SEROLOGICAL REACTIONS ON CHROMATOGRAPHIC COLUMNS

I. ANTIGEN–ANTIBODY REACTIONS

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In recent years great advances have been made in protein chromatography. The improvement of the properties of hydroxylapatite ($Ca_5(PO_4)_3OH$) which permits its successful application to the isolation and fractionation of various proteins has been reported by TISELIUS *et al.*[1] and by HJERTÉN[2]. The feasibility of chromatographic procedures employing the cellulosic ion-exchangers was reported by SOBER *et al.*[3,4], who utilized in particular the anionic exchanger diethylaminoethyl-cellulose for the fractionation of serum proteins; the cationic exchanger sulphomethyl-cellulose was introduced by PORATH[5] for the same purpose. The main object of the present communication is to describe some experiments in which the use of these new column materials for the study of antigen–antibody reaction was explored.

Mention may be made of the fact that the purification of antibodies has been accomplished by several workers by coupling antigen chemically to an insoluble carrier, allowing antibody to react with the antigen and then dissociating it from the antigen–antibody complex. This has been demonstrated by LANDSTEINER AND VAN DER SCHEER[6] who coupled diazotized stroma with haptens for the adsorption of homologous and heterologous antibodies. CAMPBELL *et al.*[7] have observed that the antigenic protein though coupled by diazo reaction to an insoluble carrier, such as *p*-aminobenzyl-cellulose, still possesses the capacity for combining with its antibody. LERMAN[8] has described the purification of antibodies on adsorbent specific for antibodies homologous to the simple hapten, *p*-azobenzenearsonate, and has achieved the separation of two distinctly different fractions of antibody by using a gradient elution with a solution of specific hapten Na arsanilate. Purification of antibodies was achieved by MANECKE AND GILLERT[9], who diazotized and coupled polyaminostyrene resin to serum proteins, and also by ISLIKER[10,11] who combined human serum albumin, and PR 8 strain of influenza A virus and group specific polysaccharide to synthetic resin. WILLIAMS AND STONE[12] used bovine serum albumin-coated cellulose for the purification of antibody and were able to remove a subtilisin-like enzyme from the carboxypeptidase preparation by antisubtilisin sera adsorbed on a subtilisin-coated cellulose column.

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ISLIKER[11] has discussed the chemical nature of antibodies in a review. In the present study, use was made of these observations for the study of antigen–antibody reaction on solid supporting media without any resort to chemical coupling.

EXPERIMENTAL

The chromoprotein R-phycoerythrin used in the present study was prepared from the red algae *Ceramium rubrum* according to TISELIUS *et al.*[1] and separated from the R-phycocyanin present in the crude extract by chromatography, using hydroxylapatite as adsorbent. The peak emerging at 0.03 *M* Na phosphate buffer, pH 6.8, was used for the present study. The eluate was concentrated by ultrafiltration in the cold, and the concentrated solution was employed for the immunization of rabbits. The concentration of proteins in the eluate was estimated by micro-kjeldahl analyses, and then made up to 1.0 g protein/100 ml.

During the immunization procedure, the method as developed by FREUND *et al.*[13,14] was followed. The adjuvant mixture was prepared by mixing thoroughly two volumes of stock PE* solution, one volume of sterilized and melted pure lanolin, and two volumes of paraffin oil of low viscosity to a viscous emulsion. Merthiolate in a proportion of 1:10,000 was added to the emulsion. The adjuvant mixture was kept in small vials at —4° until used.

Male rabbits, weighing approximately 4–5 kg each, were used for the immunization. 1.0 ml of the adjuvant mixture was injected subcutaneously in the back region twice a week for six to eight weeks. The rabbits were bled periodically from the marginal ear vein and the immune serum thus collected was tested for its potency. When the titer value of the immune serum had attained a potency sufficient for the present experimental purpose, the rabbits were bled by cardiac puncture, the sera were collected and stored at —4° in small vials with merthiolate as a preservative until used. The antibody content of pooled immune sera was estimated as described by KABAT AND MAYER[15] and the nitrogen content of the precipitate was determined by the method of LOWRY *et al.*[16]. Antisera solutions were dialysed against isotonic saline prior to use.

The amount of PE that emerged from the hydroxylapatite and SM-cellulose columns was determined by measuring the optical density of the eluate at 566 mμ[17] in a Beckman spectrophotometer model B, using a 0.5 cm cell. When a DEAE-cellulose column was used, in which case under our experimental conditions PE was strongly adsorbed by the adsorbent and γ-globulin migrated down very fast without being retained on the column, the protein concentration in the eluate was estimated by measuring the optical density at 280 mμ in a Beckman Quartz DU spectrophotometer. Simultaneously, control experiments were conducted with identical amounts of antisera alone on columns of the same size under conditions identical with those of the test. Ultracentrifugal and paper electrophoretic analyses were resorted to in order to

* Abbreviations used: PE = R-phycoerythrin; ASPE = antisera against R-phycoerythrin; SM-cellulose = sulphomethyl-cellulose; DEAE-cellulose = diethylaminoethyl-cellulose.

identify the different protein components which emerged from the column, and this helped in the selection of a suitable buffer concentration for the adsorption or desorption of γ-globulin as desired.

RESULTS

Hydroxylapatite and SM-cellulose columns

In the present study, the feasibility of observing the antigen–antibody reaction on the hydroxylapatite columns was first investigated by applying frontal analysis in the chromatography of the mixtures. PE solution was observed to emerge from the column at a low salt concentration[1], whereas γ-globulin required a very high buffer salt concentration for its elution, *e.g.* 0.2 *M* phosphate buffer and higher[1,2]. The columns (3.0–4.0 × 1.0 cm) were well packed and washed overnight with 0.03 *M* Na phosphate buffer, pH 6.8. At this particular buffer strength (0.03 *M* Na phosphate buffer, pH 6.8), PE was completely eluted within the second hold-up volume of the hydroxylapatite column used, whereas γ-globulin stayed on the column; this was confirmed by paper electrophoresis and ultracentrifugation of the eluate fractions. The PE solution was prepared by diluting the stock solution with 0.03 *M* Na phosphate buffer, and by dialysing overnight in the cold against the same buffer. Prior to application on the chromatographic column, ASPE solution (0.5 ml) was dialysed against isotonic saline overnight in the cold. When the antisera solution was found to be adsorbed on the column, three portions of 0.5 ml buffer were added to bring down the antisera adhering to the sides of the column. A dilute solution of PE was then allowed to pass through the column. The eluate was collected in 0.5 ml fractions, and the optical density at 566 mμ was then measured. Control experiments were run simultaneously on a similar column with normal rabbit sera of identical volume and protein concentration. It was observed during the elution procedure that in the case of the control experiments, PE appeared in the eluate fraction as soon as a volume of eluate equal to the hold-up volume of the hydroxylapatite column had passed out, whereas in the case of experiments where ASPE was applied prior to the addition of PE to the column, both the appearance of PE in the eluate fractions and its attainment of the original concentration were retarded. One of the typical experiments is represented in Fig. 1, where the arrow on the PE–ASPE curve indicates the break-through volume of PE. The results indicate that though the immune γ-globulin is adsorbed on the hydroxylapatite column, it still possesses the capacity for reacting with its antigen bound to its surface under the present experimental conditions.

In the next set of experiments an attempt was made to apply the elution technique as a means of determining the combining capacity of antibody adsorbed on hydroxylapatite. Antiserum (0.5 ml) was adsorbed on the column, the top of the column was washed with three portions of 1.0 ml each of the developing buffer, and then the equivalent amount of PE (0.5 ml), as determined by the precipitin reaction in free solution, was added to the column. When using hydroxylapatite columns, generally of dimensions 7.0 × 1.0 cm, 0.03 *M* Na phosphate buffer, pH 6.8, was used for the elution of PE unattached to the antibody adsorbed on the column. The rate of flow

of the developing buffer was maintained at a low value of 1.0-2.0 ml/h. Though ASPE and its equivalent amount of PE as determined by the precipitin reaction in solution were used, it was observed that under the experimental conditions described here, the antigen retention capacity of the antibody adsorbed on the column decreased con-

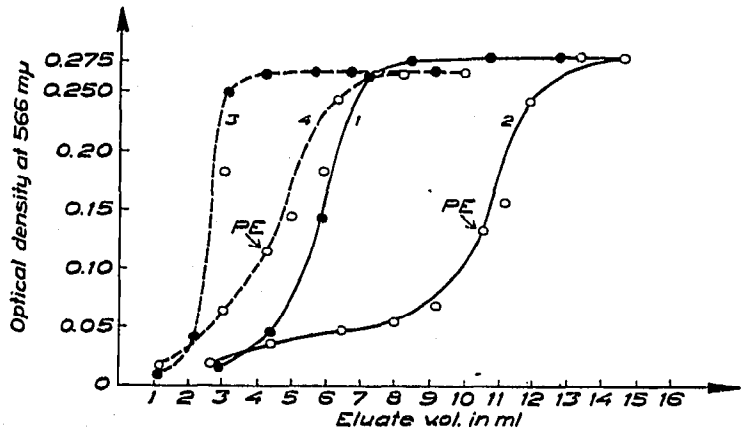

Fig. 1. Frontal analysis of the mixtures of PE and ASPE on hydroxylapatite and SM-cellulose columns. The arrow indicates the break-through volume of PE. Curves 1 and 2 were obtained with hydroxylapatite, curves 3 and 4 with SM-cellulose. Curves 2 and 4 show the behaviour of PE in presence of ASPE, and curves 1 and 3 show the behaviour of PE alone.

siderably in comparison with that evaluated by the quantitative precipitin method. The PE and ASPE solutions were both dialysed against 0.03 *M* Na phosphate buffer, concentrated to an amount comparable to that used in isotonic saline media, and the precipitin reaction was conducted in 0.03 *M* phosphate buffer. When the amounts of PE and ASPE used were the same as those applied on the chromatographic column,

TABLE I

EFFECT OF THE SUPPORTING MEDIA ON THE ANTIGEN-RETENTION CAPACITY OF ANTIBODY ADSORBED ON HYDROXYLAPATITE AND SM-CELLULOSE COLUMNS

Column operation was conducted at 20°, with a rate of flow maintained at 2 to 3 ml/h. Approximately 1-ml fractions were collected, volumes were measured, and from the optical density at 566 mμ the amount of PE recovered was calculated.

Quantity of antibody in ml of antisera	*Supporting media*	*Bound PE (mg protein N)*	*% Capacity**
0.5 ml ASPE	Isotonic saline	0.267	100.0
0.5 ml ASPE	Hydroxylapatite	0.121	45.4
0.5 ml ASPE	SM-cellulose	0.089	33.4

* The capacity of the antibody (ASPE) for retaining the antigen (PE), the antibody being fixed to the adsorbent.

the results obtained were, within the experimental error, identical with those reported in Table I. Similar experiments were also carried out with ASPE and PE on SM-cellulose columns (5.5–6.0 × 0.9 cm). Na phosphate buffer, pH 6.1, and ionic strength

0.005, was chosen for the column procedure, as it effects the complete desorption of PE from the column without affecting the γ-globulin which stays on the column. The results obtained with SM-cellulose are in accord with the observations made with hydroxylapatite columns. Table I shows that the decrease in the antigen-retention capacity of antibody varies and that this capacity is dependent on the adsorbent used. On comparing the results obtained with hydroxylapatite and SM-cellulose, it appears that the antibody when adsorbed on SM-cellulose loses more of those determinant groups that are responsible for precipitin reaction than when adsorbed on hydroxylapatite columns.

Chromatography of protein mixtures on hydroxylapatite is dependent on the salt concentration of the developing buffer[1]. Attempts were made to study the effect of varying the salt concentration on the ASPE–PE system by increasing the salt concentration stepwise or by using a higher initial concentration, the pH of the developing buffer remaining constant at pH 6.8. The test system with PE–ASPE was so chosen that the antigen–antibody ratio would be 5:1. The system with excess antigen would possibly enable the antibody molecule to come in contact with more antigen than it could possibly do at the equivalence point. It has been shown earlier that ASPE stays on hydroxylapatite column under the experimental condition of low salt concentration. It is to be expected that PE bound to ASPE would remain on the column till it is dissociated by the increasing salt concentration or a large volume of the developing buffer. Using a definite volume of the developing buffer in each increasing salt concentration step and a definite time schedule for the whole reaction, the antigen–antibody dissociation may be studied with the stepwise elution technique. ASPE was added to PE (1.0 ml) in accordance with the ratio of antigen–antibody desired, incubated at 37° for half an hour, and the mixture was then transferred to the column. The ionic strength of the mixture was maintained at the same value as that of the initial developing buffer. To avoid the complication that may arise from the presence of albumin, γ-globulin was precipitated at 1/3 saturated ammonium sulphate, pH 7.4. The precipitate was washed with 1/3 saturated ammonium sulphate, dissolved in isotonic saline, and dialysed against isotonic saline until free of ammonium sulphate.

Table III provides a comparison of the amount of PE eluted with increasing salt concentration when used alone, and with ASPE included in the system, the results in parentheses indicating the amount obtained from the PE–ASPE system. In all the cases so far studied, approximately 83–86% of the total amount of PE applied on the column was recovered. With these concentration steps, no overlapping of the peaks was observed. Furthermore, PE emerged from the column with the developing front at each step. It is apparent that retardation of PE on the column is caused by ASPE, since in absence of ASPE most of the PE emerged at 0.02 *M* Na phosphate buffer, pH 6.8. It seems that the amount of PE held by ASPE on the column in excess of that calculated from the equivalence point of the quantitative precipitin test, is in some sort of loose combination with ASPE. That this loose combination is dissociable at increasing salt concentration indicates that some relatively weak bonds are also involved in addition to some stronger linkages.

DEAE-cellulose column

In the previous section, cases have been described where γ-globulin remains stationary while PE emerges from the chromatographic column. The opposite behaviour occurs with columns of DEAE-cellulose where γ-globulin desorbs completely and PE remains immobile on the column under the experimental conditions used.

PE was found to be strongly adsorbed on a DEAE-cellulose column and could only be eluted at a buffer concentration of 0.2 *M* and beyond. The DEAE-cellulose column was prepared as described earlier. Na phosphate buffer, pH 7.0, of the following concentrations was used stepwise, *viz.* 0.005, 0.01. 0.05, 0.1, 0.2 and 0.4 *M*. A portion of the total PE applied was found to start migrating down the column after the addition of 0.2 *M* buffer. One of the typical experiments is shown in Fig. 2. The recovery of PE varied from 55 to 60%. It was of interest to investigate the feasibility

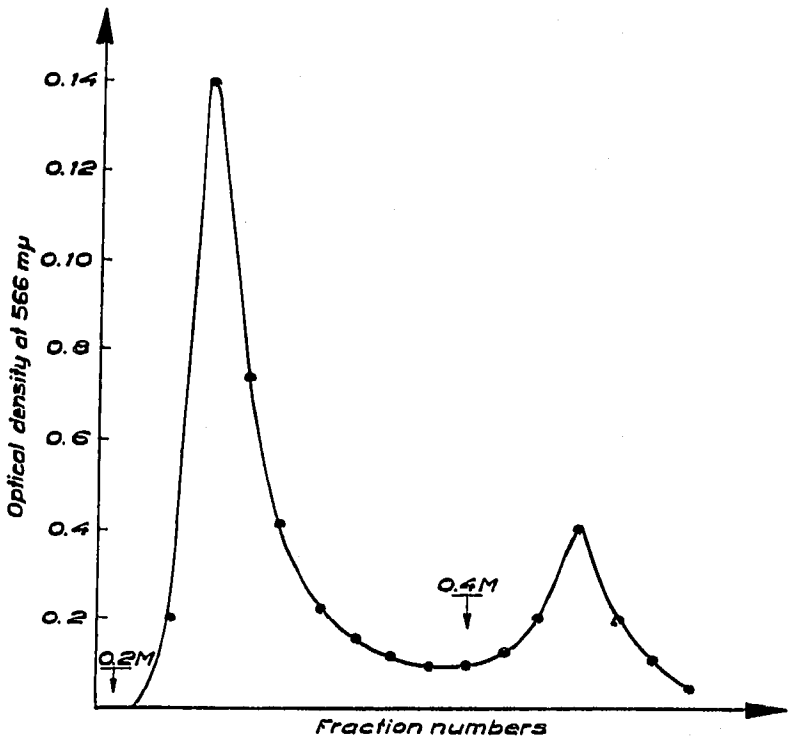

Fig. 2. Stepwise elution of PE from DEAE-cellulose column with Na phosphate buffer, pH 7.0.

of studying the antigen–antibody reaction on DEAE-cellulose columns and also to find out whether or not antigenic groups of PE were made unavailable to the immune γ-globulin as a result of its adsorption on the supporting media.

The procedure adopted in this set of experiments was almost the same as that described earlier, the exception being in the order of application of the solutions. First PE solution (0.5 ml) equivalent to 0.5 ml ASPE was added to the column and when it had been adsorbed, ASPE (0.5 ml) was applied to the column. 0.5 ml fractions were collected, the content of each tube was diluted to 4.0 ml with isotonic saline and the protein content was determined by measuring the optical density at 280 mμ. Control

experiments were run on similar columns of the same dimensions without PE being adsorbed on the column and the difference in the total optical densities revealed the approximate amount of γ-globulin bound. 0.1 *M* Na phosphate buffer, pH 7.0, was chosen as the developing buffer. On comparing the results with those of the control

TABLE II

RETENTION OF ANTIBODY BY THE ANTIGEN PE ADSORBED ON A DEAE-CELLULOSE COLUMN

First 0.5 ml of PE, and then 0.5 ml of ASPE were added to the test column. 0.5 ml of ASPE was added to the column used as the control. Single tube experiments were carried out with 30 ml of the developing buffer being used in each concentration step. Flow-rate was maintained at 2.0 to 3.0 ml/h at 20°. 0.5 ml of ASPE precipitated 0.5 ml of PE in free isotonic saline solution.

Concentration of developing buffer	*Recovery in mg protein N*	
	Test	*Control*
0.1 *M*	28.1	25.5
0.05 *M*	21.5	17.3
0.01 *M*	8.0	6.85

experiment it was found that under the experimental conditions, the recovery of 84–91% of the total protein applied could be achieved. The difference in the amount of protein eluted indicates that this amount of protein is kept bound to PE and that it cannot be eluted under the experimental conditions used. It may, however, be pointed out that no antibody was found in the effluent.

The effect of different salt concentrations was studied by means of single tube experiments, the experimental details of which are described in the following paper. 0.05 and 0.01 *M* Na phosphate buffer, pH 7.0, were employed and the recoveries achieved were 81.0 and 85.0% of the control experiment respectively. Table II indicates that though the amount of protein eluted from the column varies depending on the salt concentration of the developing buffer, yet the difference is quite significant when PE is included in the system even at a buffer concentration as high as 0.1 *M*. No attempts were made, however, to characterize the serum protein components eluted at different buffer concentrations.

DISCUSSION

The experimental results presented in this communication demonstrate that the immune γ-globulin when adsorbed on some adsorbent materials (hydroxylapatite, SM-cellulose) is still capable of combining with antigen and the same holds true in the reverse case where the antigen is adsorbed on DEAE-cellulose. This observation is in conformity with the results obtained by previous workers with immunological adsorbents where the antigen is chemically bound to an insoluble carrier. It has not yet been established with certainty how far the different constituent groups of a protein molecule influence the chromatographic behaviour on a column or how large are the combining sites, but it is obvious that the ionizing groups play an important role. In

spite of the rapid progress that has been made in the field of immunochemistry, the distribution of peptide groups or the amino acid sequence of the antibody that brings about the specificity towards its antigen is not definitely known. It has been shown by SINGER[18] that while bovine serum albumin retains most of its activity as a precipitating antigen on extensive guanidination and acetylation, antibody to bovine serum albumin loses all its activity under the same conditions of acetylation. It has been suggested by SINGER AND CAMPBELL[19] that the ε-NH_2 group of lysine of antibody and the COOH group of antigen participate in the antigen–antibody reaction. KOSHLAND *et al.*[20] have found that the procedures used, such as acetylation, iodination, photo-oxidation, destroy 75% of the activity of the antibody against negatively charged phenylarsonic acid and 10% of the activity of the antibody against the positively charged phenyltrimethylammonium group as measured by quantitative precipitin tests and equilibrium dialysis. Similarly GROSSBERG AND PRESSMAN[21] observed that acetylation of anti-*p*-azophenylarsonate and anti-*p*-azophenyltrimethylammonium antibodies causes both a reduction in number of binding sites and a change in average binding constants, and that of *p*-azobenzoate produces primarily a reduction in number of binding sites. These studies indicate that tyrosine is present in the combining site; the difference in the reactions observed may, however, be attributed to the spatial configuration of tyrosine or to its content.

The adsorption of antibody and antigen on a column blocks certain active groups but the nature and the extent of the combination are not definitely known. The present study shows that while they are adsorbed on the chromatographic column antibody molecules lose some of the combining sites. The decrease in the efficiency of an antibody in retaining its homologous antigen may originate from steric hindrance. It is obvious that the COOH groups of a protein would be attracted by the basic groups of an anion exchanger, while the amino, imino, imidazole and guanidyl groups would be directed towards the acidic groups of a cationic exchanger. A portion of the total protein may be held up on the adsorbent in such a fashion that the antigen is prevented from reaching the combining site. The experiments reported here seem to indicate that such steric hindrance may possibly be one of the causes for the decrease in retention capacity of the antibody. The experiments with the cellulosic exchangers SM- and DEAE-cellulose indicate that both the cationic and anionic groups are needed for antigen–antibody reaction. Since the experiments were conducted at pH 6.8 with hydroxylapatite, at pH 7.0 with DEAE-cellulose, and at pH 6.1 with SM-cellulose, only the imidazole groups of histidine remain fully charged. It is quite possible that histidine may play a significant role in antigen–antibody reaction although this cannot be established from the present study. Mention may, however, be made that antibody to pneumococcus polysaccharide type 3, which is polycellobiouronic acid, when likewise adsorbed on hydroxylapatite columns demonstrates the ability to combine with its homologous antigen. The dissociation of the PE–ASPE complex brought about by increasing the salt concentration indicates the presence of antigen–antibody combination of varying magnitude. The possibility of protein–protein interaction at low salt concentration should, however, be considered, though no retardation in the elution

behaviour of PE has been found in presence of normal rabbit γ-globulin. Furthermore, the total recovery of PE is approximately the same, within the experimental error (Table III), in spite of the difference in the salt concentration of the starting buffer. It would be quite interesting to pursue the study on the nature of the combination as revealed by the present study.

TABLE III

EFFECT OF SALT CONCENTRATION ON PE–ASPE COMBINATION

ASPE was added to PE (1.0 ml, O.D. 566 mμ = 6.0) maintaining the antibody : antigen ratio at 1:5, incubated at 37° for 30 min, and the mixture was then transferred to the hydroxylapatite column, 7.0 × 1.0 cm. 30 ml of Na phosphate buffer, pH 6.8, was used in each concentration step. Flow-rate was maintained at 10 ml/h,and the column operation was carried out at 3°. Figures in parentheses indicate the results obtained with ASPE–PE mixture while the others indicate PE alone. Total recovery represents the amount of PE recovered from the test system containing ASPE–PE mixture in comparison with that of PE alone.

Initial conc.	*% Recovery in concentration steps*					*Total recovery*
	0.005 M	*0.01 M*	*0.02 M*	*0.05 M*	*0.1 M*	
0.005 *M*	59.9	30.4	7.7	2.0		
	(42.5)	(20.3)	(9.9)	(11.1)		(83.8)
0.02 *M*			100.0			
			(68.5)	(8.2)	(9.0)	(85.7)
0.05 *M*				100.0		
				(67.8)	(15.7)	(83.5)

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SUMMARY

Studies have been made on the antigen–antibody reaction with PE and its antibody when the immune γ-globulin is strongly adsorbed on chromatographic adsorbents such as hydroxylapatite and sulphomethyl-cellulose. The reverse case has also been studied with the anionic cellulose exchanger diethylaminoethyl-cellulose where PE remains rather firmly bound to the adsorbent. Frontal analysis and the stepwise elution technique show that both antigen and antibody react with their complementary reactants while adsorbed on column material. Increasing the salt concentration causes some of the PE–ASPE complex to dissociate, which shows that PE–ASPE combination varies in magnitude. Some possible implications have been discussed.

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