.858	0.418	0.95	" "
	0.880	1.210	—	—	Excess lipoprotein
	1.55	1.700	—	—	" "
	2.21	1.974	—	—	" "
D	0.105	0.468	0.363	3.5	Excess antibody
	0.210	0.672	0.462	2.2	" "
	0.420	0.940	0.520	1.2	" "
	0.735	1.270	0.535	0.73	" "
	0.945	1.512	0.567	0.60	" "
	1.890	1.846	—	—	Excess lipoprotein
E	0.043*	0.112	0.069*	1.5	Excess antibody
	0.129	0.244	0.115	0.85	" "
	0.168	0.288	0.120	0.67	" "
	0.336	0.456	0.120	0.33	" "
	0.672	0.776	0.104	0.11	" "

* E lipoprotein was analyzed for the presence of HSA and the heat-labile α_2-glycoprotein by precipitation with calibrated antisera. It contained 2.5 per cent of HSA and less than 0.1 per cent of α_2-glycoprotein. The values given are corrected for this inert impurity. A, B, and D lipoproteins contained less than 1 per cent HSA and less than 0.1 per cent glycoprotein. These values are not corrected. The higher HSA content of E lipoprotein probably is a result of its isolation from plasma rather than fraction III-0.

did not precipitate all of the antibody nitrogen (Table II). Analysis of the specific precipitate for chemical constituents of the antigen, as has been done with the blood group substances (20) and with Type II pneumococcus polysaccharide (21), offered another means of determining immunochemical

homogeneity. Analyses were made to determine whether the cholesterol added in the reactions between A, D, E, and F lipoproteins and anti-A lipoprotein in antibody excess could be recovered in the immune precipitate.

Cholesterol was determined in each of the lipoprotein antigens by the method of Abell, Levy, Brodie, and Kendall (22) and the cholesterol to nitrogen ratios were calculated. Known quantities of cholesterol-containing lipoproteins were added to constant quantities of anti-A lipoprotein at room temperature and incubated at 0–2°C. overnight. The precipitates were centrifuged in the cold and washed twice with ice-cold saline.

TABLE III

Recovery of Cholesterol in the Specific Precipitate Formed by Reacting A, D, E, and F Lipoproteins with Anti-A Lipoprotein

Lipoprotein N added		Cholesterol added	Cholesterol in precipitate	Cholesterol recovered	Total N precipitated from 0.5 ml. serum	Tests on supernatant fluids
	mg.	*mg.*	*mg.*	*per cent*	*mg.*	
A	0.132	0.397	0.400	101	0.189	No lipoprotein, no antibody
D	0.076	0.655	0.655	100	0.196	Excess antibody
E	0.076	0.243	0.287	118	0.106	" "
F	0.048	0.537	0.487	91	—	" "

Controls

Antigen	Amount added	Antisera	Cholesterol added	Cholesterol in precipitate	Total N in precipitate
	mg.		*mg.*	*mg.*	*mg.*
Type-III pneumococcus polysaccharide	0.400 SIII	Anti-Type III pneumococcus	—	0	2.640
Human serum albumin	0.017 N	Antihuman serum albumin	0.600	0.007	0.238
D lipoprotein	0.071 N				

The data in Table III show that the added cholesterol was recovered in the immune precipitate. Analyses of the supernatant fluids showed no excess of antigen in any mixture. Reactions between the specific polysaccharide of Type III pneumococcus and its homologous rabbit antibody served to control the presence of cholesterol in precipitated rabbit antibody. Human serum albumin–rabbit antihuman serum albumin, precipitated in the presence of D lipoprotein, served to control non-specific absorption or occlusion of low density lipoproteins on the immune precipitates. These control reactions show that the recovered cholesterol was not derived from the rabbit antibody or from absorbed or occluded lipoproteins.

A second approach, quantitative C′ fixation in dilute systems yielding no visible precipitates, was made. This technic has been used to study cross-

reactions between hen and duck egg albumins and between Types III and VIII pneumococcus polysaccharides (23, 24). The following experiments were designed to determine the extent of cross-reaction between low density lipoproteins.

Beginning with 0.25 μg. of lipoprotein N, increasing increments of A, C, D, E, and F lipoprotein N were added to 2.2 μg. of rabbit anti-A lipoprotein in the presence of 100 to 110 $C'H_{50}$ in a volume of 10.0 ml. The mixtures were incubated at 0–2°C. for 24 hours. The diluent for C′ fixation was veronal buffer except that 0.001 M Ca^{++} was used during the course of the fixation reaction since it has been demonstrated that Ca^{++} mediates the fixation of guinea pig C′ by an immune reaction (25). For the hemolytic assay, 0.0005 M Mg^{++},

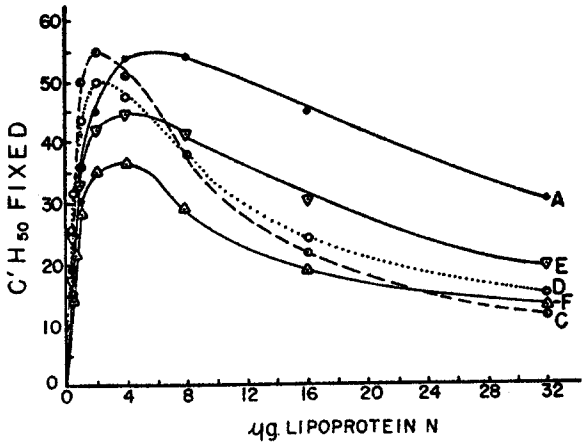

FIG. 2. Fixation of C′ by varying quantities of A, C, D, E, and F lipoproteins and 2.2 μg. of anti-A lipoprotein N at 0–2°C. for 24 hours.

and 0.00015 M Ca^{++} were incorporated in the veronal buffer (13). In a similar manner, increasing quantities of B, C, D, E, and F lipoprotein nitrogen were reacted with 2 μg. of anti-D lipoprotein nitrogen. 2.2 μg. of rabbit anti-A lipoprotein N were anticomplementary to the extent of 12 $C'H_{50}$. Since this remained constant in all C′ fixation analyses, the C′ fixed was calculated by subtracting residual hemolytic activity from that of the antibody control.

The data in Fig. 2 show that from 60 to 98 per cent of the fixable C′ combined in the heterologous reactions. Similarly, in experiments with 2.0 μg. of anti-D lipoprotein N and the heterologous lipoproteins (Fig. 3), a comparable percentage of cross-reaction occurred. These data clearly indicate the similarity of immunologic determinants in the different classes of low density lipoproteins. This cross-reaction is also apparent in the absorption of anti-A lipoprotein with E lipoprotein and the demonstration of anti-A lipoprotein activity in the supernatant fluid.

31 μg. of E lipoprotein N in a volume of 1.0 ml. was added to 0.5 ml. of anti-A lipoprotein and left at room temperature for ½ hour. After incubation at 0–2°C. overnight, the

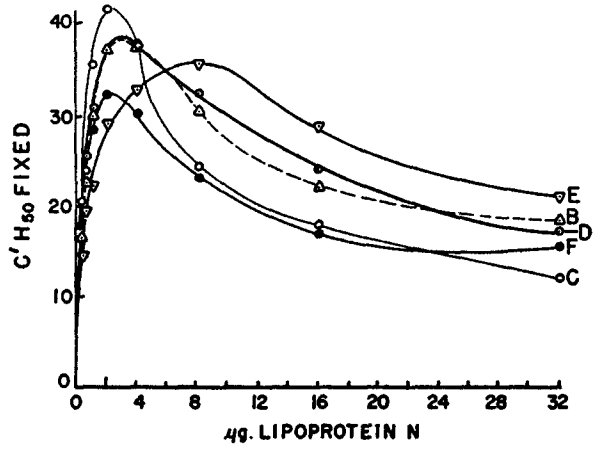

FIG. 3. Fixation of C′ by varying quantities of B, C, D, E, and F lipoproteins and 1.8 μg. of anti-D lipoprotein N at 0–2°C. for 20 hours.

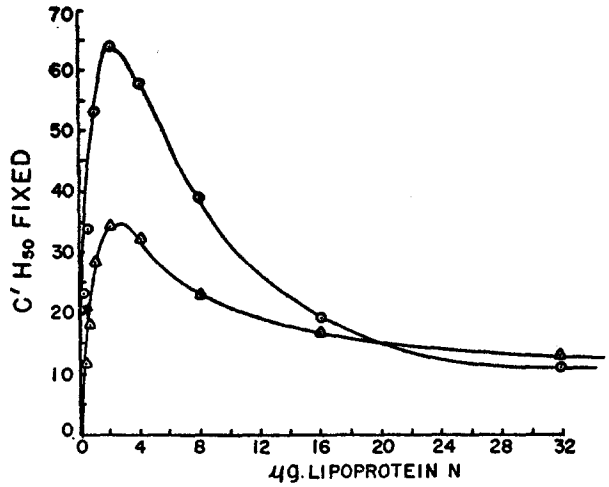

FIG. 4. Fixation of C′ by varying quantities of C lipoprotein with 2.2 μg. of anti-A lipoprotein N (○) and anti-A lipoprotein absorbed with E lipoprotein (△).

precipitate was removed, washed twice with ice-cold saline, and analyzed for N. The results showed that 25 μg. of the total 110 μg. of antibody N was removed. The supernatant fluids and control sera were centrifuged at 22,000 *G* for 1 hour and diluted with veronal buffer 1:100 with respect to the original sera. Quantitative C′ fixation was performed with

increasing increments of C lipoprotein. C lipoprotein was used as an antigen to assay for absorption of cross-reacting antibody because lipoprotein A was not available when the experiment was done. Fig. 2 shows that A and C lipoproteins fix the same amount of C′ and therefore the substitution of C for A in this experiment appears valid.

As shown in Fig. 4, the reaction of E lipoprotein with anti-A lipoprotein removed antibody reactive to C lipoprotein.

It was not known whether the antigenic similarity between low density lipoproteins resided in the lipid or protein portions of the molecule. Accordingly, A and C lipoproteins were treated with ethanol-ether mixture, 3:1 by volume, at room temperature to remove the lipids. The extracted lipids were finely suspended in saline and tested for activity by precipitation and C′ fixation. No activity was observed, which suggested that the antigenic determinants are, at least in part, in the protein portions of the molecule.

DISCUSSION

Immunochemical homogeneity, which is basic to this study, has been demonstrated by several procedures. Although both the single and double diffusion technics demonstrate a multiplicity of antibodies in the unabsorbed anti-A and anti-D lipoprotein sera, absorption with lipoprotein-free plasma removed all but the antigen-antibody system under study. It can be seen from the data in Fig. 1 that four migrating fronts developed when whole serum diffused into agar containing unabsorbed anti-D lipoprotein and that only one band developed after absorption. Studies by the double diffusion procedure showed that anti-A lipoprotein twice absorbed with lipoprotein-free plasma no longer reacted with a crystalline preparation of heat-labile α_2-glycoprotein and serum albumin and gave a single line with whole serum and all low density lipoprotein preparations tested. In analyses of precipitin supernatant fluids, antigen and antibody were never found simultaneously (Table II), which provides additional evidence that the system is immunochemically homogeneous.

Specific lipid stains have been used to demonstrate that the antigen in the immune reaction is a lipoprotein (3). In view of the contradictory reports of the presence of lipoidal material in specific precipitates (26, 27), evidence for antibody reactive to lipoproteins was obtained in the present work by the demonstration that cholesterol could be quantitatively recovered in the immune precipitate. Controls were included to demonstrate that rabbit antibody to the Type III pneumococcus contained no detectable cholesterol and to show that no cholesterol was occluded or absorbed when an unrelated precipitate was formed in the presence of lipoprotein. The data in Table III show that 101, 100, 118, and 91 per cent of the amount of the cholesterol added was recovered in the specific precipitate when A, D, E, and F lipoproteins reacted with anti-A lipoprotein in the region of antibody excess or equivalence.

Gitlin (2) observed five and six bands in the β-lipoprotein anti-β lipoprotein reaction. Grant and Berger (5) observed one band with whole serum and chicken anti-β lipoprotein. Korngold and Lipari (3) observed only one band when β-lipoprotein and anti-β-lipoprotein diffused into each other. This immunochemical homogeneity in unabsorbed antisera may be due to the relatively short course of immunization used by the latter workers. It should be pointed out that in reactions between the homologous antigen and unabsorbed antisera, fewer lines of precipitation may be observed by agar-diffusion technics than when whole serum, the source of the purified antigen, is used as the diffusing antigen. Five bands were detected by single diffusion in the reaction between whole serum and anti-A lipoprotein; only three bands appeared when the reaction was carried out on an agar plate. In this case the Oudin technic was more sensitive than the Ouchterlony. It is apparent that immunochemical homogeneity should be examined by more than one method.

The conclusion that antigenically similar determinant groups exist in the low density lipoproteins is based on the following findings: (*a*) Antibody to A lipoprotein will precipitate with different classes of lipoproteins; (*b*) Antibody to A and D lipoproteins will fix C′ when they react with the heterologous lipoproteins; (*c*) Antibody to A lipoprotein can be absorbed with heterologous lipoprotein, leaving antibody still reactive to the homologous antigen; (*d*) Reactions of identity are obtained when these different lipoproteins and antibody to A or D lipoprotein are allowed to diffuse into each other (Fig. 5), (*e*) When antibody to A lipoprotein is used to measure the diffusion coefficients of lipoproteins, the diffusion coefficients are different with each lipoprotein. This finding rules out the possibility that the cross-reaction between anti-A and lipoproteins B, D, E, and F is due to contamination with A lipoprotein. The analytical ultracentrifuge patterns, recovery of the majority of the cholesterol added as A, D, E, or F lipoprotein, and the shape of the precipitin and C′ fixation curves are further evidence against this possibility.

The quantitative precipitin data given in Table II show that 46, 74, 100, and 20 per cent of the anti-A lipoprotein reacted with A, B, D, and E lipoproteins, respectively. With respect to peak fixation of C′, anti-A lipoprotein reacted with A, C, D, E, and F lipoproteins 100, 100, 91, 82, and 67 per cent, respectively (Fig. 2), while 92, 100, 92, 87, and 77 per cent of anti-D lipoprotein reacted with B, C, D, E, and F lipoproteins, respectively (Fig. 3). Thus, the degree of cross-reaction, as measured by C′ fixation, when the antigen and antibody were allowed to react in the presence of C′ at 0°C. for from 20 to 24 hours, is greater than that obtained by precipitin studies. This is in agreement with the cross-reaction studies of Osler and Heidelberger on duck and hen egg albumins (23) and pneumococcus Type III and VIII polysaccharides (24).

It is of interest to note the relatively few antigenic sites available on the surface of these lipoprotein molecules. Thus, in the region of extreme antibody

excess, antigen-antibody ratios of 2.3, 1.8, 3.5, and 1.5 were obtained. These would correspond to molar ratios ranging between four and eight. The ratios were calculated by assuming a molecular weight of 1.3 million for A, B, and D, and of 4.2 million for E. A and B were assumed to be 26 per cent peptide, D 23 per cent, and E 18 per cent. In contrast, studies on other immune systems in which the antigen is a protein of large molecular weight, such as thyroglobulin or tobacco mosaic virus (28), show 40 and 900 molecules of antibody

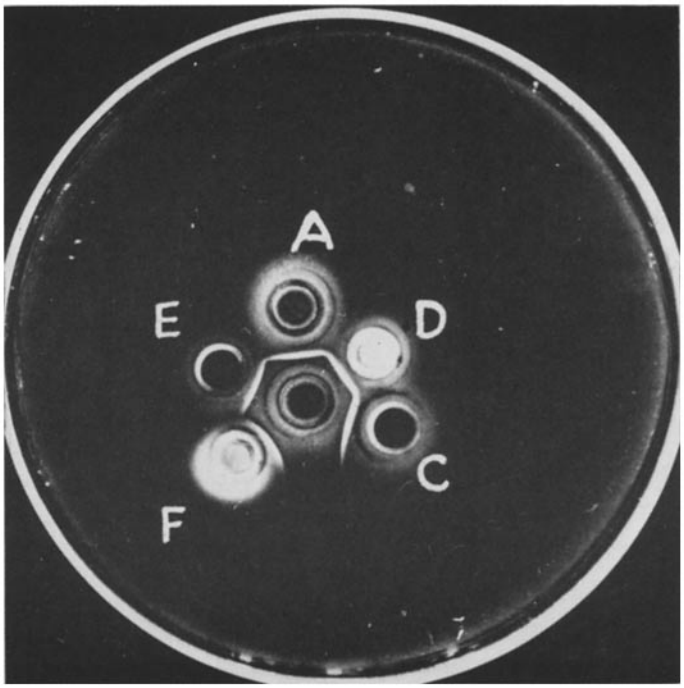

FIG. 5. Double diffusion reaction of A, B, D, E, and F lipoproteins with anti-A lipoprotein.

to each respective antigen molecule. The small number of antigenically reactive sites on the surface of these lipoproteins is probably due, in part, to the small amount of antigenic peptide at the surface of the molecule and, in part, to the spatial configuration of the lipid.

The fact that more antibody N was precipitated with a heterologous antigen was surprising (Table II). One possible explanation is that the injected lipoproteins were altered by the clearing factor mechanism of the rabbit and that antibodies were produced to these altered lipoproteins. Bragdon and Boyle (29) have shown that lipoproteins from one species of animal injected into another species may be converted into lipoproteins of a different flotation

class. An alternative possibility is that the lipid did not interfere with antibody synthesis but did with antigen-antibody interaction.

While the cross-reactions may be due to partial similarity of the protein portion of the antigen, as is probably the case in the duck-hen ovalbumin cross-reaction, it is more probable, in view of the interconversion of lipoproteins, that the protein portions of all low density lipoproteins contain identical antigenic sites and that the lipid masks portions of the peptide chain in the cross-reacting lipoproteins. This is somewhat analogous to the lecithinase–antilecithinase system in which lecithin was shown to interfere with the inhibition of lecithinase by antilecithinase when the enzyme substrate complex was added to antilecithinase (30).

The cross-reactivity indicates that all groups of low density lipoproteins will contribute in the immunologic estimation of specific low density lipoprotein classes unless antisera are suitably absorbed with all other groups of heterologous antigens. Such a procedure is probably impractical because of its complexity and, in some cases,—namely, with anti-A lipoprotein,—theoretically impossible. Thus, antisera, such as have been employed by Grant and Berger (5), will not differentiate a serum containing a high concentration of the β-lipoprotein of Oncley *et al.* (7) from a serum containing a normal concentration of β-lipoprotein and an elevation of larger lipoproteins.

It is possible that the two α-lipoproteins and the lipoproteins found in certain diseases, such as biliary cirrhosis, may constitute a separate cross-reacting system. Preparations of the two α-lipoproteins do not react with the anti-low density lipoproteins.

SUMMARY

The following human low density lipoproteins were prepared: β-lipoproteins of densities greater than 1.040 (A, B, C), a β-lipoprotein of $-S_{1.063} = 5$ (D), a lipoprotein of $-S_{1.063} = 19$ (E), and a lipoprotein of $-S_{1.063} = 70$ (F).

Data are presented which show the immunochemical homogeneity of the D lipoprotein rabbit–anti-D lipoprotein system.

Cross-reactions between antibody to A and D lipoproteins and the above lipoproteins have been demonstrated by quantitative precipitation, quantitative complement fixation, and single and double diffusion in agar.

The antigenic similarities appear to be associated with the protein portions of the molecule.

The antisera produced did not differentiate the low density lipoprotein classes.

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