STUDY OF THE ADSORPTION ON AND DESORPTION FROM POLYSTYRENE–HUMAN SERUM ALBUMIN CONJUGATES OF RABBIT ANTI-HUMAN SERUM ALBUMIN ANTIBODIES HAVING DIFFERENT SPECIFICITIES

By TOM WEBB, Ph.D., and CLAUDE LAPRESLE, M.D.

(From the Service de Chimie microbienne, Institut Pasteur, Paris)

Received for publication, March 13, 1961)

The existence in the serum of rabbits immunized with human serum albumin (H.S.A.) of anti-H.S.A. antibodies having different specificities was demonstrated by reacting such sera with enzymatically degraded H.S.A. (1).

In the present work, we have studied the adsorption and desorption of these antibodies on insoluble polystyrene-H.S.A. conjugates prepared as described by Gyenes and Sehon (2).

Materials

Polyamino-polystyrene obtained from the Norsk Hydro-electrisk, Oslo, Norway, through the courtesy of Dr. A. Sehon.

Human Serum Albumin.—Squibb fraction V prepared by ethanol fractionation and kindly given by the American Red Cross.

Degraded Human Serum Albumin.—1.5 gm H.S.A. was degraded for 6 hours at pH 3.5 and 37°C by 750 proteolytic units of the rabbit spleen protease prepared as described previously (3). Under these conditions 30 per cent of the degraded albumin is no longer precipitable by 2 per cent T.C.A. The reaction was stopped by neutralizing the solution and the degraded albumin was stored at −15°C.

Inhibitor.—One of the products of the degradation of H.S.A. was purified as described previously (4).

Rabbit Anti-H.S.A. Sera.—The sera were collected after two series of injections of H.S.A. adsorbed on alum as described previously (4).

Goat Serum Anti-rabbit Serum.—Prepared by repeated intravenous injections of rabbit serum.

Aquax.—Obtained from George T. Gurr, Ltd., London.

Methods

Preparation of Polystyrene-H.S.A. Conjugates.—

To 8 gm of polyamino-polystyrene suspended in 160 ml of 1 N HCl, 6 gm of sodium nitrite were added slowly, with stirring at 0°C. The mixture was stirred for 30 minutes and then washed successively with 500 ml of 5 per cent solution of sodium acetate, 500 ml of a 5 per cent solution of urea, 1 litre of distilled water, 500 ml of 0.15 M NaCl and finally suspended in 200 ml of 0.15 M NaCl.

This work was supported in part by grant E-3225 from the National Institutes of Health, Bethesda.

To the diazotized polystyrene 2 gm of H.S.A. was added and the mixture was stirred for 18 hours at 0°C and then washed with 500 ml of 0.15 M NaCl. The quantity of albumin fixed on the polystyrene was estimated by the difference between the amount of albumin added and the amount recovered in the washing. This quantity was found to be about 200 mg. Unreacted diazo groups were blocked by addition of glycine.

Adsorption of Rabbit Anti-H.S.A. Antibodies on the Polystyrene H.S.A.-Conjugates.—A bed of cellulose was prepared at the restricted end of a glass column and the desired amount of the polystyrene–H.S.A. conjugate was packed into the column under 1 atmosphere of pressure.

The rabbit antiserum was passed through the column at room temperature and under 1

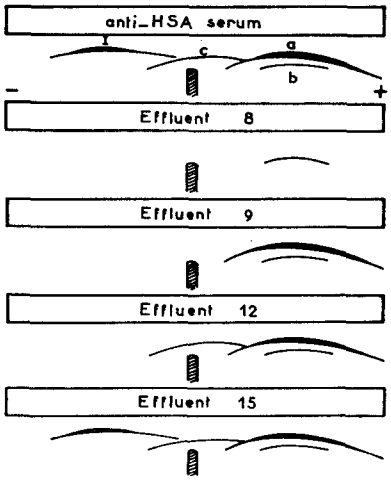

FIG. 1. Immunoelectrophoretic pattern of degraded H.S.A. revealed by rabbit anti-H.S.A. serum and several effluents from the H.S.A.-polystyrene column. *a*, *b*, *c*, and I designate different precipitin lines formed by the degraded H.S.A. with the anti-H.S.A. serum.

atmosphere of pressure. About 0.4 ml serum passed per minute. The effluents were collected and tested against undegraded and degraded H.S.A. The column was washed with 1 litre of 0.15 M NaCl which is the amount required in order that the effluents had an optical density of less than 0.025 at 280 mμ.

Desorption of Rabbit Anti-H.S.A. Antibodies from the Polystyrene-H.S.A. Conjugates.—The desorption of the antibodies from the polystyrene–H.S.A. conjugate was achieved by passing solutions of acidic pH through the column at 4°C. Two different acidic solutions were employed for the desorption of the antibodies: acetate–HCl buffer pH 3.0, 0.5 M and 0.1 N HCl solution in 0.15 M NaCl.

The eluates were collected in 5 ml fractions in tubes kept at 0°C. Their optical density was measured at 280 mμ and the pH was measured with a glass calomel electrode pH metre. The eluates were then neutralized with NaOH and stored at −15°C.

Protein Determination.—The amount of protein in the different antigen and antibody preparations was measured with the biuret reagent by reference to a standard curve made with H.S.A.

Quantitative Determination of Rabbit Anti-H.S.A. Antibodies.—Quantitative specific precipitation was done using the method of Heidelberger and Kendall (5). The quantity of

precipitate was measured with the biuret reagent as described by McDuffie and Kabat (6). The quantity of antibody precipitable was calculated from the maximum of the curve by subtracting the amount of albumin added to the antiserum at this point.

Immunoelectrophoresis.—Immunoelectrophoresis was carried out according to the method of Grabar and Williams (7) under the following conditions: agar gel at 1 per cent; sodium

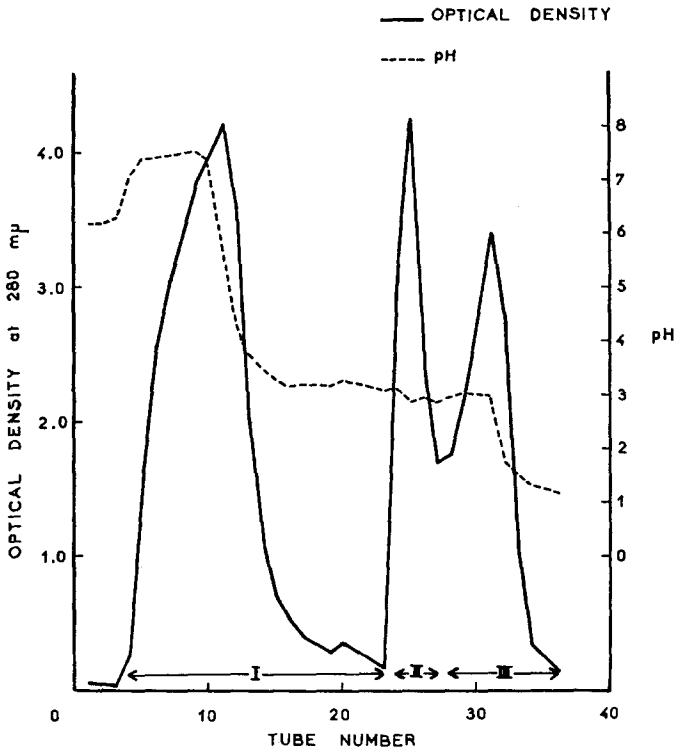

FIG. 2. Elution of rabbit anti-H.S.A. antibodies from the H.S.A. polystyrene column. The curves represent the optical density and the pH of the 5 ml eluates. Acetate buffer pH 3 was added at tube 1. 0.1 N HCl in 0.15 M NaCl was added at tube 19. Fraction I: tubes 4 to 23; Fraction II: tubes 24 to 27, fraction III: tubes 28 to 36.

veronal buffer at pH 8.2 and ionic strength 0.05 in the reservoirs and 0.025 in the gelose. V/cm: 5; duration: 4 hours.

RESULTS

Adsorption of rabbit Anti-H.S.A. Antibodies on Polystyrene-H.S.A. Conjugates.—When the serum (containing 5.05 mg/ml antibodies precipitating with H.S.A.) was passed through a column of 8 gm of polystyrene-H.S.A. conjugate, the first 52.5 ml of effluent serum (fractions 1 to 7) contain no antibodies precipitating with H.S.A. The following effluent fractions (Nos. 8 to 16) contained antibody precipitating with H.S.A. These fractions of 7.5

ml were concentrated 2.5 times by dialysis against a concentrated solution of aquax, and used to reveal the immunoelectrophoretic pattern of degraded H.S.A. Fig. 1 shows a schematic representation of the results obtained in this experiment. The original serum contains antibodies giving the 3 precipitin

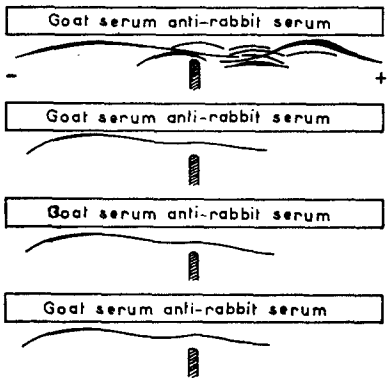

FIG. 3. From top to bottom. Immunoelectrophoretic patterns of: rabbit anti-H.S.A. serum; Fraction I; Fraction II; Fraction III.

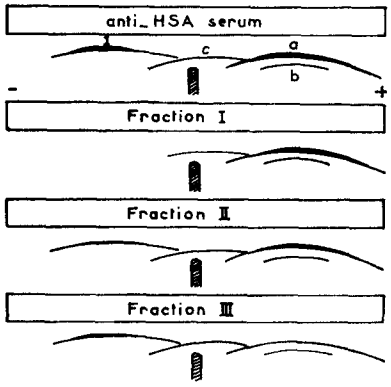

FIG. 4. Immunoelectrophoretic pattern of degraded H.S.A. revealed by rabbit anti-H.S.A. serum and the three fractions of purified anti-H.S.A. antibody

lines *a*, *b*, *c* with the degraded albumin as described previously (1). In addition, this serum contained antibodies precipitating with the fragment of albumin described recently (4), and designated as "inhibitor." The first effluent serum (fraction 8) precipitating with H.S.A. gives only the line *b* with degraded H.S.A.; effluent 9 gives both lines *a* and *b*; effluent 12 gives lines *a*, *b*, and *c*; effluent 15 lines *a*, *b*, *c*, and the precipitin line I with the inhibitor.

Desorption of Rabbit Anti-H.S.A. Antibodies from Polystyrene-H.S.A. Conjugates.—

Fig. 2 shows the optical densities of the eluates obtained when the antibodies are desorbed from the polystyrene-H.S.A. conjugate. The acetate buffer at pH 3.0 elutes as a broad peak about half of the protein recovered. Two additional peaks are eluted by the 0.1 N HCl solution. The eluates of each of the 3 peaks were grouped as shown in the figure forming fraction Nos. I, II, and III and dialyzed against 0.15 M NaCl solution.

A portion of each of these fractions was concentrated by dialysis against aquax so that the final concentration of precipitating anti-H.S.A. antibodies was approximately the same as that in the original serum.

Fig. 3 shows the immunoelectrophoretic pattern given by each of these concentrated fractions revealed by a goat anti-rabbit serum. Each eluate gives only one line which is identical with the line given by the γ-globulins of the original rabbit serum.

TABLE I

Composition of the Three Fractions of Purified Antibodies

Fraction	I	II	III
Total protein *mg*	139	53	60
Total antibody precipitating with H.S.A. *mg*	115	40	39
Total antibody precipitating with inhibitor *mg*	0.35	2.83	11.35

Fig. 4 shows the immunoelectrophoretic pattern of degraded H.S.A. when it is revealed by the original rabbit anti-H.S.A. serum and by each of these concentrated fractions. These fractions give the same lines as the original antiserum but the density of the lines are not the same and varies from one fraction to the other. The line given by the "inhibitor" is more dense from fraction I to III and inversely, the line *a* given by fraction III is much weaker than the line *a* given by fractions I and II.

For each fraction, the protein content, the quantity of antibody precipitable by H.S.A. and the quantity of antibody precipitable by the "inhibitor" was measured and the results are shown in the table. This table shows two things: (*a*) the amount of antibody precipitable by H.S.A. is less than the amount of protein contained in each fraction and the proportion of these precipitating antibodies diminishes from fractions I to III. (*b*) Only traces of antibody precipitating with the "inhibitor" are present in fraction I. These antibodies were eluted by 0.1 N HCl and are contained principally in fraction III.

DISCUSSION

The conjugation of antigens to an insoluble matrix by diazo linkages has already been used for the isolation of purified antibodies (8). Both cellulose (9, 10) and polystyrene (11) have been found adequate as the insoluble matrix.

Gourvitch *et al.* (10) reported that as columns of horse serum–albumin–cellulose conjugates approached saturation by the corresponding rabbit antibodies, the antibody content rises in successive fractions of the effluent indicating a gradual saturation of the specific sorbent. In our experiments such effluents have been examined for their content of rabbit anti-H.S.A. antibodies of different specificities. Using these effluents to reveal the immunoelectrophoretic pattern of degraded HSA it has been demonstrated that the first effluents precipitating with H.S.A. contained only some of the different anti-H.S.A. antibodies present in the original antiserum. As the column approaches saturation, the other antibodies of different specificities appeared successively until the effluents gave (with degraded H.S.A.) the same immunoelectrophoretic pattern as the original rabbit anti-H.S.A. serum. This result shows that the saturation of the different determinant groups of the albumin molecules is obtained by the passage through the column of different amounts of the anti-H.S.A. serum. The amount of serum required for the saturation of a given determinant group depends most probably on the ratio: concentration of corresponding antibody in the immune serum/the number of the determinant group available on the sorbent. Our results show that this ratio is different for the different determinant groups of the H.S.A. molecule.

In the experiments of Gourvitch *et al.* (10) rabbit anti-horse serum albumin antibodies were eluted from horse serum albumin–cellulose conjugates by solutions of 1 per cent NaCl of increasing acidity. They found that the bulk of the antibodies were eluted between pH 3.8 and 3.6 with additional amounts at pH 3.4 and 3.2. After elution at pH 3.2 a solution of 5 per cent NaCl 0.1 N HCl caused only a small additional release of protein. They also found that different eluates did not give identically shaped precipitin curves with horse serum albumin and concluded that this observation was due to some kind of antibody heterogeneity.

In our experiments it was found that after desorption of the anti-H.S.A. antibodies by an acetate buffer (0.5 M) at pH 3.0 an almost equal amount of antibody was desorbed by 0.1 N HCl in 0.15 M NaCl. The apparent discrepancy between our results and those of Gourvitch *et al.* (10) may be due to the fact that different antigens were used or to the different experimental conditions employed.

By using two solutions of different pH for the elution of the rabbit anti-H.S.A. antibodies these antibodies were fractionated into 3 different fractions. Each of these fractions contained only γ-globulins as shown by immunoelectrophoretic analysis and each contained γ-globulins of the same wide range of mobilities as the γ-globulins of the original serum.

By testing these three fractions with degraded H.S.A. and with the fragment of H.S.A. called the "inhibitor", we have been able to demonstrate that antibodies having different specificities require different pH for maximal elution.

This suggests that the pH required for the elution of antibodies depends at least partially upon their specificity.

There is in each fraction some protein which is not precipitated by H.S.A. at the equivalence point. This protein is most probably anti-H.S.A. antibody since it is γ-globulin which has been fixed on the H.S.A.-polystyrene conjugate. The non-precipitation of these antibodies can be explained by assuming that the H.S.A. behaves as a monovalent antigen *vis à vis* these isolated antibodies as Raynaud and Relyveld (12) have suggested to explain the non-precipitation of neutralizing antitoxins.

SUMMARY

An insoluble specific adsorbent for anti-human serum albumin antibodies was prepared by coupling human serum albumin (H.S.A.) to polystyrene by azo bonds. Rabbit anti-H.S.A. immune serum was passed through a column of the adsorbent. It was shown that different volumes of the immune serum were required for the saturation of the different determinant groups of H.S.A. by their corresponding antibodies.

The elution of the anti-H.S.A. antibodies adsorbed on the column was achieved by passing successively through the column an acetate buffer pH 3.0 and a solution of 0.1 N HCl in 0.15 M NaCl. The antibodies were eluted in three different fractions, each of which was composed of γ-globulins only. These three fractions contained different proportions of antibodies of different specificities.

We wish to thank Mrs. J. Cavillon and Miss N. Motel for their technical assistance.

BIBLIOGRAPHY

1. Lapresle, C., Etude de la dégradation de la sérumalbumine humaine par un extrait de rate de lapin. II. Mise en évidence de trois groupements spécifiques différents dans le motif antigénique de l'albumine humaine et de trois anticorps correspondants dans le sérum de lapin anti-albumine humaine, *Ann. Inst. Pasteur*, 1955, **89**, 654.
2. Gyenes, L., and Sehon, A. H., Preparation and evaluation of polystyrene antigen conjugates for the isolation of antibodies, *Canad. J. Biochem. Physiol.*, 1960, **38**, 1235.
3. Lapresle, C., and Webb, T., Study of a proteolytic enzyme from rabbit spleen, *Biochem. J.*, 1960, **76**, 538.
4. Lapresle, C., and Webb, T., Etude de la dégradation de la sérumalbumine humaine par un extrait de rate de lapin. VII. Isolement et propriétés d'un fragment d'albumine humaine, *Ann. Inst. Pasteur*, 1960, **99**, 523.
5. Heidelberger, M., and Kendall, F. E., A quantitative study of the precipitin reaction between Type III pneumococcus polysaccharide and purified homologous antibody, *J. Exp. Med.*, 1929, **50**, 809.
6. McDuffie, F. C. and Kabat, E. A., A comparative study of methods used for

analysis of specific precipitates in quantitative Immunochemistry, *J. Immunol.* 1956, **77,** 193.

7. Grabar, P., and Williams, C. A., Jr. Méthode immuno-électrophorétique d'analyse de mélanges de substances antigéniques, *Biochem. et Biophysica Acta,* 1955, **17,** 67.
8. Isliker, H. C., Chemical nature of antibodies, *Advances Protein Chem.*, 1957, **12,** 388.
9. Campbell, D. H., Luescher, E., and Lerman, L. S., Immunologic adsorbents (I) Isolation of antibody by a cellulose-protein antigen, *Proc. Nat. Acad. Sc.*, 1951, **37,** 575.
10. Gourvitch, A. E., Kapner, R. B., and Nezlin, R. S., Isolation of antibodies by means of cellulose fixed antigens and a study of their properties, *Biokhimyia*, 1959, **24,** 144.
11. Gyenes, L., Rose, B., and Sehon, A. H., Isolation of antibodies on antigen-polystyrene conjugates, *Nature*, 1958, **181,** 1465.
12. Raynaud, M., and Relyveld, E. La réaction toxine-antitoxine diphtérique, *Ann. Inst. Pasteur*, 1959, **97,** 636.