Separation of Antigens by Immunological Specificity

USE OF CELLULOSE-LINKED ANTIBODIES AS IMMUNOSORBENTS

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1. Immunosorbents were prepared by coupling activated aminocellulose with the γ -globulin concentrates of antisera prepared against ovalbumin and human serum albumin. 2. The immunosorbents were low in solubility, but high in capacity for homologous antigens. 3. The high specificity of these immunosorbents was demonstrated by their use in fractionating various mixtures of fluorescent ovalbumin, ^{131}I -labelled human serum albumin, lysozyme and ribonuclease.

Initial stages of attempts to use serological specificity for the fractionation of protein antigens have been described (Smith, Tozer, Gallop & Scanes, 1962). Individual antigen-rabbit antibody complexes were obtained from the interaction of mixtures of antigens with mixtures of corresponding antibodies by using a modified gel-diffusion technique. These complexes were then injected into rabbits to produce antibodies specific to the original antigens (Smith, Gallop & Tozer, 1964). The next stage would be to use these monospecific antibodies to fractionate mixtures of soluble antigens either directly by combining with the antigen required, which subsequently might be recovered from the antigen-antibody complex, or indirectly by removing unwanted antigens from solution. No general solution of the direct method has yet been found but methods of removing unwanted antigens could be achieved by converting these antibodies into insoluble immunosorbents, and two methods for accomplishing this are described below and in the accompanying paper (Stephen, Gallop & Smith, 1966).

In contrast with the many attempts to use solid-phase antigens for the specific adsorption of antibodies from sera (for review see Sehon, 1963), relatively few experiments have been made on the reverse process in which insoluble antibodies have been used for the specific adsorption of antigens from solution. Cellulose-antigen-antibody complexes, capable of adsorbing a further amount of original antigen owing to the bivalence of the antibody, have been described. In these complexes the primary cellulose-antigen combination was either physical (Williams & Stone, 1957) or covalent (Gurvich & Drizlikh, 1964). Insoluble aggregates of

rabbit antibody and goat anti-(rabbit antibody) have been used to adsorb antigens on to the rabbit antibody (Kahn, Leskowitz & Lowell, 1962). McDuffie & Sutherland (1963) coagulated antigen-antibody complexes by heating and by chemical treatment, and claimed that these preparations were capable of specific adsorption and subsequent desorption of the homologous antigen. Antibodies have also been linked covalently to diazotized amino-polystyrene (Kent & Slade, 1960) and to cellulose (Jagendorf, Patchornick & Sela, 1963), but with both these preparations the adsorption of antigen was not specific. Recently DeCarvalho, Lewis, Rand & Uhrick (1964) have developed immunosorbents in which bisdiazotized benzidine was used as a cross-linking agent for immune γ -globulins, and Olovnikov (1964) has made similar preparations with 4,4'-diaminodiphenylamine.

The present paper describes the properties of immunosorbents prepared by covalently linking antibody to cellulose. The method is based on that of Gurvich, Kuzovleva & Tumanova (1961) for fixing antigens to cellulose for the specific adsorption of antibodies. The next paper (Stephen *et al.* 1966) describes a method for preparing immunosorbents by the oxidative cross-linking of thiolated antibodies to form disulphide-linked polymers.

MATERIALS

Buffers

The following buffers were used: barbiturate buffer for electrophoresis, pH 8.6 and 10.05 (1.84 g. of barbituric acid/l., 10.31 g. of sodium barbiturate/l.); phosphate-buffered saline (1), pH 7.0 (4.25 g. of NaCl/l., 2.44 g. of KH_2PO_4 /l., 8.09 g. of Na_2HPO_4 /l.); phosphate-buffered saline (2), pH 7.0, same as for (1), with the addition of 11.7 g. of NaCl/l.; phosphate buffer, pH 6.2 (23.8 g. of Na_2HPO_4 ,

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12H₂O/l., 10.4 g. of NaH₂PO₄·2H₂O/l., 1.0 g. of NaCl/l.); borate buffer, pH 8.8 (12.37 g. of H₃BO₃/l., 1.92 g. of NaOH/l.).

Antigens

Fluorescent ovalbumin. This was prepared as described by Smith *et al.* (1962), and further purified. An 18 ml. sample (4.2%, w/v) was passed through a column (2.5 cm. diam. × 100 cm. high) of Sephadex G-25 (Pharmacia, Uppsala, Sweden) equilibrated in barbiturate buffer, pH 8.6 and I 0.05, after which curtain electrophoresis (Spinco model C.P.) in the same buffer [fluorescent ovalbumin preparations diluted to 1.4% (w/v) in barbiturate buffer] yielded three fractions. The first and second fractions, which contained non-protein fluorescent material and protein of low fluorescence respectively, were discarded. The third fraction, consisting of fluorescent labelled protein, was dialysed against phosphate-buffered saline (1), filtered through Millipore filters and stored at 4°.

¹³¹I-labelled human serum albumin. This was obtained from The Radiochemical Centre, Amersham, Bucks. Samples were used within 1 month of labelling.

Lysozyme and substrate. Lysozyme (egg white), obtained from Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks., was further purified by passing a 15 ml. sample (20%, w/v) in phosphate-buffered saline (2) through a column (2.5 cm. diam. × 100 cm. high) of Sephadex G-75 that had been equilibrated in the same buffer. The lysozyme activity was contained in the second of the two protein fractions that emerged from the column. The purified lysozyme was filtered through Millipore filters and stored at 4°. *Micrococcus lysodeikticus* was grown on tryptic meat-agar slopes at 37° overnight. The organisms were harvested, washed three times in 0.9% NaCl, once in water and then freeze-dried.

Ribonuclease and substrate. A chromatographically homogeneous sample of ribonuclease was purchased from Seravac Laboratories. Yeast RNA was purchased from the same source and its acid-solubility was approx. 50% as determined by the method of Wade (1961). Preparations of RNA free from pigment, and of low acid-solubility, were obtained by a single-stage process. NaHCO₃ (10%, w/v) was added slowly to RNA (2.5 g.) in phosphate-buffered saline (2) (25 ml.) until the solution of RNA was complete and the pH neutral. Phosphate-buffered saline (2) was then added to give a volume of 50 ml. This solution was fractionated on a column (7 cm. diam. × 18 cm. high) of Sephadex G-200 equilibrated in phosphate-buffered saline (2) at room temperature. The fraction emerging between 200 and 320 ml. was dialysed against water and freeze-dried. The product, which was obtained in low yield (20%), had an acid-solubility of not more than 10% and a distribution coefficient (*K_d*) in the Sephadex G-200 system between 0 and 1.

Antibodies

Rabbit anti-ovalbumin and rabbit anti-(human serum albumin). These antibodies were prepared by a modification of the method of Smith *et al.* (1964) as follows. Four intracutaneous injections of immune precipitate [100 μg., 100 μg., 200 μg. and 400 μg. in 0.2 ml. of adjuvant (Freund & McDermott, 1942)] were given on days 1, 3, 5 and 10 respectively. These injections were followed by intravenous injections (1 mg. of immune precipitate/0.1 ml. of 0.9%

NaCl) on days 41, 43 and 45. Rabbits were bled on day 50 and γ-globulin concentrates were prepared as described by Smith *et al.* (1962). Approx. 100 ml. of concentrate was obtained from the serum (800 ml.) from 20 rabbits. Many batches of materials have been prepared. A typical batch of anti-ovalbumin concentrate contained 23.2 mg. of antibody/ml. and 123.0 mg. of total protein/ml. Corresponding values for a typical batch of anti-(human serum albumin) concentrate were 16.3 mg. of antibody/ml. and 133.0 mg. of total protein/ml.

METHODS

Estimation of fluorescent ovalbumin. Fluorescence was measured with a Locarte Mk. 4 fluorimeter (Locarte Co., London, S.W. 7) with primary filter LF3 (monochromatic, 436 mμ) and secondary filter LF7 (≥ 510 mμ).

Estimation of ¹³¹I-labelled human serum albumin. Radioactivity of samples (0.5 ml.) was measured with a coincidence counter (Liquid Measuring Head 2022, and Coincidence Control Unit 2032; Isotope Developments Ltd., Reading) with a cylindrical plastic scintillator (NE102; Nuclear Enterprises Ltd., Edinburgh), 2 in. diam. × 2 in. high, drilled to take capped polythene specimen tubes (external dimensions, 8 mm. diam. × 40 mm. high). The ¹³¹I-labelled human serum albumin was diluted appropriately with non-iodinated human serum albumin so that approx. 0.5 μC of ¹³¹I was used in each experiment. Dead-time corrections were made but decay corrections were avoided by using a portion of the experimental sample as reference standard throughout each experiment.

Estimation of lysozyme. This was based on the method of Prasad & Litwack (1963). A suspension of freeze-dried *M. lysodeikticus* (50 mg./250 ml.) in phosphate buffer, pH 6.2, was kept at 0°. Samples of lysozyme (1–5 μg. in 1 ml. of phosphate buffer, pH 6.2) were added to samples [5 ml. in optically matched tubes (¾ in. × 6 in.)] of the *M. lysodeikticus* suspension. The mixtures were shaken and left at room temperature for 15 min. The degree of lysis was measured by the change in *E*₆₅₀ in a Spectronic 20 spectrophotometer (Bausch and Lomb Inc., Rochester, N.Y., U.S.A.).

Estimation of ribonuclease. Freeze-dried RNA (see the Materials section) was dissolved in sodium acetate, pH 5.0 and I 0.1, to form a solution having *E*₂₆₀^{4m} 320; this varied ± 5% with different preparations. Samples of ribonuclease (1–5 μg.) in 1 ml. of water were added to the RNA solutions (2 ml. at 0°), and the mixtures kept at 0° for 3 hr. A linear relationship was established between the concentration of ribonuclease and the amount of acid-soluble nucleotides released, as measured by the method of Wade (1961).

Estimation of protein. This was measured by the Kjeldahl method of digestion followed by direct nesslerization, or by measuring *E*₂₈₀. In both cases standard curves were constructed by using the appropriate protein.

Estimation of antibody. This was done as described by Smith *et al.* (1962).

Analytical electrophoresis. Standard procedures (Smith, 1960) for electrophoresis on cellulose acetate strips (Oxo Ltd., London) in barbiturate buffer, pH 8.6 and I 0.05, were adopted. The strips were dried and stained with Nigrosine [0.02% (w/v) in acetic acid (2%, v/v)]. A 1 μg. amount of protein applied in 10 μl. could be detected.

Concentration of protein samples. This was accomplished by the method of Kohn (1959). Commercial polyethylene

glycol was dialysed for 5 days against running water and freeze-dried before use.

Preparation of the cellulose-immunosorbents. The method used was a modification of that described by Gurvich *et al.* (1961). Cotton wool (Regaid; Boots Pure Drug Co., Nottingham) (130 g.) was steeped in ethanol (4.0 l.) overnight, mixed with acetyl chloride (315 ml.) and heated on a water bath under reflux for 2–3 hr. The ethanolysed cellulose (50 g.) was then treated with *m*-nitrobenzyltrimethylpyridinium chloride [7 g. in 100 ml. of sodium acetate (2.0%, w/v)] at 80° and stirred until dry, and then heated for 40 min. at 125°. After washing with water, drying, washing with benzene and redrying, the product was reduced to the corresponding amino compound by treatment at 50–60° (for 30 min. with stirring) with 300 ml. of Na₂S₂O₄ (15%, w/v). The product was washed with water, acetic acid (30%, v/v) and then with water, and dried.

This material (3.3 g.) was dissolved in a cuprammonium solution [Cu(OH)₂ (5 g.) treated with a solution of sucrose (1.1 g. in 43 ml. of water) and aq. NH₃ (sp.gr. 0.88, 66 ml.)] and the concentration of NH₃ was adjusted by the addition of more aq. NH₃ (sp.gr. 0.88) (133 ml.). The aminocellulose was reprecipitated, after shaking for 1–2 min., by adding water (133 ml. at 80°), rapidly cooling to 4° and adding cold H₂SO₄ (10%, v/v) until the blue colour was discharged. The precipitated cellulose was thoroughly washed until washings were neutral. The activated aminocellulose was diazotized by treatment with NaNO₂ (13 g.) in 666 ml. of HCl (10%, v/v) at 0° for 30 min. with stirring. After washing, the diazotized aminocellulose was immediately suspended in borate buffer, pH 8.6 and I.O.48 (150 ml.), and treated with γ -globulin concentrate (3.3 g. of protein). Water was added to give a final volume of 600 ml. and the reaction allowed to proceed with gentle shaking at 4° overnight. The preparation was then centrifuged, the supernatant removed, the precipitate resuspended in 600 ml. of phosphate-buffered saline (1) and recentrifuged. This procedure was repeated once with 600 ml. of phosphate-buffered saline (1), twice with similar volumes of sodium acetate, pH 3.0 and I.O.2, and finally twice more with phosphate-buffered saline (1).

The protein uptake of diazotized cellulose (100 g.) in 20 experiments with anti-ovalbumin and anti-(human serum albumin) γ -globulin concentrates was 18–35 g. (equivalent to 3–8 g. of antibody).

Immunosorbent columns. The immunosorbent derived from 3.3 g. of activated aminocellulose and containing 100–250 mg. of coupled antibody was mixed with swollen Sephadex G-25 (18 g.; coarse grade equilibrated with appropriate buffer) and the slurry made into a column (4 cm. diam. $\times$ 10 cm. high). The column was washed with phosphate-buffered saline (1) at 4° (flow rates 7–10 ml./hr.) until the protein content of the effluent reached an equilibrium value of not more than 3 μ g./ml. (the sample for this analysis was derived from a large volume of effluent that had been concentrated by freeze-drying after preliminary dialysis against water).

A mixture (1:1, w/w) of the homologous antigen and a heterologous antigen was added to the column. The quantity of homologous antigen was related to the amount of antibody bound to the cellulose and was 50–100% of the amount that would have reacted at optimum proportions if the antibody had been in solution [typical antigen/antibody ratios (w/w) were 1:10.3 for ovalbumin/anti-ovalbumin and 1:5.9 for human serum albumin/anti-(human serum

albumin)]. The columns were eluted with phosphate-buffered saline (1) and 5 ml. fractions were collected. The protein profile of the effluent was followed by measuring *E*₂₈₀. Those fractions comprising the main protein peak were pooled and concentrated by the method of Kohn (1959). Sometimes a small precipitate formed during concentration and this was removed by centrifugation. The recovery of heterologous antigen in this peak was measured together with its contamination with homologous antigen.

The degree of purity of the final preparation of heterologous antigen was determined in three ways. First, the amount of extraneous protein derived from the column was estimated from the difference between the sum of the heterologous and homologous antigens as estimated from label or enzyme measurements, and the total protein as determined by nitrogen measurements. Secondly, the material was examined by cellulose acetate electrophoresis under conditions in which a 5% contamination with globulin would be detected. Thirdly, the precipitin analysis of the recovered antigen was compared with that of the original antigen.

RESULTS

The results of using immunosorbents derived from either anti-ovalbumin or anti-(human serum albumin) to separate their homologous antigens from equal mixtures with ¹³¹I-labelled human serum albumin and fluorescent ovalbumin are shown in Table 1. Also included in Table 1 are the results obtained with similar preparations derived from the inert γ -globulin of unimmunized rabbits.

Similar immunosorbents were used to separate fluorescent ovalbumin and ¹³¹I-labelled human serum albumin from lysozyme (see Table 2) and from ribonuclease (see Table 3).

There was one important change in experimental procedure. The columns were made, washed and eluted with phosphate-buffered saline (2), which contained more sodium chloride than phosphate-buffered saline (1). This increase in salt concentration was necessary to depress reversible adsorption of the basic proteins on the column material.

Cellulose acetate electrophoresis of the recovered antigens supported the evidence shown in Tables 1–3 for low contamination by column protein and homologous antigens. The recovered heterologous antigens (fluorescent ovalbumin, ¹³¹I-labelled human serum albumin, lysozyme and ribonuclease) were compared with the original antigens by precipitin analyses. In all cases the weights of antigen-antibody precipitates obtained under optimum conditions were the same for equal weights of recovered and original antigen.

DISCUSSION

The two cellulose-linked immunosorbents prepared from anti-ovalbumin and anti-(human serum albumin) γ -globulin concentrates have been used successfully on a preparative scale to remove

Table 1. *Separation of fluorescent ovalbumin from ¹³¹I-labelled human serum albumin*

This Table summarizes the results of three experiments (each representative of ten similar experiments) with immunosorbents prepared from anti-ovalbumin and anti-(human serum albumin) and 'inert' rabbit γ -globulin respectively. The columns were prepared and used as described in the text. A mixture containing 9.0 mg. of fluorescent ovalbumin and 9.0 mg. of ¹³¹I-labelled human serum albumin was used in each experiment.

Immunosorbent	Anti-ovalbumin	Anti-(human serum albumin)	'Inert' globulin
Nature of column			
Protein (mg.) coupled to 3.3 g. of diazocellulose	716	660	616
Antibody (mg.) coupled to 3.3 g. of diazocellulose	110	93	0.0
Total antigen eluted			
Fluorescent ovalbumin (% of initial)	3.7	99.0	99.5
¹³¹ I-labelled human serum albumin (% of initial)	94.9	5.5	92.5
Heterologous antigen in main peak			
Recovery (%)	68.9	87.7	78.5*
			73.0†
Contamination of heterologous by homologous antigen (%)	1.7	5.0	—
Contamination of heterologous antigen by column protein (%)	0.0	1.0	3.0

*Fluorescent ovalbumin.

† ¹³¹I-labelled human serum albumin.

Table 2. *Separation of fluorescent ovalbumin and ¹³¹I-labelled human serum albumin from lysozyme*

This Table summarizes the results of two experiments (each representative of three similar experiments) with immunosorbents prepared from anti-ovalbumin and anti-(human serum albumin) respectively and lysozyme as heterologous antigen in each case. The columns were prepared and used as described in the text.

Immunosorbent	Anti-ovalbumin	Anti-(human serum albumin)
Nature of column		
Protein (mg.) coupled to 3.3 g. of diazocellulose	1140	1183
Antibody (mg.) coupled to 3.3 g. of diazocellulose	273	250
Mixture of antigens added to columns		
Fluorescent ovalbumin (mg.)	13.2	—
¹³¹ I-labelled human serum albumin (mg.)	—	11.9
Lysozyme (mg.)	13.2	11.9
Total antigen eluted		
Fluorescent ovalbumin (% of initial)	0	—
¹³¹ I-labelled human serum albumin (% of initial)	—	4.3
Lysozyme (% of initial)	88.0	92.0
Heterologous antigen (lysozyme) in main peak		
Recovery (%)	77.0	89.0
Contamination of heterologous by homologous antigen (%)	0.0	1.4
Contamination of heterologous antigen by column protein (%)	2.0	0.0

their homologous antigens from three other antigens, two of which were highly basic proteins.

During preliminary investigations of the preparation of insoluble cellulose-linked proteins, two cellulose intermediates were studied, *p*-aminobenzylcellulose (Campbell, Leuscher & Lerman, 1951) and *m*-aminobenzylloxymethylcellulose (Gurvich *et al.* 1961). High protein uptakes were

achieved only with the latter, which was adopted for subsequent work. Two sources of ethanolysed cellulose powder were investigated. The product obtained from cotton wool (Regaid; Boots Pure Drug Co.) was preferred to commercial ethanolysed cellulose powder (Munktel, Sweden). Optimum conditions for immunosorbent production varied with the nature of the cellulose. For example, when

Table 3. *Separation of fluorescent ovalbumin and ¹³¹I-labelled human serum albumin from ribonuclease*

This Table summarizes the results of two experiments (each representative of three similar experiments) with immunosorbents prepared from anti-ovalbumin and anti-(human serum albumin) respectively and ribonuclease as heterologous antigen in each case. The columns were prepared and used as described in the text.

	Immunosorbent	...	...	...	Anti-ovalbumin	Anti-(human serum albumin)
Nature of column						
Protein (mg.) coupled to 3.3 g. of diazocellulose					1010	966
Antibody (mg.) coupled to 3.3 g. of diazocellulose					220	173
Mixture of antigens added to column						
Fluorescent ovalbumin (mg.)					22.1	—
¹³¹ I-labelled human serum albumin (mg.)					—	19.8
Ribonuclease (mg.)					22.1	19.8
Total antigen eluted						
Fluorescent ovalbumin (% of initial)					0.0	—
¹³¹ I-labelled human serum albumin (% of initial)					—	1.2
Ribonuclease (% of initial)					91.0	98.0
Heterologous antigen (ribonuclease) in main peak*						
Recovery (%)					91.0	98.0
Contamination by homologous antigen (%)					0.0	1.2
Contamination by column protein (%)					0.0	0.0

* The ribonuclease emerged in a relatively sharp peak; all fractions were pooled for analysis.

the starting material was Munktell powder, maximum protein capacities could only be achieved by selecting a temperature of 100° for the decomposition of the pyridinium salt, and a solution time of at least 2 hr. in the ammoniacal copper solution. Corresponding data for Regaid cellulose were 125° and 1–2 min. respectively. The physical nature of the cellulose preparations was such that admixture with a solid diluent (Sephadex G-25) was necessary to obtain adequate flow rates.

The specificity of the immunosorbents was high as measured by the total recoveries of the heterologous antigens, and only with lysozyme did these fall below 90%. This apparent non-specific adsorption was probably because it was so basic (pI 11.05). However, this effect was small compared with the results obtained by Jagendorf *et al.* (1963), who showed that their bromoacetylcellulose-anti-ovalbumin preparation adsorbed 30 times as much ribonuclease (pI 9.45) as ovalbumin. In our experiments the degree of non-specific adsorption of lysozyme was considerably diminished by increasing the ionic strength of the eluting buffer, indicating that the adsorption was reversible and due neither to the denaturation of lysozyme nor to its covalent union to the cellulose via unblocked coupling centres.

The insolubility of these immunosorbents is of vital importance to their usefulness in purifying antigens. If the preparations were too soluble the operation would become in principle no different to attempting the separation of two antigens by

precipitating one directly with its homologous γ -globulin concentrate and thereby contaminating the other with 'inert' globulins. The evidence in Tables 1–3 and the results of cellulose acetate electrophoresis and precipitin analyses of the recovered products indicate that the degree of insolubility of the immunosorbents was sufficient for their use in this field. However, it should be pointed out that the protein content of the effluent from an immunosorbent column before loading with the antigens is dependent on the flow rate. For example, if the column was kept overnight there was a significant increase in the protein content of the initial effluent; however, on continuing elution the protein content was rapidly decreased to the usual equilibrium value of 1–3 μ g. of protein/ml.

It is hoped to develop further this method of purifying antigens and to attempt to desorb homologous antigens from the columns, thus obtaining pure antigen and at the same time regenerating the immunosorbent for further use. It should be emphasized that the method does not require the pre-existence of pure antigens to obtain monospecific antisera (Smith *et al.* 1962, 1964).

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