SEROLOGICAL REACTIONS ON CHROMATOGRAPHIC COLUMNS

II. HAPTEN–ANTIBODY REACTIONS

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In the preceding communication[1] it has been reported that immune γ-globulin adsorbed on solid supporting media, such as hydroxylapatite and the cellulose ion-exchanger sulphomethyl-cellulose, is capable of combining with the homologous antigen and that the reverse also prevails, with the antigen being adsorbed on the anionic exchanger diethylaminoethyl-cellulose. The present communication describes an investigation carried out with the antibodies against different polysaccharides, such as soluble pneumococcus polysaccharides and dextran. The observation that the antibody ASS_3** against S_3 adsorbed on a solid supporting medium combines with the antigen has led to the study being extended to the reaction of the antibody with its natural hapten, *e.g.*, glucose etc. Attempts have been made to assess the amount of simple haptens, such as glucose, cellobiose and galactose, that remain bound to the antibody by employing column operation on hydroxylapatite. A few preparations were made by oxidizing cellulose and these were employed for the inhibition of the precipitin reaction of S_3 with its homologous antiserum. Inhibition of the reaction of S_2 by low molecular weight oligosaccharides obtained from the dextran hydrolysate is reported. Chromatography was also employed to study the competitive binding of the different haptens for the antibody site.

MATERIALS AND METHODS

The antisera against the different pneumococcus polysaccharides were received from the State Serum Institute, Copenhagen, Denmark, through the courtesy of Dr. ERNA LUND. Pneumococcus polysaccharides types 2, 3 and 8 were received from Prof. MICHAEL HEIDELBERGER, Rutgers University, N.J. The different low molecular weight fractions isolated from dextran hydrolysate were kindly supplied by Dr. P. FLODIN and DR. K. GRANATH, Pharmacia Inc., Uppsala. Human antidextran serum was provided by Prof. ELVIN A. KABAT, Columbia University, New York.

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** Abbreviations: ASS_x = rabbit antisera against pneumococcus type specific polysaccharide, S_x, x being the type specific number; AS_{Dex} = human antisera against dextran; $dextran_{3,500}$ and $dextran_{41,500}$ = dextran fractions of molecular weight 3,500 and 41,500 respectively.

The procedure followed for the inhibition assay consisted in the addition of known amounts of a hapten present in a definite volume to 0.5-ml portions of antisera and the incubation of the resulting mixture for half an hour at 37°. A suitable quantity of antigen was then added and the volume was made up to 2.0 ml with 0.15 *M* saline and again incubated at 37° for half an hour. After the incubation, the tubes were kept in a cold room at 3°, and mixed well twice daily for five days. A control containing antigen and antibody without the inhibitor was run along with each set of experiments. The precipitates were dissolved in 1.0 *N* NaOH and the nitrogen content was estimated using a suitable aliquot according to LOWRY *et al.*[2]. The percentage inhibition as accomplished by the hapten was calculated on the basis of the nitrogen content in the precipitates obtained from the control and the test.

To assess the amount of a natural hapten bound by the antibody, short columns of different adsorbents were employed. The flow-rate was generally kept low, about 1.5–2.0 ml/h, and the column operation was carried out at room temperature, 20°. Fractions, approximately 0.5 ml each, were collected using a time-operated fraction collector. Each fraction was made up to a volume of 2.0 ml, and estimated quantitatively with 0.5-ml aliquots. Lower oligosaccharides were estimated according to the method of DISCHE[3] as modified by GURIN AND HOOD[4], and the uronic acids by the carbazole method as described by DISCHE[5]. To enhance the rate of reaction between hapten and antibody, 0.5-ml portions of antisera dialysed against 0.15 *M* saline were incubated with a known quantity of carbohydrate hapten for half an hour at 37° prior to their application to the column. Each experiment with the antibody and hapten using an adsorbent column was preceded by a control experiment where only the hapten was run on the same column under identical experimental conditions.

For the experiments with a radioactive tracer, 1-^{14}C-glucose, antisera (0.5–1.0 ml) were incubated with 0.5 ml of glucose solution containing 5.0 μmole of non-labelled glucose as a carrier and an activity of 5000–6000 c.p.m., control experiments being carried out with 1-^{14}C-glucose solution alone omitting the antibody. The volume of each eluate fraction was measured and duplicate samples of 0.5 ml each were used for the activity measurements. The radioactivity was determined with a windowless gas-flow counter. A comparison with the control provided the information about the hapten binding of the antibody or the competitive binding of haptens for the combining site.

A few preparations were made to obtain the cellohexoses and cellobiouronic acids, using cellulose as the starting material. No attempt was made to obtain the individual products as pure ones. The crude products containing the different cellohexoses and/or cellobiouronic acids were used for the study of the inhibition reaction and of the hapten-binding reaction.

Preparation No. I. Cellulose was oxidized by gaseous NO_2 in a static chamber, washed free of acid and dried according to the method described by YACKEL AND KENYON[6], and used as such.

Preparation No. II. Cellulose was acetylated according to the method of DICKEY AND WOLFROM[7]. The mixture of acetylated cellohexoses was freed of acids, dried,

treated with barium methylate in the usual manner, and then subjected to gaseous NO_2 oxidation[6].

Preparation No. III. Oxidized cellulose containing 10–12% COOH groups was subjected to acetylation and deacetylation[7], and the final product was treated with barium methylate.

Preparation No. IV. The acetylated cellohexoses were prepared[7], and treated with barium methylate in the usual manner.

RESULTS

Reaction of pneumococcus polysaccharide with its homologous antibody fixed on an adsorbent

It was considered interesting to study the effect of pneumococcus polysaccharide on the chromatographic behaviour of its homologous antiserum adsorbed on a hydroxylapatite column. Two identical columns, 25 × 1.0 cm, were washed overnight with the starting buffer, 0.01 *M* Na phosphate buffer, pH 6.8. It may be mentioned that with the ionic strength of the developing buffer used here, γ-globulin stays immobilized on hydroxylapatite column[8]. 2.0 ml of ASS_3 were dialysed against 0.15 *M* saline, and were found to be equivalent to 300 μg of S_3 by the normal precipitin reaction. The protein solution was allowed to become adsorbed on the columns and then S_3 (600 μg in 1.0 ml of 0.01 *M* Na phosphate buffer) was added to one column and allowed to adsorb. Both the columns were kept at that stage for 24h before the usual elution procedure was undertaken. The following buffer concentration steps were employed, *viz.* 0.01, 0.025, 0.05

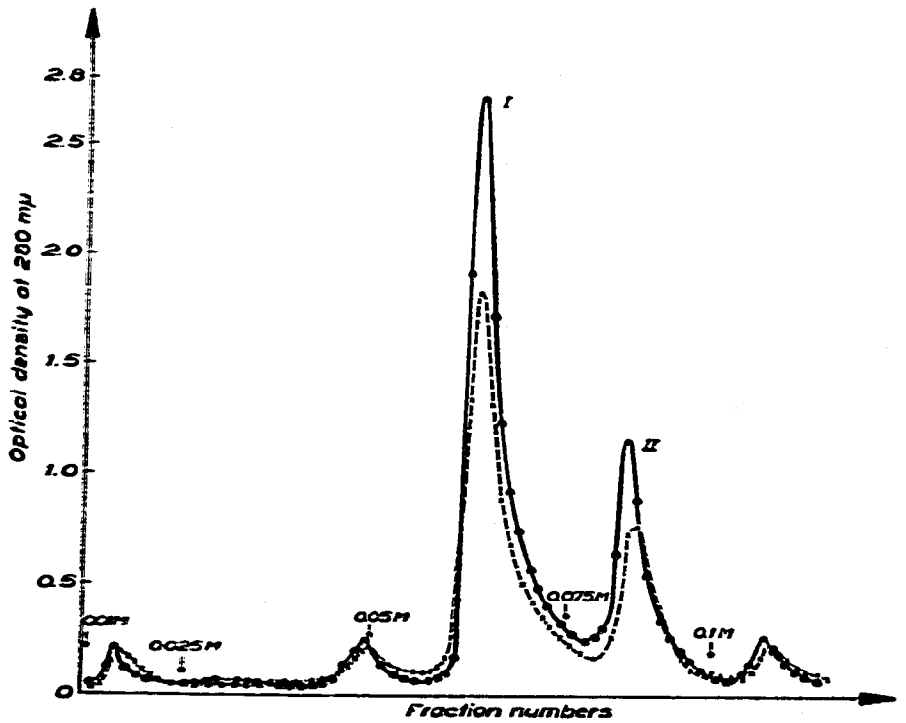

Fig. 1. The effect of S_3 on the chromatographic behaviour of ASS_3 adsorbed on a hydroxylapatite column. ×---× represents the results obtained with ASS_3 and S_3. ●—● represents the results obtained with ASS_3 alone.

0.075, 0.1 and 0.2 *M*. 5.0-ml fractions were collected from each column and the optical density at 280 mμ was plotted against the fraction numbers. Fig. 1 indicates that though ASS_3 is adsorbed on the hydroxylapatite column, it is capable of combining with the antigen. As a result of the reaction, a large part of protein remained immobile on the column and could not be eluted by the experimental procedures employed. The difference in the area under the different peaks indicates the amount of globulin remaining on the column. As shown in Fig. 1, peak I consists mostly of γ-globulin with a little albumin (6.3 and 4.5 S_{20} respectively), whereas peak II contains only γ-globulin (6.6 S_{20}). These results imply that some combining sites of the adsorbed ASS_3 molecule remain free and combine with S_3 molecules, possibly forming a precipitate similar to that found in the solution. An extension of this observation has been utilized for the study of hapten-binding by the antibody.

Reaction of hapten and antibody as studied by column techniques

It is known from the investigations of various workers that though a natural hapten representing one of the constituents of the basic unit of an antigen may bring about an inhibition in the antigen–antibody reaction, yet in order to be efficient, it should possess a certain multiple of the basic antigenic structure. While studying the inhibition of the reaction of dextran (1,6 linkages) with AS_{Dex}, KABAT[9] observed that the maximum inhibition of the specific precipitation could be attained with isomaltotriose and isomaltotetraose. From this he concluded that the dimensions of the minimum groupings on an antibody molecule required for the combination are of the order of three to four glucose units. Although this view may hold good with the straight chain uncharged polyglucoses such as dextran, it seems difficult to test this idea with the protein antigen because of the meagre knowledge about the molecular configuration of the amino acid sequence of the antigenic determinant groups of a particular protein. Most of the studies of this kind have been carried out with artificial haptens introduced into a protein carrier (for reviews *cf.* LANDSTEINER[10], and PRESSMAN[11]). A hapten representing a small chemical grouping when attached to a protein carrier may induce an antibody specificity, and it has been demonstrated by BREINL AND HAUROWITZ[12], and also by MARRACK AND SMITH[13] that when a simple hapten is dialysed between normal serum and hapten homologous antiserum, the hapten distribution favours the antiserum phase.

During the present investigation, it was observed that glucose, cellobiose, and glucuronic acid could bring about a partial inhibition in the precipitin reaction of ASS_3 with S_3 for example; the maximum inhibition achieved was 11.4% with 10 μmoles of cellobiose. The structure of S_3 has, however, been established as glucopyranosyluronic acid-4-β-D-glucose, wherein D-glucose is linked to C-3 of glucuronic acid while the later is in turn linked to C-4 of a second D-glucose residue[14–16]. The observation that the antibody, while adsorbed on an adsorbent column, still has some free combining sites, together with the observation that under the present experimental conditions the low molecular weight substances pass through hydroxylapatite column without being adsorbed whereas γ-globulin remains fixed on the column, provided an

impetus to investigate the capability of ASS_3, while adsorbed on the supporting media, for retaining one of the natural haptens from a dilute solution of the hapten in a suitable buffer. Short columns, 3.0–4.0 × 1.0–1.2 cm, were washed overnight with 0.03 *M* Na phosphate buffer, pH 6.8. Antiserum (0.5 ml) was added to each column, and a dilute solution of glucose, cellobiose and glucuronic acid, in 0.03 *M* Na phosphate buffer, pH 6.8, was passed separately through the treated column. 0.5-ml fractions were collected, and sugar or uronic acid was estimated quantitatively by the carbazole method. Some of the typical results are shown in Fig. 2a and b. The differences observed

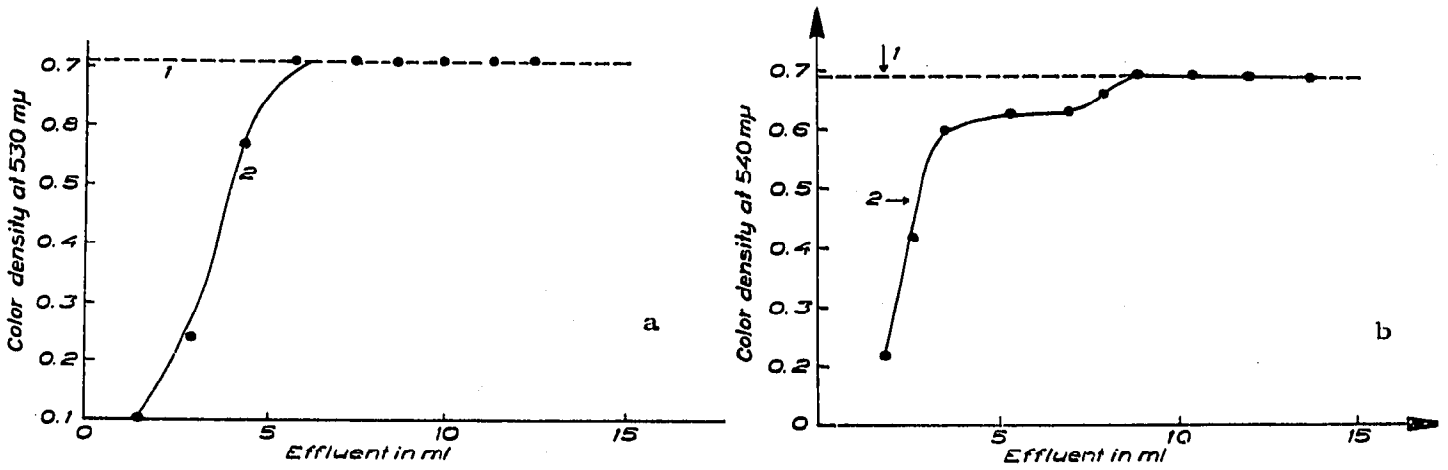

Fig. 2. Frontal analysis experiments of ASS_3 and hapten on hydroxylapatite. (a) Reaction of ASS_3 with glucuronic acid. Curve 1 denotes the O.D. at 530 mμ of the developing glucuronic acid solution; curve 2 denotes the same in the presence of ASS_3. (b) Reaction of ASS_3 with glucose. Curve 1, the developing glucose solution only; curve 2, with ASS_3 adsorbed on the column.

were not conspicuous enough to allow any definite conclusion, but it appeared that this small difference might originate from the sluggish reaction between the hapten and antibody, and might also be due to a reduction in the number of available antigen-combining sites of the antibody as a result of its adsorption on an adsorbent. An attempt was made to find a better way of studying the reaction by incubating the antiserum with a known quantity of monosaccharide or disaccharide prior to its application to the adsorbent column. A comparison was made by running a control experiment on the column with normal rabbit serum and the hapten, and the hapten alone, which was eluted completely with 0.03 *M* Na phosphate buffer, pH 6.8. Then the incubated mixture containing antiserum and the same amount of hapten as in the control was run on the same column under identical conditions. The results are shown in Fig. 3. The difference in the amount of glucose emerging from the column may be considered as the amount held up by ASS_3. It was thus found that out of 1800 μg glucose used for the reaction, 277 μg was retained by 0.5 ml ASS_3. When normal rabbit serum was employed instead of ASS_3, under identical experimental conditions, both the curves became identical. Furthermore, quantitative assay showed that glucose was not retained by normal rabbit serum. A dextran hydrolysate of molecular weight 2400[17] was employed to investigate whether a non-specific reaction might take

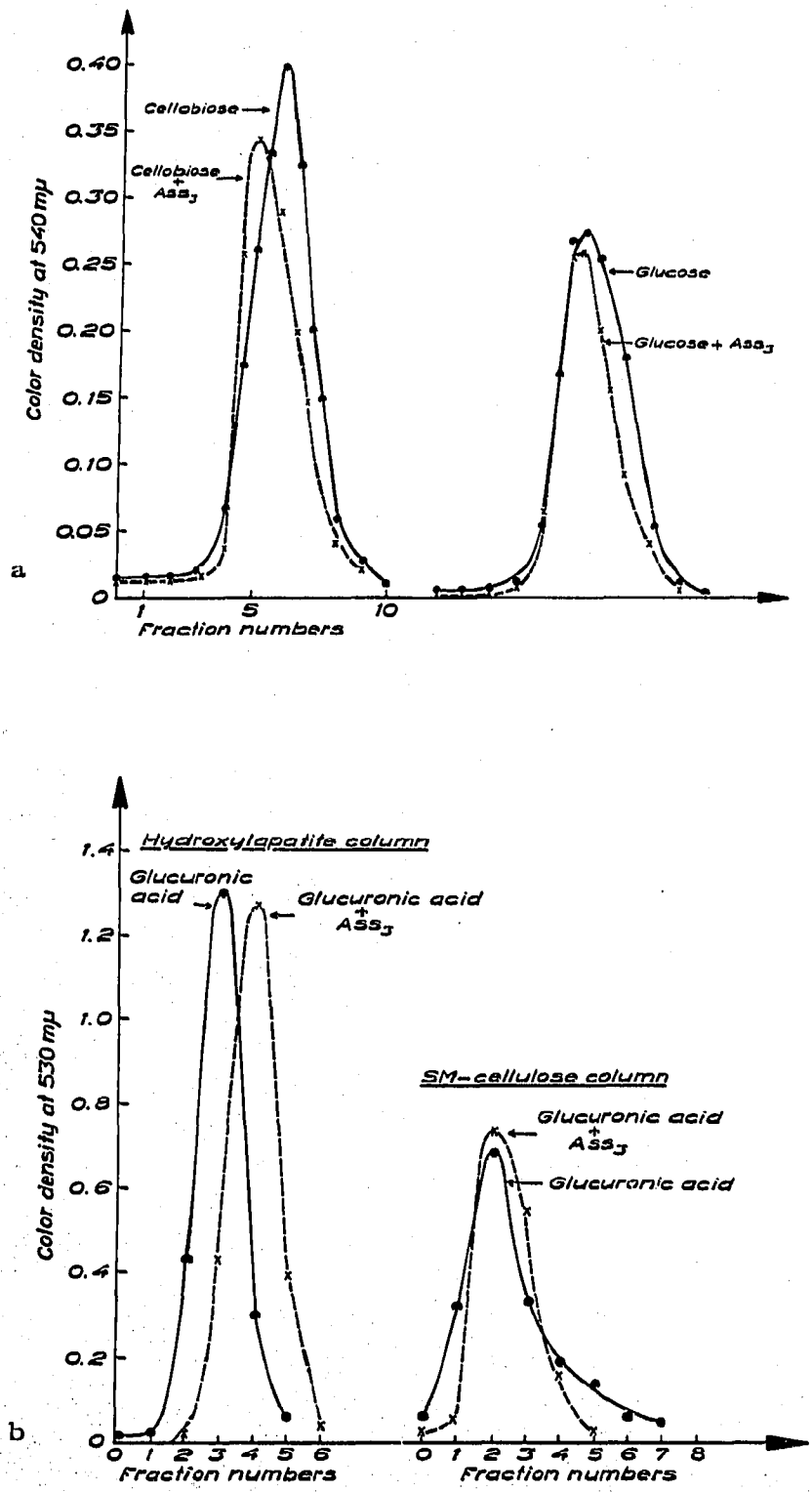

Fig. 3. (a) The retention of haptens, glucose and cellobiose by ASS_3 adsorbed on a hydroxylapatite column. (b) The behaviour of ASS_3 toward glucuronic acid when adsorbed on hydroxylapatite columns, and SM-cellulose columns.

place between ASS_3 and the dextran fraction. This dextran fraction contained about 95–96% of 1,6 linkages, as determined by the periodate oxidation method[17]. 0.5 ml of dialysed ASS_3 was incubated for 30 min with 5.0 μmoles of dextran fraction in 0.5 ml 0.03 *M* Na phosphate buffer, pH 6.8, prior to its application to the hydroxylapatite column. Quantitative estimations of the fractions revealed that identical amounts were recovered from the column both in the case of dextran alone, and of dextran and ASS_3.

Though glucuronic acid is one of the basic constituents of S_3, and though it partially inhibits the precipitin reaction of ASS_3 with S_3 in 0.15 *M* saline solution, it was observed that during the chromatographic procedure employed here glucuronic acid was not retained by ASS_3. When 0.5 ml ASS_3 was incubated with 10 μmoles glucuronic acid in 0.5 ml 0.03 *M* Na phosphate buffer, pH 6.8, and subjected later to column operation on hydroxylapatite, quantitative estimation revealed that the glucuronic acid employed was completely recovered in the effluent, the quantity being identical to that of the control. It was of interest to find out whether or not ASS_3 would behave in a similar manner on the cationic cellulose ion-exchanger sulphomethyl-cellulose as it does on the hydroxylapatite column. ASS_3 (0.5 ml) was incubated with 5 μmoles of glucuronic acid contained in 0.5 ml Na phosphate buffer, pH 6.1, ionic strength 0.025, and then the incubated mixture was applied to the column, the hold-up volume of the column being determined by using a dilute phycoerythrin solution. An identical quantity of glucuronic acid run earlier on the same column served as a control. 0.5-ml fractions were collected and estimated in the usual manner. It was found, in analogy with the results on the hydroxylapatite column, that ASS_3, while adsorbed on sulphomethyl-cellulose, could not retain glucuronic acid, but it was observed that glucose was retained by ASS_3 though the reagent blank was higher due to the presence of cellulose particles in the effluent.

Attempts were made to find out whether or not the antibodies against other pneumococcus polysaccharides behave similarly when adsorbed on hydroxylapatite. ASS_2 was treated with 10 μmoles of glucose in the manner described earlier. It has been shown by STACEY[18] that all the glucose molecules in the S_2 molecule are in the form of 1,4,6 branch points - that is bound in glycosidic linkage at 1-, 4- and 6-positions, and it has been suggested that all the rhamnose is linked in positions 1 and 3[19]. 318 μg glucose was retained by 0.5 ml ASS_2 out of 1800 μg employed for the reaction. A similar retention of hapten was also observed with ASS_8. JONES AND PERRY[20] have established the structure of S_8 as the repeating unit of O-β-D-glucopyranosyluronic acid-(1,4)-O-β-D-glucopyranosyl-(1,4)-O-α-D-glucopyranosyl-(1,4)-O-β-D-galactopyranosyl-(1,4)-. 0.5 ml of ASS_8 was found to retain 434 μg out of 3420 μg glucose added. Confirmatory experiments of a similar nature were undertaken by incubating α-D-glucose (5 μmoles in 0.5 ml buffer), containing 1-^{14}C-glucose as tracer, with ASS_2 and ASS_8 (0.5 ml each) under identical experimental conditions as described earlier. The results are shown in Fig. 4b, which are similar to those shown in Fig. 4a. Measurement of the ^{14}C activity in the eluate fraction showed that when antibody is included in the system, there is a considerable difference in the activity of the effluent.

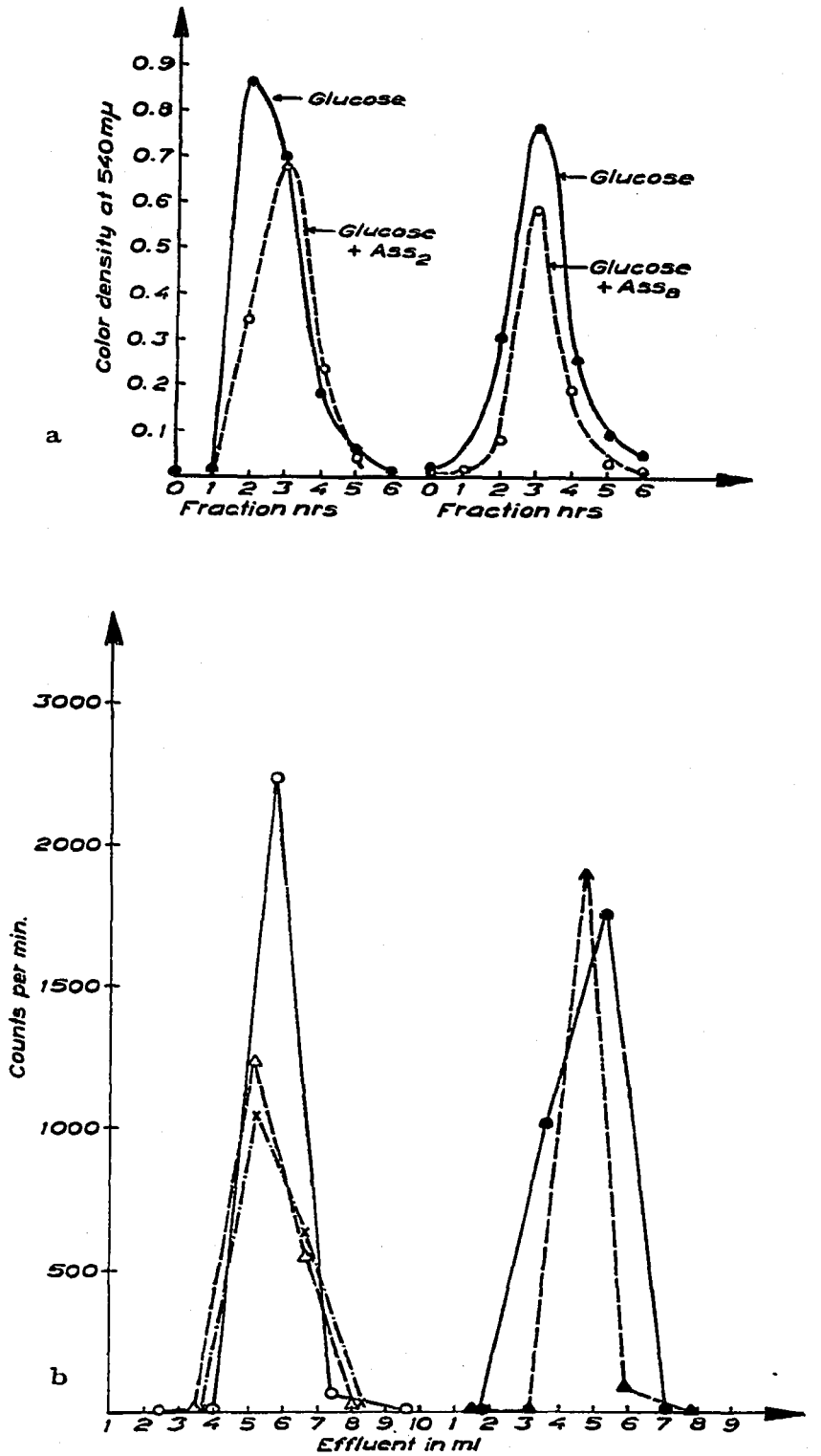

Fig. 4. (a) The retention of glucose by ASS_2 and ASS_8 adsorbed on hydroxylapatite columns. (b) The retention of glucose by ASS_2 and ASS_8 adsorbed on hydroxylapatite columns as revealed by measuring the activity of 1-^{14}C-glucose in the effluent. O—O the activity of 1-^{14}C-glucose with 5 μmoles of glucose; Δ- - -Δ the activity of 1-^{14}C-glucose with 5 μmoles of glucose in the presence of ASS_8; $\times$-·-·-$\times$ the activity of 1-^{14}C-glucose with 5 μmoles of glucose and galactose each in presence of ASS_8; ●—● 1-^{14}C-glucose with 5 μmoles of glucose, control of the experiment with ASS_2; ▲- - -▲ 1-^{14}C-glucose with 5 μmoles of glucose in presence of ASS_2.

Experiments with radioactive tracers also showed that no dissociation of the hapten–antibody complex occurs under the experimental condition, although dissociation occurs at a salt concentration higher than 0.2 *M* Na phosphate buffer. D-Galactose (5 μmoles in 0.5 ml buffer) appears to help the binding of glucose by ASS_8. 0.5 ml α-D-glucose (5 μmoles) containing 1-^{14}C-glucose was incubated with ASS_8 and ASS_{20} (1.0 ml), and the volume was made up to 6.5 ml with 0.005 *M* Na phosphate buffer, pH 6.8. A control containing glucose only, and the test solution containing glucose and antisera were spun at 56,100 r.p.m. in a Spinco model E Ultracentrifuge, rotor J, for 5 h at 20°. The supernatants from the control had an activity of (a) 926, and (b) 758 c.p.m./ml; the test with ASS_8 had (a) 798, and (b) 522 c.p.m./ml, and with ASS_{20}, (b) 676 c.p.m./ml respectively.

During the present investigation, it was observed that the haptens employed passed the hydroxylapatite columns without being retained. Quantitative determination of the eluate fractions and the experiments with radioactive glucose (Fig. 4 b) showed that the simple haptens were completely desorbed from the column within the second hold-up volume quantity of eluate, the hold-up volume of hydroxylapatite columns being determined with methyl orange. Hence, the total eluate equalling three times the hold-up volume of a particular volume was collected in a tube, and estimations of the haptens were carried out on a suitable aliquot of such a collected volume. Some of the results are shown in Table I. When similar experiments were carried out

TABLE I

RETENTION OF HAPTENS BY THE ANTIBODIES AS MEASURED BY THE SINGLE TUBE EXPERIMENTS ON HYDROXYLAPATITE COLUMN

0.5 ml of antiserum dialysed against 0.15 *M* saline was incubated with the hapten (5 μmoles in 0.5 ml buffer) at 37° for 30 min, prior to their application to the column. The difference in the amount of sugar recovered from the control and the test columns was reported as the amount of sugar bound.

Hapten	*μmoles*	*Antisera*	*O.D. 540 mμ*	*μmoles sugar bound*
D-Galactose	5		0.78	
D-Galactose	5	ASS_8	0.68	0.74
D-Cellobiose	10		0.40	
D-Cellobiose	10	ASS_8	0.36	1.90
L-Rhamnose	20		0.46	1.73
L-Rhamnose	20	ASS_2	0.42	

with ASS_2, ASS_3, and ASS_8, and glucuronic acid in concentrations of 5 and 10 μmoles on a hydroxylapatite column, the results obtained confirmed the earlier observations that glucuronic acid is not retained by the antibody adsorbed on such a column.

Reactions of antibodies against pneumococcus polysaccharides with cellulose oxidation products

In the first set of experiments for the inhibition assay, 0.5 ml of ASS_3, and 150 μg S_3 in 0.5 ml of 0.15 *M* saline were used. As described earlier, the antisera were incubated

first with the inhibitor, and then again with S_3. In the second set, the amount of S_3 was decreased from 150 μg to 90 μg, the other conditions being kept constant. The same procedure was carried out when the inhibition assay of the precipitin reaction of ASS_2 with S_2 by the cellulose oxidation products and by the oxidation products of dextran fraction was studied. According to DICKEY AND WOLFROM[7], the products obtained by acetolysis of cellulose range in degree of polymerization from 1 to 6 glucose units. Oxidation by NO_2 preferentially attacks the primary hydroxyl groups of cellulose to yield oxidized materials containing chains of anhydroglucose and anhydroglucuronic acid units[21]. Table II shows that the preparation containing larger amounts of uronic

TABLE II

STUDIES ON THE INHIBITION IN THE PRECIPITIN REACTION OF ASS_3 WITH S_3, AND OF ASS_2 WITH S_2

Cellulose oxidation products	*Test system*	*mg polysaccharide added*	*% Inhibition*	
			I[a]	*II*[b]
I	$ASS_3 + S_3$	1.77	30.0	—
		3.58	43.2	22.8
		5.37		47.8
II	$ASS_3 + S_3$	1.15	23.2	—
		2.3	27.9	—
		2.3	27.9	—
		4.6	31.2	—
		5.75		16.0
III	$ASS_3 + S_3$	1.6	27.9	—
		3.25	35.9	—
		6.5	43.8	30.0
		8.1		31.0
IV	$ASS_3 + S_3$	1.09	27.4	—
		2.18	30.3	—
		4.36	34.4	—
		5.45	—	14.0
I	$ASS_2 + S_2$[c]	3.58	20.0	
II	$ASS_2 + S_2$	4.6	8.2	
III	$ASS_2 + S_2$	6.5	2.0	
IV	$ASS_2 + S_2$	4.36	4.0	
Oxidized dextran (14–15% COOH)	$ASS_2 + S_2$	0.048	6.4	
		0.096	4.1	
		0.240	1.1	

[a] 0.5 ml ASS_3 + 150 μg S_3 in 0.5 ml 0.15 *M* saline.
[b] 0.5 ml ASS_3 + 90 μg S_3 in 0.5 ml 0.15 *M* saline.
[c] 0.5 ml ASS_2 + 180 μg S_2 in 0.5 ml 0.15 *M* saline.

acids inhibits the precipitin reaction to a greater extent. The greater inhibition brought about by the cellulose oxidation product preparation No. I in the reaction of ASS_2 with S_2, suggests that some of the glucuronic acids in the S_2 molecules are linked in a manner similar to that in cellobiouronic acid. Single tube chromatographic experi-

ments as described earlier were conducted with preparation No. I, and No. III, and the oxidized dextran, and ASS_2, ASS_3 and ASS_8, to find out whether or not the oxidized products are retained by the antisera. These experiments showed that identical amounts could be recovered from the control and the test experiments.

Effect of dextran hydrolysates on the cross-reaction of dextran and different pneumococcus polysaccharide antisera

The configurational requirements and the approximate dimensions of the combining site of an antibody molecule have been reported by KABAT[9]. Similarly, the well-known cross-reaction of ASS_2 and dextran was chosen for the study of the inhibition in the cross-reaction[22]. The molecular weight of the different dextran hydrolysates was determined by carrying out end group analysis using the periodate method[17]. The inhibition in the reaction of ASS_2 with S_2 was studied with these dextran fractions and at the same time the inhibition in the cross-reaction of ASS_2 with dextran was carried out as described earlier. The dextran sample (95% 1,6 linkages) had the following specification[5]: M_w 143,000, M_n 106,000, and η 0.38. As communicated by DR. FLODIN, fraction VIII contained isomaltose, isomaltotriose, isomaltotetraose, and isomaltopentaose, and fraction VII contained isomaltotriose, isomaltotetraose, and upwards. Fractions VI to I did not contain oligosaccharides lower than isomaltopentaose. Table III shows that with the increase in the molecular weight of these fractions, that is with

TABLE III

INHIBITION IN THE PRECIPITIN REACTION OF ASS_2 WITH S_2, AND IN THE CROSS REACTION OF ASS_2 WITH DEXTRAN, AND OF ASS_{20} WITH DEXTRAN BY DEXTRAN FRACTIONS

0.5 ml of antisera was used for the inhibition assay. 180 μg of S_2 and dextran fractions, 0.2 and 0.4 μmole, were used for the system with ASS_2 and S_2. 20 μg of dextran and dextran fraction, 0.4 μmole, were used during the inhibition assay of the cross reaction of ASS_2 and dextran, and ASS_{20} and dextran.

Dextran fractions	*Mol.wt.*	*% Inhibition*				
		(ASS_2 + S_2) with		*(ASS_2 + dextran) with*	*(ASS_{20} + dextran) with*	
		0.2 μmole	*0.4 μmole*	*0.4 μmole*	*0.2 μmole*	*0.4 μmole*
I	4300–5000	5.4	9.0	55.6	74.5	35.7
II	2900–4300	7.0	2.0	—	47.7	41.0
III	2200–2900	7.0	6.0	54.8	53.6	60.5
IV	1680–2200	3.9	5.4	54.8	52.6	55.8
V	1500–1680	1.0	1.0	53.0	45.6	56.6
VI	1290–1500	7.3	6.1	51.0	42.4	—
VII	950–1290	4.2	1.7	49.3	41.8	53.0
VIII	400–950	0.0	0.0	34.0	41.0	23.6

the increase in the molecular dimensions, the efficiency of producing an inhibition increases. It is quite plausible that in order to reach the active sites of antibody, which are complementary to the antigen determinant group, without considerable structural distortion, the inhibitor should possess a larger dimension. Inhibition in the cross-reaction may be brought about even by the lower molecular weight fractions,

though the efficiency is more apparent with the higher molecular weight ones. Inhibition in the precipitin reaction of ASS_2 with S_2 brought about by oxidized dextran appears to result from the glucose residues rather than from the glucuronic acid residues as compared with the results obtained with cellulose oxidation products. Results obtained with the reaction of ASS_{20} with dextran in the presence of the different fractions substantiates the findings obtained with ASS_2.

Competitive binding of haptens

Competitive binding of haptens has been studied by KARUSH[23], and NISONOFF AND PRESSMAN[24] who employed the equilibrium dialysis technique. The relative combining affinity could be determined by measuring the ability of a competing or related hapten to displace a bound reference hapten from the antibody. The chromatographic techniques described earlier in this paper have now been successfully employed for the study of such phenomena, with systems containing ASS_2, ASS_{20}, and AS_{Dex}, and dextran fractions of molecular weight 3,500 and 41,500. Antisera (0.5 ml) dialysed overnight in the cold against 0.15 *M* saline were incubated with α-D-glucose (5.0 μmole in 0.5 ml 0.005 *M* Na phosphate buffer, pH 6.8) containing 1-^{14}C-glucose. Then the competing hapten was added to the mixture, which was again incubated for 30 min. The mixture was then chromatographed as usual at room temperature, and approximately 4.0-ml fractions were collected. Measurement of the radioactivity in the effluent revealed the trend of the reaction, and this is presented in Table IV. The greater displacement of glucose from its bound state by $dextran_{41,500}$ in comparison with $dextran_{3,500}$ substantiates the earlier observations (Table III). When ASS_{20} was incubated first with the competing hapten $dextran_{3,500}$, the inhibition was found to be consider-

TABLE IV

DISPLACEMENT OF GLUCOSE BOUND TO ANTIBODY BY COMPETITIVE HAPTENS

AS_{Dex} (1.0 ml), ASS_2 (0.5 ml), and ASS_{20} (0.5 ml) were incubated first with reference hapten glucose, and then with competing haptens. The radioactivity reported under $dextran_{3,500}$ and $dextran_{41,500}$ represents ^{14}C in excess of that obtained with the control comprising glucose and antisera. Hydroxylapatite columns, 7.0 × 1.0 cm, and 0.005 *M* Na phosphate buffer, pH 6.8, were used for the column operation.

Antisera	*Glucose*		*$Dextran_{3,500}$*		*$Dextran_{41,500}$*	
	conc. μg	*c.p.m.*	*concn. μg*	*c.p.m.*	*concn. μg*	*c.p.m.*
AS_{Dex}	450	1929	7	665	4.15	286
			14	768	8.30	1535
AS_{Dex}	450	1727	7	661	4.15	188
			14	798	8.30	1260
ASS_2	900	4831	70	699	41.5	3139
ASS_{20}	900	1727	70	2537*		
				737**		
ASS_{20}		1152	70	1516*		
				943**		

* ASS_{20} (0.5 ml) was incubated with $dextran_{3,500}$ first, and then with 1-^{14}C-glucose.
** ASS_{20} (0.5 ml) was incubated with a mixture of $dextran_{3,500}$ and 1-^{14}C-glucose.

able. The results indicate that either some glucose molecules could reach the antibody sites to be bound there, or some dextran$_{3,500}$ molecules are displaced by glucose molecules. It may, however, be mentioned that similar investigations may be extended to other systems, and that the competitive binding of galactose, mannose, glucose-1-phosphate, glucose-6-phosphate, α-methyl glucoside, α-D-glucose, and β-D-glucose for the antibody site of ASS_3 and of ASS_8, has been studied. The results obtained will be reported elsewhere.

DISCUSSION

The experimental results presented here demonstrate that chromatographic experiments on hydroxylapatite can be successfully utilized to determine the binding of haptens such as glucose by the antibodies. The antibody–hapten reaction as investigated in the present communication does not give us information about the absolute amount of hapten bound to the antibody, but it should provide us with knowledge about the relative amounts of different haptens retained by the antibody. In this respect, the behaviour of glucuronic acid seems very perplexing. Attempts made to test the fractions obtained from the cellulose oxidation products, where the ratio of hexoses to uronic acid is higher than that in the cellobiouronic acid, gave results similar to those obtained with glucuronic acid alone. The only plausible explanation that may be advanced is that the very site where glucuronic acid remains attached is also the site for the attachment of the adsorbent with γ-globulin. It may, however, be surmised that during the operation, the glucuronic acid molecule is replaced, and freed from the antibody. This suggestion finds support from the experiments conducted with aldobiouronic acids and the other antisera. It may, however, be pointed out that the surfaces of globular proteins possess negatively charged groups ($RCOO^-$) of aspartic and glutamic acid residues, and positively charged (RNH_3^+) groups of lysine and arginine residues, and in addition, there are the polar phenolic hydroxyls of tyrosine and imidazole groups of histidine residues. It may be assumed that if the basic groups of antibody be blocked by the cationic exchanger, then naturally the glucuronic acid molecule will not be able to stay there. This idea suggests, however, that the affinity of the basic groups of antibody for acidic groups of cationic exchanger are stronger than that for the carboxyl groups in glucuronic acid. It has been reported by SINGER[25] that bovine serum albumin retains most of its activity as a precipitating antigen on extensive guanidination and acetylation, whereas antibodies to bovine serum albumin lose all their activity under the same conditions of acetylation. This suggests strongly that the participation of (RNH_3^+) groups of antibody molecules is involved in the antigen–antibody reaction, since acetylation as carried out under the conditions mentioned was fairly specific for free amino groups.

The advantage of the chromatographic method for hapten–antibody reaction studies is that the procedure provides fairly accurate information within a short period, and that the effluent obtained is free of any interfering protein or antibody. It may, however, be pointed out that 0.5 ml of ASS_3 neutralized 150 μg of S_3 which contained approximately 75 μg of glucose according to its established structure[14,15]. However, in

chromatographic experiments it was found that it retained 277 μg of glucose which is about 3.7 times higher than the theoretical value. The multivalent nature of antibody may account for the reactivity in excess of the theoretical value, as there are more free sites available than those necessary for the precipitin reaction. Exposure of larger surface or, in other words, unfolding of the molecule may also increase the reactivity. An anomaly has also been observed in the ratio of hapten bound by ASS_2 and ASS_8. ASS_2 retains 318 μg glucose, whereas it retains only 313 μg rhamnose, which constitutes approximately 50% of the S_2 molecule. In the case of the ratios of galactose, glucose, and cellobiose bound by ASS_8 the findings were similar.

To inhibit an antigen–antibody reaction, the inhibitor should possess a minimum structural dimension, in order to be able to compete for the active site on the antibody surface. The cellobiouronic acids will naturally be more efficient than the cellohexoses in connection with the precipitin reaction of ASS_3 with S_3, and this has been confirmed during the present investigation. The cross-reaction of dextran with ASS_2 has been inhibited quite efficiently by the fractions containing low molecular weight oligosaccharides. But when these fractions were employed to inhibit the reaction of ASS_2 with S_2, only the fractions containing larger molecules gave rise to some sort of inhibition. This indicates that to inhibit a precipitin reaction, an inhibitor which would cross react must have larger dimensions. This has been further confirmed during the investigation on the competitive binding of haptens.

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SUMMARY

Antisera such as ASS_3, ASS_8, and ASS_2 are capable of retaining the simple haptens, glucose, cellobiose and galactose, the antibody being fixed on a solid supporting medium such as hydroxylapatite or sulphomethyl-cellulose. The amount of the simple natural hapten bound to the antibody has been determined by using a chromatographic column. Column operation has been carried out for the study of the competitive binding of haptens.

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