STUDIES ON THE ANTIGENICITY OF β- AND α_1-LIPOPROTEINS OF HUMAN SERUM*

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An antigenic difference has been shown between low and high density lipoproteins (1–3). Antigenic heterogeneity of low density lipoproteins has been reported by Gitlin (4). Aladjem, Lieberman, and Gofman (3) and Aladjem and Campbell (5) have obtained similar results by absorption, precipitin, and agar diffusion techniques. The data, however, of Korngold and Lipari (6) and Levine, Kauffman, and Brown (1) favor the antigenic homogeneity of this lipoprotein class. Absorption and quantitative precipitin analysis (1) and agar diffusion techniques (1, 6) were used in these experiments.

The antigenicity of high density lipoproteins also has been investigated. Antigenic heterogeneity has been reported (3), whereas the data of DeLalla, Levine, and Brown (2) support the homogeneity of these lipoproteins.

It is hard to explain these conflicting findings. However, methods of separation and purification of the lipoprotein classes used for antiserum production, time of standing, and immunizing procedure were different.

Agar immunoelectrophoresis has been recently proposed by Grabar and Williams (7) as a method for characterization of antigenic proteins. Separation of the different antigenic components is first achieved by electrophoresis in agar gel. Homologous antiserum is then added to the gel; at the point in which antigen and specific antibody meet, a visible line of precipitation forms. The number of these lines is supposed to be proportional to the number of antigens present in the unknown solution (8).

Agar immunoelectrophoresis was used in the present studies to determine the number of antigenic components of β- and α_1-lipoproteins separated by ultracentrifugation from normal human serum, and the antigenic capacity of the lipide-free residue after delipidation of the lipoprotein.

Methods

β-lipoproteins of fresh normal sera were separated by centrifugation for 18 hours at density 1.063, the chylomicrons having been removed by a previous centrifugation at 30,000

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R.P.M. and density 1.003 for 2 hours. β-Lipoprotein concentrate, overlaid in a preparative ultracentrifuge tube with a sodium chloride solution of 1.063 density, was respun for an additional 18 hours. α_1-Lipoprotein samples (Experiments 2, 4, and 6) were concentrated by ultracentrifugation for 18 hours from sera at density 1.21 after removal of the low density lipoprotein fractions at density 1.063. α_1-Lipoprotein concentrate, overlaid with potassium bromide solution at density 1.21, was respun for 18 hours. In another experiment (No. 7) the time of ultracentrifugation for the separation of the α_1-lipoprotein fraction was increased to 48 hours. α_1-Top fractions, overlaid with *potassium bromide* solution of density 1.21, were spun for an additional 24 hours.

Samples of β- and α_1-lipoproteins were dialyzed overnight against sodium chloride M/15 and then stored at +3°C. until used (no longer than 48 hours). Homogeneity of samples under investigation was checked in the analytical ultracentrifuge.

α_1-lipoprotein samples were delipidized according to the technique previously described (9). When this procedure was applied to β-lipoprotein samples, the protein residue was insoluble in water. The following procedure was therefore used: 2 ml. of β-lipoprotein concentrate was extracted in cold ether at −50°C. (freezing mixture, ethyl alcohol–dry ice). After standing 1 hour at this low temperature, the sample was kept at +3°C. for 24 hours. It was then continuously extracted by ether for 24 hours using a liquid-liquid extractor to which a water jacket was applied to maintain the temperature around +5°C. At the end a solid emulsion was observed between the water and ether layers. Only the water phase was analyzed immunochemically.

Nitrogen content was determined according to Pregl's (10) modification of the Kjeldahl method.

The agar immunoelectrophoresis technique of Grabar and Williams (7) was used. A solution of 1.5 per cent agar (Bacto-agar Difco) in a veronal sodium buffer, pH 8.2, was employed as a stabilizing medium. Glass plates (18 × 8 cm.) were used as a support of a 4 mm. thick layer of the buffered agar. The central trough (25 × 3 mm.) was filled with antigen solutions of nitrogen content between 100 to 200 μg. The electrophoretic separation was obtained in 6 hours with a potential gradient of 5.6 v./cm. and a current of 40 milliamperes.

For each sample a duplicate run was performed. The first plate was stained either with Amidoschwartz (protein staining) or with Sudan black (lipoprotein staining) according to the procedure described by Uriel and Grabar (11) to identify the position of the different boundaries; the second agar plate was used for immunoprecipitin study. A trough (80 × 3 mm.) was cut in a direction perpendicular to the central trough at a distance of 10 mm. and filled with 0.1 to 0.2 ml. of antiserum.

Horse anti-normal human serum, obtained from Pasteur Institute, was used in all our experiments. Its nitrogen content was 1.34 per cent.

Antigen-antibody system was kept in a humid chamber at room temperature for a period of 4 to 7 days. Precipitation lines were stained either with Sudan black or azocarmine according to Uriel and Grabar (11).

Agar double diffusion, as described by Ouchterlony (12), was also performed. The same buffered agar as for immunoelectrophoresis was used. Round wells, 10 mm. in diameter, were cut in the agar layer 2 mm. thick. Proper amounts of antigen and antibody were added: antigen 30 to 50 μg. N, antibody 250 to 500 μg. N. The best antigen-antibody nitrogen concentration ratio was 1:9. The lines of precipitation formed were stained in the same way as were those in immunoelectrophoresis.

Absorption Experiments.—Immune serum was absorbed with either crystalline serum albumin or normal human serum deprived of the lipoprotein fractions by ultracentrifugation. Albumin or serum lipoprotein–free material was added to the immune serum until no further precipitation occurred in the precipitation tube. The absorption procedure was con-

sidered satisfactory when, by the Ouchterlony technique, antiserum absorbed with albumin or with lipoprotein-free serum did not show precipitation lines respectively against serum crystalline albumin or serum proteins deprived of albumin.

EXPERIMENTS AND RESULTS

When fresh normal human serum was analyzed by agar electrophoresis the presence of five main boundaries was detected by staining of protein: albumin, α_1- and α_2-globulin moved towards the anode, β- and γ-globulin towards the cathode. By lipide staining two main boundaries were detected, both located between the point of application and the anode, one was between the α_1- and α_2-globulin, the second one in the albumin zone.

Immunochemical Analysis of Mixed α_1- and β-Lipoproteins.—In these experiments an α_1-lipoprotein preparation (Experiment 2) was used. When a mixture of α_1- and β-lipoproteins was examined electrophoretically, the position of the boundaries corresponded to those observed in the agar electrophoretic pattern of whole normal serum stained for lipides. The β-lipoprotein fraction moved closer to the α_2-globulin area, showing clear evidence of a small, diffuse zone of precipitation denoting instability of the β-lipoprotein preparation in agar.

The immunoelectrophoretic pattern of one of the α_1- plus β-lipoprotein mixtures is shown in Fig. 1. Three different precipitation lines can be observed: a straight line corresponding to the β-lipoprotein boundary, a curved one corresponding to the α_1-lipoprotein, and a third line not corresponding to any visible boundary of the electrophoretic pattern, which was found later to be due to traces of albumin present in our α_1-lipoprotein preparation.

Immunochemical analysis of the α_1- plus β-lipoprotein mixture by the Ouchterlony technique showed the presence of two precipitation lines differing in position and shape (see Fig. 2 *b*); the faster moving antigenic component was the one corresponding to α_1-lipoprotein. A narrow, white ring was observed around the well containing the β-lipoprotein solution, again denoting the instability in agar of this serum protein fraction.

Immunochemical Analysis of β-lipoprotein.—After fractionation by agar electrophoresis a small, white precipitate was observed in the region of α_2-globulin (Fig. 3). Addition of antiserum to the agar plate after electrophoresis showed a straight precipitation line perpendicular to the stained boundary after 1 day of standing at room temperature. When the period of standing in the humid chamber was prolonged up to 7 days, no extra lines of precipitation were observed.

Immunoelectrophoretic analysis of the delipidized lipoprotein sample was not entirely satisfactory. Delipidation had a marked denaturing effect on the protein moiety. Only 25 per cent of the nitrogen content of the original lipoprotein sample was recovered in the water phase, which showed about 10 per

cent of the original lipide phosphorus content and no appreciable amount of cholesterol. This low protein content prevented satisfactory physicochemical characterization of the water-soluble components. However, agar electrophore-

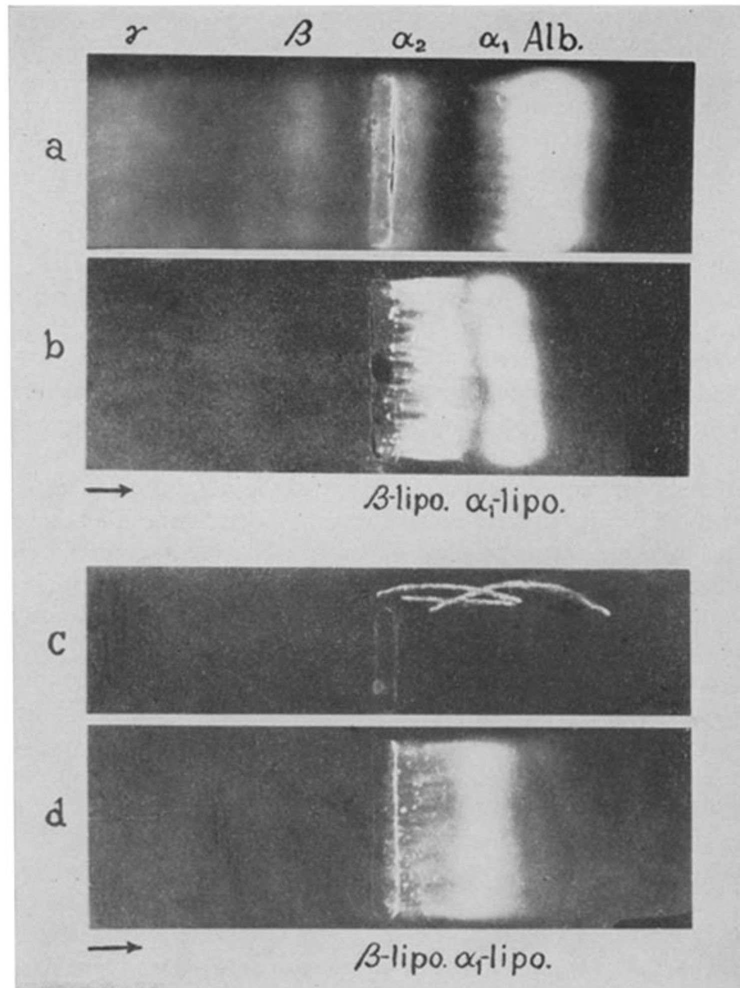

FIG. 1. Agar electrophoresis of normal human serum and of a mixture of α_1- plus β-lipoprotein. (*a*) Human serum stained with Amidoschwartz. (*b*) Human serum stained with Sudan black. (*c*) Immunoelectrophoresis of a mixture of α_1- plus β-lipoprotein. (*d*) Same as (*c*) stained with Sudan black.

sis of the delipidized material did not show change in mobility in comparison to the corresponding boundary in the original lipoprotein sample, and addition of antiserum brought out a single precipitation line similar to the one shown by the original sample.

A single antigenic component was also found on analysis by the Ouchterlony technique (see Fig. 2). It was observed, however, that periods of standing up to 4 weeks could induce formation of four to six visible lines in the agar, presumably related to drying of the agar. These lines did not stain with Sudan black or azocarmine.

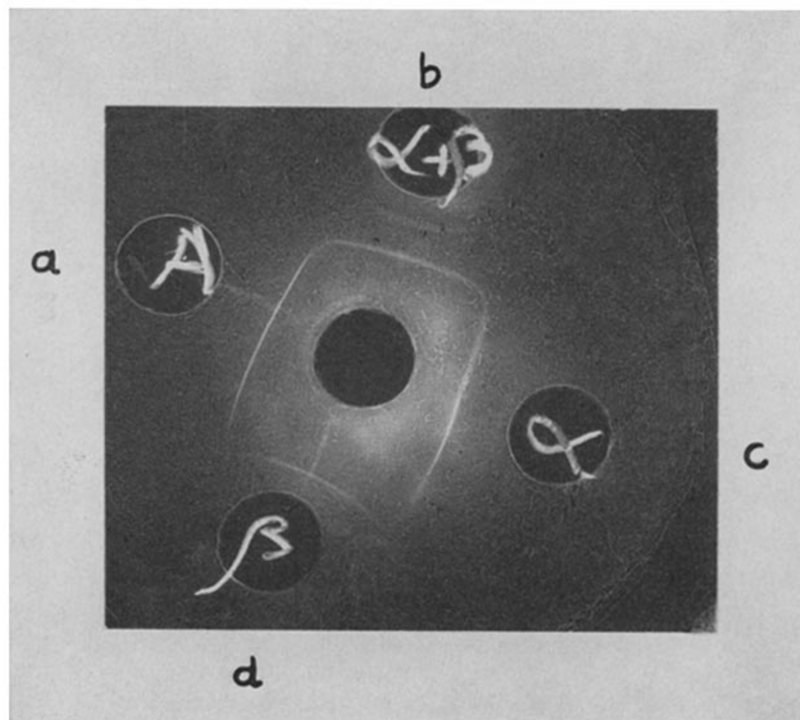

FIG. 2. Ouchterlony technique applied to albumin and different lipoprotein fractions. Central well, antiserum. (*a*) Albumin. (*b*) α_1- plus β-lipoprotein. (*c*) α_1-Lipoprotein. (*d*) β-Lipoprotein. The plate was stained with azocarmine after standing a week at room temperature.

Immunochemical Analysis of α_1-Lipoprotein.—Preparations 2, 4, and 6 were used in these experiments. Agar electrophoresis showed a broad boundary in the albumin zone. Immunoelectrophoresis elicited two distinct curved precipitation lines which crossed, one corresponding to the stained α_1-lipoprotein boundary and a second one ahead (see Fig. 4 *a*).

When a delipidized α_1-lipoprotein sample was analyzed (Fig. 4 *b*), two boundaries were demonstrated by agar electrophoresis: one migrated slowly from the point of application towards the anode, while the second small component migrated at the same rate as albumin. Addition of antiserum to the plate re-

sulted in two lines of precipitation with the same shape and position as those observed in the original α_1-lipoprotein sample.

Crystalline serum albumin was added (100 μg. N) to the α_1-lipoprotein

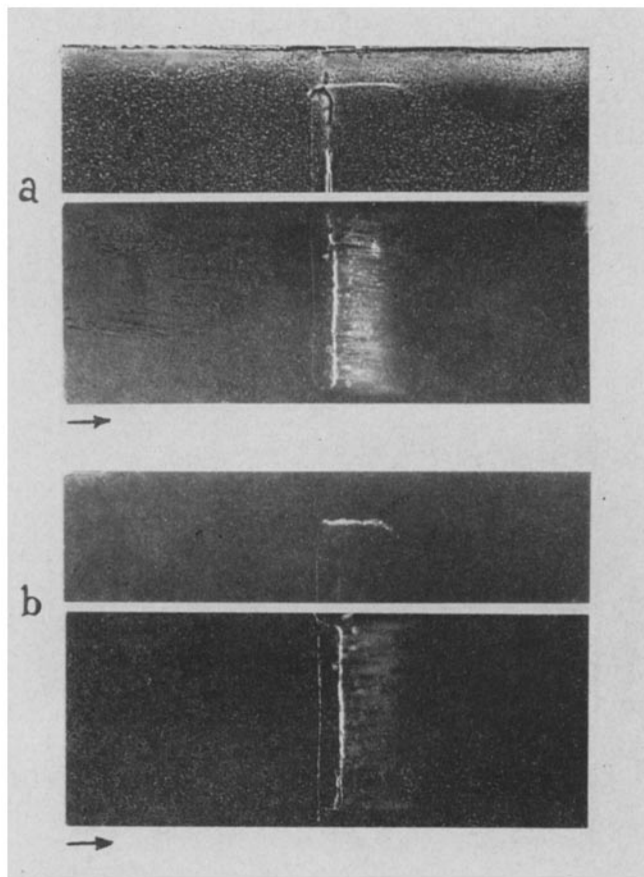

FIG. 3. Agar immunoelectrophoresis of (*a*) β-Lipoprotein sample. (*b*) Delipidized β-lipoprotein.

and its delipidized sample. α_1-Lipoprotein plus albumin gave the same immunoelectrophoretic pattern as seen in Fig. 4 *a*, except that a broader boundary was observed. Delipidized α_1-lipoprotein plus albumin (see Fig. 4 *c*) showed by agar electrophoresis two distinct boundaries in the same position as Fig. 4 *b*. Addition of antiserum demonstrated two lines of precipitation very similar to those observed in Figs. 4 *a* and 4 *b*.

The Ouchterlony technique was applied to samples of albumin, delipidized

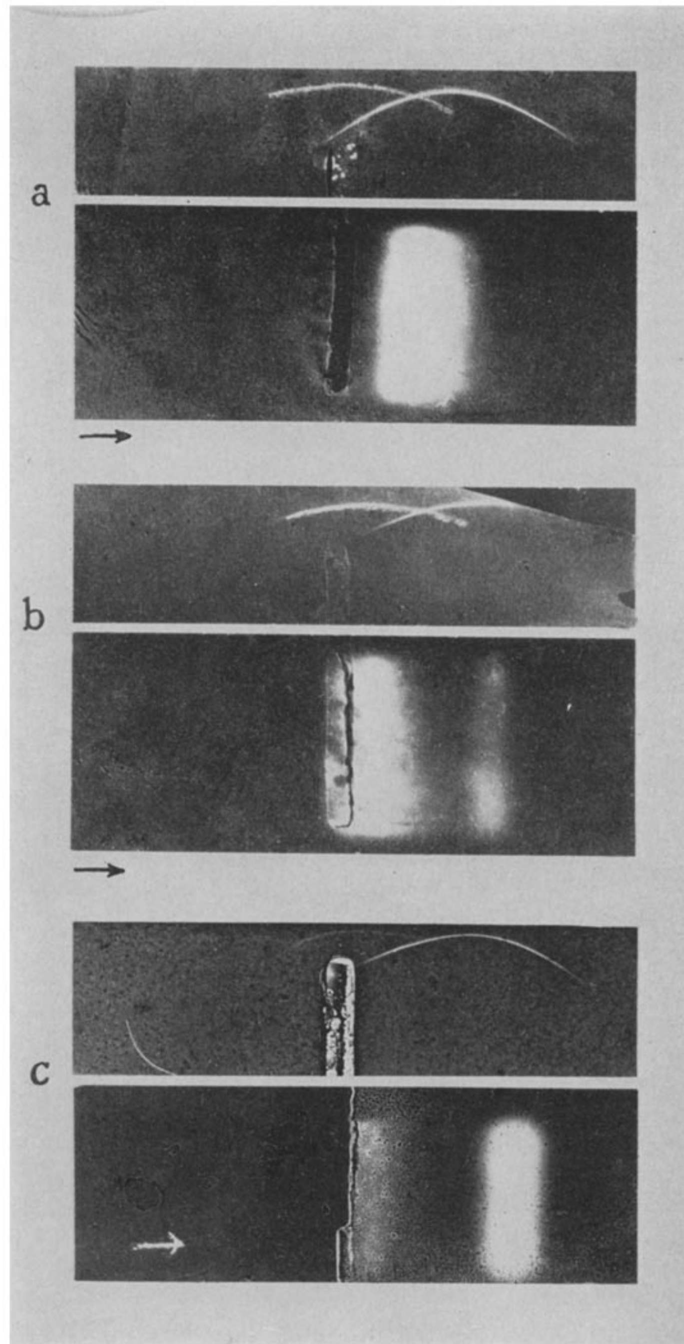

FIG. 4. Agar immunoelectrophoretic analysis of α_1-lipoprotein. Effect of addition of albumin. (*a*) α_1-Lipoprotein. (*b*) Delipidized α_1-lipoprotein. (*c*) Delipidized α_1-lipoprotein plus albumin.

α_1-lipoprotein, and a mixture of albumin–α_1-lipoprotein. The plate was stained with azocarmine after standing 1 week at room temperature (Fig. 5). All the antigens studied showed a single line of precipitation and the same rate of diffusion. In Fig. 5 the line corresponding to the albumin solution appears faint

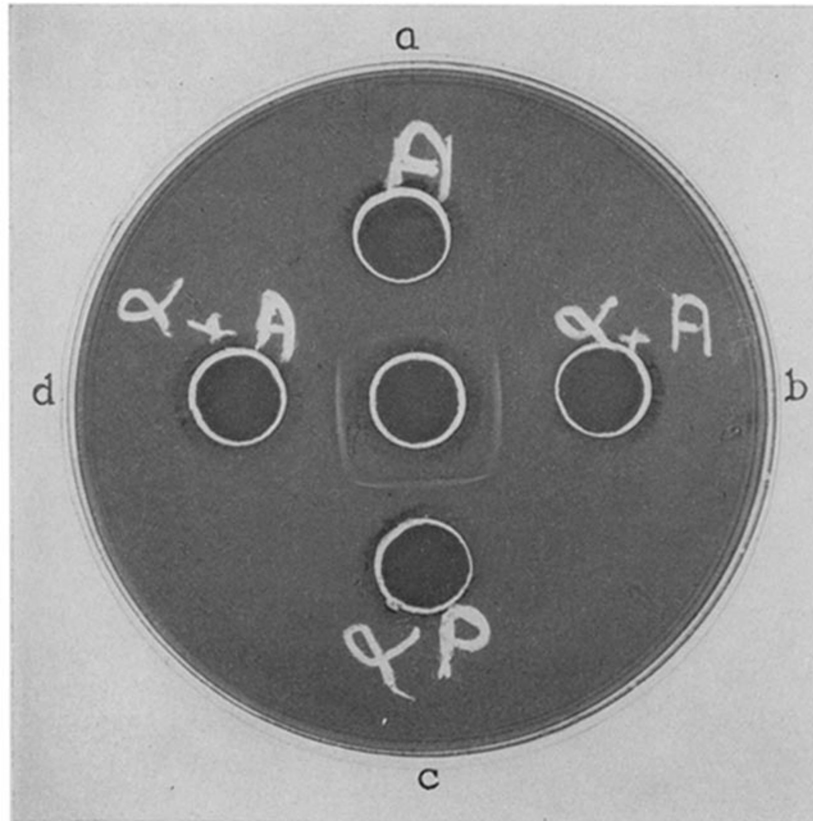

FIG. 5. Ouchterlony techinque applied to samples of albumin, delipidized α_1-lipoprotein, and a mixture of albumin plus α_1-lipoprotein. Central well, antiserum. (*a*) Albumin. (*b*) Mixture of α_1-lipoprotein plus albumin. (*c*) Delipidized α_1-lipoprotein. (*d*) Same as (*b*).

in comparison to the other lines of precipitation: a process of partial solubilization of the precipitate had taken place, related to an antigen excess. This line of precipitation was at its maximum after the first 48 hours of standing, and subsequently diminished.

Immunochemical analyses of α_1-lipoprotein preparation 7 (separated by ultracentrifugation for a total period of 48 hours) showed results which differed from those obtained with other α_1-lipoprotein samples (Fig. 6). After fractiona-

tion by agar electrophoresis a single boundary was detected on staining for protein. Addition of antiserum induced formation of a very prolonged, single line of precipitation, starting from the point of application and extending towards the anode (Fig. 6 *a*). The delipidized sample also showed a single

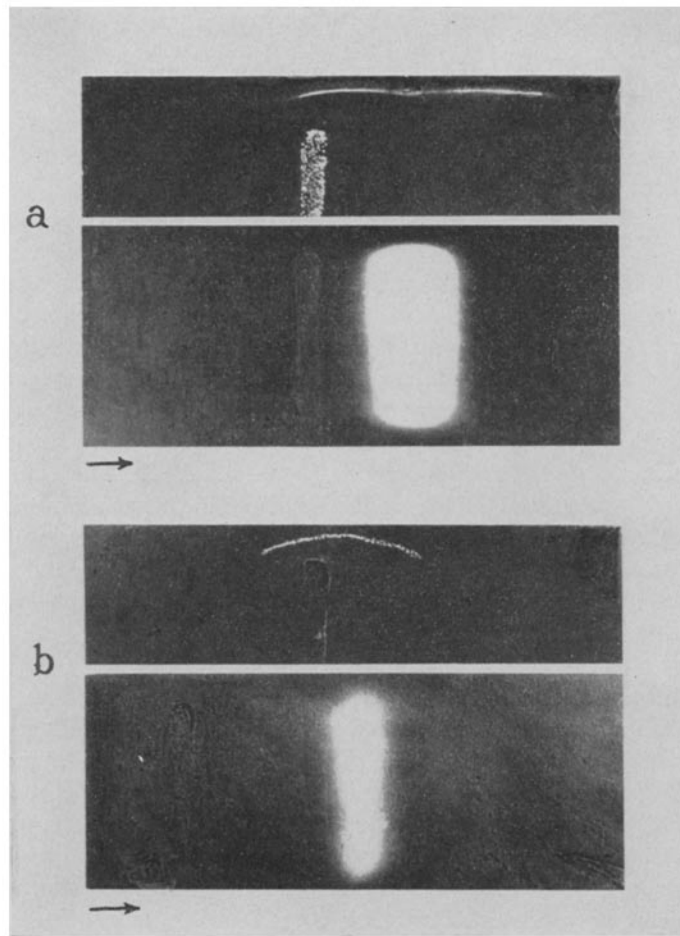

FIG. 6. Agar immunoelectrophoresis of a purified α_1-lipoprotein sample (No. 7). (*a*) α_1-Lipoprotein sample. (*b*) Delipidized α_1-lipoprotein.

boundary by agar electrophoresis; its position, however, was very close to the point of application, showing a decreased mobility in comparison with the non-delipidized α_1-lipoprotein sample (Fig. 6 *b*). Addition of antiserum elicited a single line of precipitation similar in shape to the one demonstrated in the original lipoprotein sample.

Experiments with Absorbed Antisera.—When antiserum absorbed with albumin was used, α_1-lipoprotein samples 4 and 6, (which gave two lines of precipitation against total immune serum), elicited only one line in a position corresponding to the α_1-lipoprotein boundary shown by agar electrophoresis. Identical results were obtained when an antiserum, previously absorbed with lipoprotein-free serum, was added to the same α_1-lipoprotein preparation.

DISCUSSION

The agar electrophoresis method showed that lipoproteins move faster in gel than on paper or by free electrophoresis. When a mixture of α_1- and β-lipoproteins was analyzed, their position was respectively in the area of albumin and α_2-globulin. These data confirm those previously reported by Uriel and Grabar (11). β-Lipoprotein solutions seemed, however, to be quite unstable in the gel medium used. Boyle (13) has recently proposed a method for determination of serum β-lipoprotein levels by using a solution of K-agar in suitable buffer.

By agar immunoelectrophoresis, human serum α_1- and β-lipoproteins were shown to be antigenically different; this was confirmed by the agar double diffusion technique of Ouchterlony. These findings are in agreement with those reported by Aladjem *et al.* (3) and Levine *et al.* (1, 2).

We have also shown that β-lipoprotein (S_f 0–20, *i.e.* $-S$ 25–70) samples are antigenically homogeneous. A single precipitation line, after addition of antiserum, was observed with both techniques of double diffusion in agar and immunoelectrophoresis. The small, water-soluble, phosphorus-containing protein residue after delipidation, was antigenically identical to the original β-lipoprotein sample. Unfortunately, much of the protein residue, because of its insolubility, was not available for study.

The demonstration that serum β-lipoproteins have a single antigenic determinant is in agreement with the data of Levine *et al.* (1) and Korngold and Lipari (6), and contrasts with those of Gitlin (4), Aladjem, Lieberman, and Gofman (3), and Aladjem and Campbell (5). Possible explanations for the antigenic complexity shown by these authors (4, 3, 5) could be either impurities present in the lipoprotein preparations or physicochemical alterations of these lipoproteins resulting from standing in the refrigerator with consequent change in antigenic activity. The use of whole serum as an immunizing agent is safer from this viewpoint, since it is known that serum has a stabilizing effect on lipoproteins.

Antigenic complexity of β-lipoprotein samples was also shown by Aladjem and Campbell (5) by the Ouchterlony technique. However, this was noted only in agar plates after a period of several weeks. Physical changes easily occur in the agar resulting in the formation of false precipitation lines. We have observed this phenomenon in some of our plates after 4 weeks. From four to six lines of precipitation appeared, of which only one was stained either by azocarmine or Sudan black.

Immunochemical analysis of α_1-lipoprotein preparations showed the presence of two different components by means of agar immunoelectrophoresis. The second line found was due to the presence of albumin, occurring as an impurity in the α_1-lipoprotein samples separated by ultracentrifugation for 18 hours. This interpretation was supported by the following findings: (1) Addition of crystalline serum albumin to the α_1-lipoprotein and to its delipidized sample did not change the result of the immunochemical reaction; (2) when an antiserum absorbed either with albumin or lipoprotein-free serum was used for immunoprecipitin reaction, α_1-lipoprotein preparations elicited only one precipitation line corresponding to the α_1-lipoprotein boundary.

When albumin and α_1-lipoprotein were mixed and analyzed by agar electrophoresis, a single, broad boundary was observed. Slight differences in mobility of the two serum fractions may account for the broadening of the boundary and also for the difference in position of the two corresponding precipitation lines.

The albumin boundary, which did not appear when agar electrophoresis was performed on the whole α_1-lipoprotein sample, was shown to be present in the corresponding delipidized preparation. Following delipidation, the mobility of the protein residue of the α_1-lipoprotein sample was reduced; albumin, with a mobility unaffected by this process, appeared, therefore, as a distinct boundary.

When α_1-lipoprotein samples were prepared by ultracentrifugation for a period of 3 days, no trace of albumin was detected immunochemically.

We mentioned that samples of α_1-lipoprotein analyzed by agar electrophoresis showed decreased mobility in comparison with the original sample of lipoprotein. This finding contrasts with the results of our previous study (9), which showed no change in mobility in free electrophoresis. However, the peculiar behavior of the lipoproteins in agar does not allow a direct comparison between the two methods.

Because of its high sensitivity immunoelectrophoresis provides more rigorous criteria of purity for antigenic proteins. As little as 100 to 200 μg. of an antigenic protein can be detected. Neither ultracentrifugation nor free electrophoresis reaches this sensitivity. Indeed, some of our α_1-lipoprotein preparations, which were found to have albumin as an impurity by immunoelectrophoresis, appeared to be homogeneous when analyzed by ultracentrifugal and free electrophoretic methods.

The fact that delipidation did not change the antigenic activity of a lipoprotein molecule, supports the view that the protein moiety carries the antigenicity of the lipoprotein. Kunkel (14) has shown that removal of 90 per cent of lipides from a lipoprotein preparation does not affect its antigenicity. More recently, DeLalla, Levine, and Brown (2) have shown that high density lipoproteins extracted with a mixture of hot alcohol and ether yield a partially denatured protein; and a water-soluble residue with unchanged antigenic activity.

We have already mentioned that a mixture of albumin and α_1-lipoprotein produced only a single boundary on agar electrophoresis. To this boundary Uriel and Grabar (11) gave the name of lipoalbumin in the belief that albumin might be the protein moiety of this lipoprotein molecule. Our data, however, rule out this possibility. "Lipoalbumin" was found to be a mixture of albumin and α_1-lipoprotein, each one of which was antigenically distinct. This result supports the hypothesis that albumin and the protein moiety of α_1-lipoprotein are distinct proteins.

SUMMARY

The techniques of agar immunoelectrophoresis and agar double diffusion were applied to the study of the antigenicity of β- and α_1-lipoproteins separated by ultracentrifugation from normal human sera. The effects of delipidation were also investigated.

It was shown that β- and α_1-lipoproteins are antigenically distinct. For each class of lipoprotein studied, a single antigenic component was demonstrated. In some, but not all, preparations of α_1-lipoprotein a second, small antigenic component was detected, and identified as albumin. Absorption with lipoprotein-free serum or albumin removed this component without changing the lipoprotein band.

Delipidation did not affect the antigenicity of either β- or α_1-lipoproteins.

Immunoelectrophoresis, because of its high sensitivity and specificity, provides an additional criterion of purity for antigenic proteins in addition to the data that can be obtained from ultracentrifugal and free electrophoretic analysis.

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