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Isolation and Study of Rabbit Antibodies Specific for Certain of the Antigenic Groups of Human Serum Albumin

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A fragment of human serum albumin called 'inhibitor' was shown by Lapresle & Webb (1960a) to carry only some of the antigenic sites of the albumin molecule. In the present work, we have studied the isolation and properties of rabbit anti-(human serum albumin) antibodies specific for these sites. The antibodies were isolated by specific adsorption and subsequent elution from insoluble 'inhibitor'-benzylcellulose conjugates.

MATERIALS

Polyaminopolystyrene was kindly given by the Norsk Hydro Elektrisk, Oslo (Norway).

p-Aminobenzylcellulose was obtained from Touzart et Matignon, Paris, France.

Diethylaminoethylcellulose (DEAE-cellulose) and carboxymethylcellulose (CM-cellulose) (Selectacel) were obtained from Brown and Co. and were used in columns provided with a cooling jacket kept at 4°.

Sephadex G-200 and Sephadex G-100 were obtained from Pharmacia, Uppsala, Sweden. They were used in columns at room temperature.

The molarity of the potassium phosphate buffers used is expressed with respect to phosphate and the pH was measured with a pH-meter.

Human serum albumin (Squibb fraction V), prepared by ethanol fractionation, was kindly given by the American Red Cross.

Degraded human serum albumin was prepared as follows. A portion (2.5 g.) of human serum albumin was degraded (in the presence of 1 mm-cysteine) for 6 hr., at pH 3.5

and 37° by 1250 proteolytic units of rabbit cathepsin D prepared as described by Lapresle & Webb (1960b, 1962).

Rabbit anti-(human serum albumin) sera were prepared as follows. Rabbits were immunized by intravenous injections of human serum albumin adsorbed on alum and the sera were collected after either 3 weeks or 6 weeks of immunization, as described by Lapresle & Webb (1960a).

METHODS

Preparation of 'inhibitor'. The fragment of human serum albumin called 'inhibitor' can be detected by electrophoresis in agar gel, as shown by Lapresle & Webb (1960a). This property was used to detect the presence of 'inhibitor' in the various fractions obtained during the following purification.

Step I. A solution (50 ml.) containing 2.5 g. of degraded human serum albumin was dialysed against 5 mm-potassium phosphate buffer, pH 8.0, and passed through a column (50 cm. long × 2 cm. diam.) of 15 g. of DEAE-cellulose equilibrated with the same buffer. Under these conditions 'inhibitor' is eluted by the passage of the same buffer, but most of the degraded albumin stays on the column. All eluates having an extinction at 280 mμ of more than 0.1 were pooled and the pH of the mixture was adjusted to 6.55.

Step II. The pooled eluates obtained in step I were passed through a column (30 cm. long × 2 cm. diam.) of 6 g. of CM-cellulose equilibrated with 5 mm-potassium phosphate buffer, pH 6.55. The column was washed with the same buffer and a first fraction not containing the inhibitor was discarded. A second fraction containing

'inhibitor' was then eluted by the passage of the same buffer containing NaCl (0.05M). Some other material remained on the cellulose. The eluate fractions containing 'inhibitor' were pooled, dialysed against water and freeze-dried.

Step III. The freeze-dried preparation of 'inhibitor' was dissolved in 4 ml. of 0.05M-potassium phosphate buffer, pH 8.0, containing NaCl (0.15M) and passed through a column (40 cm. long $\times$ 2.5 cm. diam.) of Sephadex G-100 equilibrated with the same buffer. 'Inhibitor' was eluted by washing the column with the same buffer.

Preparation of 'inhibitor'-polystyrene conjugates. 'Inhibitor' was conjugated with the diazotized polystyrene in the proportion 4.2 mg. of 'inhibitor'/1 g. of polystyrene, as described for human serum albumin-polystyrene conjugates by Webb & Lapresle (1961).

Preparation of 'inhibitor'-benzylcellulose conjugates. To 6 g. of *p*-aminobenzylcellulose suspended in 160 ml. of 0.2N-HCl, 1.2 g. of sodium nitrite was added slowly with stirring at 0°. The mixture was stirred for 30 min. and then washed successively with 500 ml. of 5% (w/v) sodium acetate, 500 ml. of 5% (w/v) urea, 1 l. of distilled water and 500 ml. of 0.15M-NaCl, and finally suspended in 200 ml. of 15 mM-potassium phosphate buffer, pH 6.75.

To the diazotized cellulose, different quantities of 'inhibitor' from 3.2 mg. to 5.5 mg./g. of *p*-aminobenzylcellulose were added. Under these conditions, after 18 hr. of stirring at 4°, all the 'inhibitor' was taken up by the diazotized benzylcellulose. Unchanged diazo groups were then blocked by the addition of glycine.

Isolation of antibodies. For the adsorption of the antibodies, both the 'inhibitor'-polystyrene and 'inhibitor'-benzylcellulose complexes were packed into glass columns as described by Webb & Lapresle (1961) for albumin-polystyrene conjugates. In both cases, rabbit anti-(human serum albumin) was passed through the column and the amount of antibody specific for 'inhibitor' in the effluents was measured with tanned red cells sensitized with 'inhibitor'. The amount of serum passed through was such that the titre of the effluents was the same as that of the original antiserum. This indicated that the column was saturated with these antibodies. The columns were washed with 0.15M-NaCl until the extinction of the effluents at 280 m μ was less than 0.015.

Desorption of the antibodies was achieved by passing 0.5M-glycine-HCl buffer, pH 1.0, through the column at 4°. Eluate fractions (10 ml.) were collected and neutralized immediately with NaOH. The eluate fractions containing protein were pooled, dialysed against water and freeze-dried for storage. To use the immuno-adsorbents again they were neutralized by suspension in 0.5M-potassium phosphate, pH 8.0, washed with 0.15M-NaCl and repacked into the column.

Electrophoresis and immunoelectrophoresis. Electrophoresis in agar gel and immunoelectrophoresis were carried out according to the method of Grabar & Williams (1955) under the following conditions: agar gel at 1%; sodium veronal buffer at pH 8.2 and *I* 0.05 in the reservoir, and at pH 8.2 and *I* 0.025 in the gel; either 5v/cm. for 4 hr. or 7v/cm. for 1 hr.

Ultracentrifugation. Various solutions of purified antibodies, human serum albumin-antibody complexes and 'inhibitor'-antibody complexes were prepared in 5 mM-potassium phosphate, pH 8.0, containing NaCl (0.15M),

incubated at 37° for 1 hr. and kept at 4° for 48 hr. before centrifugation. These solutions were centrifuged at 20° and at 59780 rev./min. in a Spinco model E ultracentrifuge. The sedimentation constants were calculated by the method described by Schachman (1959).

Gel-filtration. The isolated antibodies, dissolved in 0.05M-potassium phosphate buffer, pH 8.0, containing NaCl (0.15M), were filtered through a column (80 cm. long $\times$ 2 cm. diam.) of Sephadex G-200 prepared with the same buffer. The antibodies were eluted by washing with the same buffer.

Passive haemagglutination. The tanned-red-cells technique of Boyden (1951) was used under the conditions described by Borduas & Grabar (1953). The tanned red cells were sensitized with either 100 μ g. of human serum albumin or 100 μ g. of 'inhibitor'/ml. of red cells and tested with either serial 1:2 dilutions of purified antibodies or serial 1:2 dilutions of whole antiserum.

RESULTS

Preparation of 'inhibitor'. The preparation of 'inhibitor' obtained by chromatography on DEAE-cellulose contained traces of other components as shown by electrophoresis in agar gel. These other components were removed by subsequent chromatography on CM-cellulose.

By filtration on Sephadex G-100, 'inhibitor' was eluted as a single peak, and a small amount of other material, not detectable by electrophoresis in agar gel, was eluted after the passage of 'inhibitor'. It was possible by this method to prepare 60–70 mg. of 'inhibitor' from 2.5 g. of human serum albumin. This preparation of 'inhibitor' had the same immunological properties as the preparations described by Lapresle & Webb (1960a).

Isolation of antibodies. When the anti-(human serum albumin) serum was passed through the columns of either 'inhibitor'-polystyrene or 'inhibitor'-benzylcellulose, all the effluent fractions (1 ml.) agglutinated red cells sensitized with human serum albumin, showing that there were anti-(human serum albumin) antibodies that were not adsorbed on the columns. In each case the first effluents did not agglutinate tanned red cells sensitized with 'inhibitor'. Subsequent effluents agglutinated the red cells sensitized with 'inhibitor' and the titre of the agglutination increased gradually until it was the same as that of the original antiserum, indicating that the column was saturated by anti-(human serum albumin) antibodies specific for 'inhibitor'. The amount of antiserum required for the saturation varied from one serum to another, but for a given serum was consistently much smaller for the 'inhibitor'-polystyrene columns than for the 'inhibitor'-benzylcellulose columns. This indicates that the adsorption capacity of the 'inhibitor'-polystyrene column was much lower than that of the 'inhibitor'-benzylcellulose column.

Table 1. *Preparation from rabbit anti-(human serum albumin) sera of antibodies specific for 'inhibitor'*

Different amounts of 'inhibitor' were coupled by diazo-linkages to the two types of insoluble supports. Rabbit anti-(human serum albumin) sera were passed through columns of these complexes. The antibodies adsorbed were eluted with 0.5 M-glycine-HCl, pH 1.0.

Type of insoluble support	Column no.	Amount of insoluble support (g.)	Amount of inhibitor conjugated (mg.)	Amount of antibody recovered (mg.)	Antibody recovered (mg.) Inhibitor conjugated (mg.)
Polystyrene	1	4	25	7.0	0.28
	2	8	50	11.5	0.24
	3	8	50	13.0	0.26
Benzylcellulose	4	6	33	33	1.00
	5	6	30	29	0.96
	6	6	20	22.4	1.11

The results for the desorption of antibodies from several columns of either 'inhibitor'-polystyrene or 'inhibitor'-benzylcellulose are shown in Table 1. The amount of 'inhibitor' conjugated to polystyrene was greater than the amount conjugated to benzylcellulose, yet the total amount of antibody recovered from the 'inhibitor'-polystyrene columns was much lower than that recovered from the 'inhibitor'-benzylcellulose columns. The amount of antibody recovered for a given amount of 'inhibitor' was constant for each type of 'inhibitor' conjugate and four times greater for 'inhibitor'-benzylcellulose than for 'inhibitor'-polystyrene columns.

Since the amount of antibody recovered from the 'inhibitor'-benzylcellulose columns was greater than that from the 'inhibitor'-polystyrene columns, the former were chosen for the preparation of the antibodies used in the other experiments described in the present work. The column of 'inhibitor'-benzylcellulose could be used repeatedly (at least ten times). There was an initial decrease of about 30 % in the amount of antibody recovered when the column was used for the second time, but thereafter the amount of antibody recovered did not diminish appreciably. This latter observation indicates that all the antibodies are desorbed from the column by 0.5 M-glycine-hydrochloric acid buffer, pH 1.0.

Physicochemical properties of the isolated antibodies. (a) Electrophoresis and immunoelectrophoresis. Fig. 1 shows the electrophoretic and immunoelectrophoretic patterns in agar gel given by the isolated antibodies. The electrophoretic pattern shows that the major constituent of the isolated antibodies is γ -globulins. There are also other constituents having mobilities of β - and α -globulins. The immunoelectrophoretic analysis shows that isolated antibodies give two lines of precipitation: one of these lines is given by the γ -globulins and it extends into the zone of β - and α -globulins; the other line in the β -globulin zone

Fig. 1. (a) Photometric tracing of the electrophoretic pattern in agar gel given by isolated antibodies. (b). Immunoelectrophoretic pattern of isolated antibodies. R, Starting reservoir.

and close to the reservoir is given by β_2 -macroglobulin.

(b) Ultracentrifugation. Ultracentrifugal analysis of the isolated antibodies showed the presence of two components (pattern E in Fig. 3). One, which represented about 90 % of the total protein, had a sedimentation constant of 6.3s under the experimental conditions used. The other one, which represented about 10 % of the total protein, had a sedimentation constant of 17.1s under the same conditions.

(c) Gel-filtration on Sephadex G-200. Fig. 2 shows the filtration pattern on Sephadex G-200 given by the isolated antibodies. The material was eluted in three main peaks. The eluate fractions comprising each of these three peaks were pooled as shown in the Figure. Fraction I contained 5.55 mg. of protein and was shown by immunoelectrophoresis to be β_2 -macroglobulin. Fraction II contained 40 mg. of protein and was shown by immunoelectrophoresis to be γ -globulins. Ultracentrifugal analysis of this fraction showed a single component having a sedimentation constant of

6-3s under the experimental conditions used and at a protein concentration of 7 mg./ml. Fraction III did not contain protein and gave no line in immunoelectrophoresis.

Immunological properties of the isolated antibodies. (a) Specific precipitation with human serum albumin and 'inhibitor'. All the immune sera used for the isolation of these antibodies gave precipitates with human serum albumin and some gave precipitates with 'inhibitor', as described by Lapresle & Webb (1960a). Whichever type of serum was used, the isolated antibodies did not give precipitates with either human serum albumin or 'inhibitor'.

(b) Passive haemagglutination. The original immune sera were tested in serial dilutions by tanned red cells sensitized with either 'inhibitor' or human serum albumin. As found by Lapresle & Webb (1960a), the titre expressed as the last dilution giving a positive reaction was much greater (20-80 times) with human serum albumin than with 'inhibitor'. Table 2 shows the results of the agglutination by the isolated antibodies of tanned red cells sensitized with either 'inhibitor' or human serum albumin. The minimum quantity of antibody required for a positive agglutination with red cells sensitized with either 'inhibitor' or

human serum albumin was the same or slightly smaller (2-4 times) with 'inhibitor' than with human serum albumin.

Ultracentrifugal analysis of antigen-antibody complexes. The ultracentrifugal patterns given by the isolated antibodies mixed with human serum albumin or inhibitor are given in Fig. 3. The con-

Table 2. *Passive haemagglutination titre of isolated antibodies specific for 'inhibitor'*

The tanned red cells were sensitized with 100 μ g. of either 'inhibitor' or human serum albumin. They were tested with serial 1:2 dilutions of different antibody preparations.

Antibody prepn.	Minimum quantity of antibody giving positive reaction (μ g.)	
	Red cells sensitized with 'inhibitor'	Red cells sensitized with human serum albumin
1	0.8	1.6
2	0.4	0.8
3	0.2	0.8
4	1.4	1.4

Fig. 2. Gel-filtration on Sephadex G-200 of isolated antibodies. A solution (5 ml.) of isolated antibodies (10 mg./ml.) was applied to a column (80 cm. long $\times$ 2 cm. diam.). The column was washed with 0.05M-phosphate buffer, pH 8.0, containing NaCl (0.15M). The volume of each eluate fraction was 5 ml.

Fig. 3. Ultracentrifugal diagrams of isolated antibodies and mixtures of isolated antibodies with human serum albumin or 'inhibitor'. The concentrations of human serum albumin, 'inhibitor' and antibody, and the sedimentation constants of the observed components, are given in Table 3. The arrows indicate the direction of the sedimentation. The times after full speed was attained were as follows: A and B, 42 min.; C and D, 48 min.; E, F, G and H, 32 min.

Table 3. *Sedimentation constants of components observed by ultracentrifugation*

The solutions were prepared in 5 mM-potassium phosphate, pH 8.0, containing NaCl (0.15M), incubated at 37° for 1 hr. and kept at 4° for 48 hr. before centrifugation. They were centrifuged at 20° and at 59780 rev./min. in a Spinco model E ultracentrifuge. The molecular weights used for the calculation of the molar ratios were as follows: 160000 for the antibodies; 70000 for human serum albumin; 15000 for 'inhibitor'. The sedimentation constants were calculated from the peaks and are given without correction for protein and salt concentrations.

Pattern	Concn. of antigen (mg./ml.)		Concn. of antibody (mg./ml.)	Antigen:anti-body molar ratio	Sedimentation constants of observed components (s)			
	Human serum albumin	'Inhibitor'			Free antigen			
A	—	—	1.50	—	—	6.6		
B	1.45	—	1.50	2	4.2	8.4	11.0	
C	2.90	—	1.50	4.5	4.6	8.5	11.3	
D	8.50	—	1.50	13.5	4.0	7.9		
E	—	—	5.50	—	—	6.3		17.1
F	1.45	—	5.50	0.5	—	6.3	10.8	15-20
G	—	1.00	4.7	2	1.4	6.3	9.7	
H	—	0.25	4.7	0.5	—	6.5	9.5	

centrations of human serum albumin, 'inhibitor' and antibody, and the sedimentation constants of the observed components, are given in Table 3.

Pattern A was given by a solution of isolated antibody containing only one component which had a sedimentation constant of 6.6s.

Pattern B was given by the same solution of antibody mixed with an approximately equal amount of human serum albumin. There are three components; the lightest one, with a sedimentation constant of 4.2s, represented free albumin. There were two types of human serum albumin-antibody complexes present in approximately equal amounts and having sedimentation constants of 8.4s and 11.0s. There was no peak corresponding to free antibody.

Patterns C and D were given by mixtures of the same solution of antibody and greater amounts of albumin and showed the presence of the same three components as in pattern B. Comparison of patterns B, C and D shows that, with increasing amounts of human serum albumin, the amounts of free albumin and of the lighter human serum albumin-antibody complex increased, and that the amount of the heavier human serum albumin-antibody complex decreased to a trace in pattern D.

Pattern E was obtained with a different solution of antibody. This solution contained a major component having a sedimentation constant of 6.3s and a minor component sedimenting at 17.1s.

Pattern F was obtained when this antibody solution was mixed with human serum albumin to give a human serum albumin:antibody molar ratio of 0.5. There were two major constituents having sedimentation constants of 6.3s and 10.8s, and other minor constituents with constants between 15 and 20s. There is no free albumin.

Patterns G and H were given by isolated antibodies mixed with 'inhibitor'. The solution of antibody used was fraction II obtained by gel-filtration (Fig. 2). When the 'inhibitor':antibody molar ratio was 2 (pattern G), there were three components. One, which remained near the meniscus and had a sedimentation constant of between 1 and 2s, represented free 'inhibitor'. The other two components had sedimentation constants of 6.3s and 9.7s.

When the same amount of antibody was mixed with a smaller amount of 'inhibitor' to give a molar ratio of 0.5, pattern H was obtained. There were two major peaks having sedimentation constants of 6.5s and 9.5s, traces of faster-sedimenting material and no free 'inhibitor'.

DISCUSSION

Previous studies have shown that the fragment of albumin called 'inhibitor' contains only some of the antigenic groups of the albumin molecule (Lapresle & Webb, 1960a). Moreover, this fragment can be prepared in substantial amounts and in a pure form. It can be fixed by diazotization on polystyrene (Gyenes & Schon, 1960) or benzylcellulose (Campbell, Luescher & Lerman, 1951). As the benzylcellulose proved to be more efficient, it has been used for the preparation of an insoluble 'inhibitor' conjugate specific for some of the anti-(human serum albumin) antibodies.

The antibodies isolated from the 'inhibitor'-benzylcellulose do not give precipitates with either human serum albumin or 'inhibitor' but do agglutinate red cells sensitized with human serum albumin or 'inhibitor'. This agglutination indicates that the antibodies were still capable of reacting with the antigens and suggests that they were not

univalent (Gyenes & Sehon, 1962). The fact that the minimum amounts of antibody required to agglutinate red cells sensitized with human serum albumin or 'inhibitor' are of the same order shows that the isolated antibodies react with human serum albumin only at the antigenic sites common to those of 'inhibitor'.

The antigen-antibody complexes formed by either human serum albumin or 'inhibitor' and the isolated antibodies were studied by ultracentrifugal analysis.

In the series of experiments shown in Fig. 3 (patterns B, C and D) human serum albumin was mixed with antibody to give molar ratios of from 2 to 13.5. In all three cases there was free antigen and no free antibody. This shows that all the antibody molecules are capable of reacting with human serum albumin, and that for these antigen-antibody ratios there is an excess of antigen. Under these conditions there are two types of antigen-antibody complexes that have sedimentation constants similar to the α and b complexes observed with an excess of antigen by Singer & Campbell (1952) with rabbit anti-(bovine serum albumin) antibodies.

The lighter complex, which increases with the amount of human serum albumin mixed with the antibodies, represents the complex having the greatest antigen:antibody molar ratio. Therefore its composition permits estimation of the valence of the antibody. With the greatest excess of antigen (pattern D) this complex is the only one formed in a significant amount and therefore contains nearly all the antibody. The amount of human serum albumin present in the complex can be estimated by the difference between the area given by the complex in pattern D and the area given by the antibody alone in pattern A. From this amount of human serum albumin and the amount of antibody the antigen:antibody molar ratio was found to be 1.7. It is not possible to deduce the composition of this complex without knowledge of its molecular weight. However, the most likely composition of the complex compatible with the antigen:antibody molar ratio of 1.7 and the sedimentation constant of 8.5s is two molecules of human serum albumin combined with one molecule of antibody. This composition is in agreement with the composition of the α complex found by Singer & Campbell (1952) and indicates that the isolated antibodies are bivalent.

Since the lighter complex contains one molecule of antibody, the other complex, which has a lower antigen:antibody molar ratio and a greater molecular weight, must contain at least two molecules of antibody. This implies that the isolated antibodies can react with at least two sites on the human-serum-albumin molecules. As the anti-

bodies are bivalent and if one assumes that all the antibody sites are saturated because this complex is formed in the presence of free antigen, the most probable composition compatible with the sedimentation constant of 11.3s is three molecules of human serum albumin combined with two molecules of antibody. This composition is again in agreement with that proposed for the b complex by Singer & Campbell (1952).

The experiment shown in pattern F was done with a mixture of two molecules of antibody/molecule of human serum albumin. There is an excess of antibody, as shown by the presence of free antibody and the absence of free albumin. The major complex formed under these conditions has a sedimentation constant of 10.8s. Again, this complex, having a lower antigen:antibody molar ratio than the light complex formed in an excess of antigen, must contain at least two molecules of antibody attached to one molecule of human serum albumin. The composition of this complex cannot be calculated owing to the presence of other faster-sedimenting complexes, but its sedimentation constant is compatible with a composition of one molecule of human serum albumin combined with two molecules of antibody.

The faster-sedimenting material can be due in part to complexes formed with 19s antibody. However, it cannot be formed exclusively by this type of complex as it is present in too great an amount and some of it sediments less rapidly than the 19s antibodies. The existence of this material is in agreement with the fact that the isolated antibodies are bivalent and capable of reacting with at least two sites on the human-serum-albumin molecules, thus permitting the formation of chains of alternating antigen-antibody molecules.

Pattern G was obtained when 'inhibitor' was mixed with the antibody to give an antigen:antibody molar ratio of 2. As with human serum albumin in the same molar ratio, there were free antigen and two faster-sedimenting components. The lighter one had a sedimentation constant of 6.3s similar to that of free antibody. However, since 'inhibitor' has a low molecular weight (about 15000; Lapresle & Webb, 1960a) and since it is known (Singer & Campbell, 1952; Becker, 1953) that the frictional ratio of complexes of rabbit antibody with antigen are greater than that of the antibody alone, the sedimentation constant of this complex is also compatible with a composition of either one or two molecules of 'inhibitor' combined with one molecule of antibody. If one assumes that the antibodies react with 'inhibitor' in the same manner as with human serum albumin, the composition of two molecules of 'inhibitor' combined with one molecule of antibody is the most probable. The heavier component has a sedimentation con-

stant of 9.7s, which, owing to the low molecular weight of 'inhibitor', is compatible only with a complex containing two molecules of antibody. Its probable composition is three molecules of 'inhibitor' combined with two molecules of antibody, since it is formed in the presence of free antigen.

Pattern H was obtained with 'inhibitor' mixed with the antibody to give an antigen:antibody molar ratio of 0.5. As with human serum albumin in the same molar ratio, no free antigen was observed. There were two major components; the lighter one had a sedimentation constant of 6.5s which, as explained above, could represent free antibody or antibody in a complex with one or two molecules of 'inhibitor'. It is only by analogy with the result obtained with human serum albumin that one can suppose that this component corresponds to free antibody. The heavier component had a sedimentation constant of 9.5s, which is compatible only with a complex containing two molecules of antibody. If the lighter component corresponds to free antibody, the composition of the heavier one should be one molecule of 'inhibitor' combined with two molecules of antibody. There are also faster-sedimenting minor components which correspond to traces of heavier complexes.

The experiments with 'inhibitor' confirm, therefore, that the isolated antibodies are capable of reacting with two sites on the antigen molecules. These two sites presumably have different specificities and therefore the isolated antibodies would consist of two kinds of antibody having different specificities. Work in progress with the degradation products of 'inhibitor' is in agreement with this presumption.

Under the experimental conditions used the isolated antibodies do not give precipitates with either of the antigens used. They form soluble short-chain aggregates analogous to those obtained with bivalent antibody and bivalent haptens (Epstein, Doty & Boyd, 1956; Epstein & Singer, 1958). If one assumes that specific precipitation is due to the formation of a three-dimensional 'lattice' then with bivalent antibody the antigen must be at least tervalent. The observation that the isolated antibodies do not give precipitates with human serum albumin or 'inhibitor' can be explained by the fact that the antibodies are bivalent and react with probably only two sites on the antigen molecules.

Some of the rabbit anti-(human serum albumin) sera used gave precipitates with 'inhibitor'. The fact that the antibodies isolated from these sera did not give precipitates cannot be explained by an alteration in their properties caused by the low pH used for their elution, as Webb & Lapresle

(1961) have shown that antibody that is precipitated with 'inhibitor' can be eluted from albumin-polystyrene conjugates at the same pH. Another possible explanation for this fact could be that 'inhibitor' is fixed to the benzylcellulose in such a way that one of the anti-(human serum albumin) antibodies normally reacting with the inhibitor is not adsorbed on the 'inhibitor' conjugate. However, no evidence could be obtained in favour of this explanation, as the first effluents did not agglutinate red cells sensitized with 'inhibitor' and did not give precipitates with 'inhibitor' when mixed in various proportions with the isolated antibodies.

The isolated antibodies are composed of γ -globulins of a wide range of mobilities and β_2 -macroglobulins; this shows that antibodies specific for only a few of the antigenic sites of a protein molecule can still be heterogeneous with respect to their antigenic and physicochemical properties.

SUMMARY

1. An insoluble adsorbent specific for certain anti-(human serum albumin) antibodies was prepared by coupling to *p*-aminobenzylcellulose a fragment of the human-serum-albumin molecule called 'inhibitor'.

2. Rabbit anti-(human serum albumin) antibodies reacting with 'inhibitor' were specifically adsorbed on the 'inhibitor'-benzylcellulose conjugates and were desorbed by glycine-hydrochloric acid buffer, pH 1.0.

3. The isolated antibodies did not give precipitates with human serum albumin or 'inhibitor' but did agglutinate red cells sensitized with human serum albumin or 'inhibitor'. They formed with human serum albumin or 'inhibitor' soluble complexes which have been studied by ultracentrifugal analysis.

4. These antibodies were shown to be bivalent and to react with at least and probably only two sites on the human-serum-albumin or 'inhibitor' molecules.

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The Biosynthesis of Streptomycin

INCORPORATION OF ^{14}C -LABELLED COMPOUNDS INTO STREPTOSE AND *N*-METHYL-L-GLUCOSAMINE

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Although the structure of streptomycin has been known for some time, the process of its biosynthesis by *Streptomyces griseus* has not yet been elucidated. Blumsom & Baddiley (1961) examined extracts of *S. griseus* for nucleotide-sugar compounds which may be involved in synthesis of the streptose and *N*-methyl-L-glucosamine moieties of streptomycin. They found thymidine diphosphate rhamnose a possible intermediate for streptose, but no compounds more closely related to streptomycin were identified. Other workers have grown *S. griseus* in the presence of labelled compounds and examined the incorporation of the isotope into the streptomycin produced. Such experiments were first carried out by Karow, Peck, Rosenblum & Woodbury (1952), who showed that [$^{14}\text{C}_6$]glucose was incorporated into the streptomycin molecule. Hunter & Hockenhull (1955) carried out a similar experiment and degraded the streptomycin. They found that the streptidine, streptose and *N*-methyl-L-glucosamine residues had similar specific activities, showing that they were all equally derived from glucose. Hunter, Herbert & Hockenhull (1954) grew *S. griseus* in the presence of $^{14}\text{CO}_2$ and found that streptomycin subsequently isolated was labelled almost exclusively in the guanidine residues. The specific activity of the resulting streptomycin was considerably decreased by the presence of arginine in the growth medium, which suggests that arginine can act as a source of the guanidine in the antibiotic. Hunter & Hockenhull (1955) also added ^{14}C -labelled *N*-methyl-L-glucosamine to the growth medium and found that the *N*-methyl-L-glucosamine residue in the streptomycin contained

a considerable amount of isotope. Silverman & Rieder (1960) studied the formation of this amino sugar residue and obtained results that indicate that it is formed from glucose without breakage of the hexose carbon chain.

The source of the *N*-methyl group of *N*-methyl-L-glucosamine has not hitherto been studied directly, although it is known that L-methionine can stimulate the production of streptomycin (Galanina & Agatov, 1959; Majumdar & Kutzner, 1962). The *S*-methyl group of methionine seemed a likely source of the methyl group, and for this reason we have studied the incorporation of radioactivity from [$\text{Me-}^{14}\text{C}$]methionine into the streptomycin molecule. We have also studied the formation of the streptose moiety of streptomycin from labelled compounds. [^{14}C]Formate was used as a possible precursor of the *C*-formyl group of streptose, and specifically labelled glucose was used to discover the source of the main carbon chain.

MATERIALS AND METHODS

Production of streptomycin. *Streptomyces griseus* (Glaxo strain L118) was grown on slopes composed of: bacto-agar (Difco Laboratories), 2%; yeast extract (Difco), 1%; soya peptone (Oxoid), 2%; glycerol, 3%. The spores produced were used to inoculate 20 ml. of a medium composed of: nutrient broth no. 2 (Oxoid), 2.5%; glucose, 1%. The culture was grown for 24 hr. at 28° on a shaker (140 oscillations/min.). A 5 ml. inoculum was then transferred to a 500 ml. conical flask containing 50 ml. of Carvajal's (1957) medium, 0.5 g. of soya-bean meal and the radioactive compound (0.3 mc for the formate experiment; 0.08 mc for the other experiments). The culture was grown for a