Separation of Antigens by Immunological Specificity

USE OF DISULPHIDE-LINKED ANTIBODIES AS IMMUNOSORBENTS

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1. γ -Globulin concentrates of antisera prepared against ovalbumin and human serum albumin were thiolated and cross-linked to form insoluble polymers. 2. These immunosorbents were of low solubility and of high capacity for homologous antigen. 3. The high specificity of these immunosorbents was demonstrated by fractionation of various binary mixtures of fluorescent ovalbumin, ^{131}I -labelled human serum albumin, lysozyme and ribonuclease.

The preceding paper (Gallop, Tozer, Stephen & Smith, 1966) described the preparation and use of cellulose-linked anti-ovalbumin and anti-(human serum albumin) for the specific removal of their respective homologous antigens from mixtures of antigens. The present paper describes immunosorbents prepared without recourse to linkage with insoluble matrices. The preparation of these immunosorbents arose from attempts to use the method of Singer, Fothergill & Shainoff (1960), but for fractionation of antigens rather than for separating antibodies. These authors thiolated ovalbumin, bovine serum albumin and ribonuclease and found that the products could still specifically precipitate antibodies to the parent proteins. Subsequently the bifunctional organomercurial compound 3,6-bis(acetoxymethylmercurimethyl)dioxan was used to precipitate thiolated antigen from acid solutions of thiolated antigen-antibody complexes to leave pure antibody in free solution. Theoretically, pure antigen could be obtained by using the same system if the antibody, and not the antigen, were the thiolated species. This approach was examined. γ -Globulin concentrates containing antibodies to human serum albumin were thiolated, precipitated with ^{131}I -labelled human serum albumin at optimum proportions and the complexes dissolved in the glycine-sulphuric acid system described by Singer *et al.* (1960). However, after precipitation of the thiolated protein with the mercury compound only 1-2% of the total antigen remained in solution. In this work it was noticed that the thiolated antibodies aggregated when exposed to air or on concentration of solutions with polyethylene glycol, and this led to the deliberate introduction of

intermolecular disulphide bonds to form insoluble antibodies and to their use as immunosorbents.

MATERIALS AND METHODS

The materials and methods used in these experiments were the same as those described in the preceding paper (Gallop *et al.* 1966) with the following additions.

Buffers. The following buffers were used: phosphate buffer, pH 7.0 and 10.4 (4.76 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}/\text{l.}$, 2.5 g. of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}/\text{l.}$); phosphate buffer, pH 7.0 and 10.05, obtained by dilution; carbonate buffer, pH 10.6 (170 g. of $\text{K}_2\text{CO}_3/\text{l.}$, 15 g. of $\text{NaHCO}_3/\text{l.}$); sodium acetate-HCl solution, pH 1.8 (100 ml. of 0.1 M-sodium acetate, 1 ml. of conc. HCl).

3,6-Bis(acetoxymethylmercurimethyl)dioxan. This compound was synthesized as described by Singer *et al.* (1960) and was used as titrant in thiol group titrations (see below).

N-Acetylthiomocysteine thiolactone. A sample was purchased from Schwarz Bioresearch Inc., Mount Vernon, N.Y., U.S.A.

Cross-linking solution. The aqueous solution contained (per 100 ml.) $\text{K}_3\text{Fe}(\text{CN})_6$ (1.82 g.), NH_4Cl (4.86 g.) and aq. NH_3 (sp.gr. 0.88) (7.1 ml.).

Thiol group titrations. The colorimetric titration described by Singer *et al.* (1960) was used to assay the thiol content of the substituted globulins. The stoichiometry of the reaction was assumed to be:

Preparation of the immunosorbents. A 10 ml. sample of γ -globulin concentrate (150 mg. of protein/ml.), N-acetylthiomocysteine thiolactone (60 mg. dissolved in 1 ml. of water) and carbonate buffer, pH 10.6 (10 ml.), were pre-cooled to 0° and then mixed. The reaction was allowed to proceed for 35 min. at 0°, when it was stopped by the addition of phosphate buffer, pH 7.0 and 10.4 (40 ml.). The reaction mixture was then passed through a column (2.5 cm. diam. $\times$ 100 cm. high) of Sephadex G-25 previously equilibrated with phosphate buffer, pH 7.0 and 10.05. The

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thiolated protein emerged at the void volume of the column free from *N*-acetylhomocysteine, a hydrolytic by-product of the reaction, and was freeze-dried. The product contained 8–9 thiol groups/mol. of globulin but this could be varied since it was a function of the ratio of thiolactone to protein concentration. The yield of thiolated protein was consistently high (90–95%).

The method for cross-linking was based on that described by Benesch & Benesch (1958) for gelatin. Phosphate buffer, pH 7.0 and 1.0 M, was added to the freeze-dried thiolated protein (4 ml. to 1 g. of protein) to form a paste. An excess (approximately 2 ml.) of the cross-linking solution was added to the paste, which rapidly gelled to form a product with a rubber-like consistency. After standing at 0° for about 1 hr. with occasional stirring, the aggregated polymer was dispersed in phosphate-buffered saline (2) (30 ml.) by homogenization in a glass tissue-grinder. The suspension was then centrifuged and the supernatant removed; the protein was washed in phosphate-buffered saline (2) (30 ml.), left for 30 min. and recentrifuged. This process was repeated (with homogenization when necessary to disperse residual clumps) until successive supernatants contained less than 10 µg. of protein/ml. The preparation was then washed twice with sodium acetate-HCl solution, pH 1.8 (30 ml.), and twice with phosphate-buffered saline (2) (30 ml.). The yield of protein in the cross-linking stage varied in the range 50–90%.

Immunosorbent columns. The washed suspension of disulphide-linked protein was mixed with 4 times its settled volume of Sephadex G-200 suspension. The Sephadex had been swollen in water, washed with boiling water (to remove soluble material that absorbed at 280 mµ) and then equilibrated with phosphate-buffered saline (2). The mixed slurry was poured into a column of suitable dimensions (1 g. of disulphide-linked protein with its appropriate amount of Sephadex G-200 filled a column of 2.5 m. diam. to a height of 10 cm.) and further washed with phosphate-buffered saline (2) until the protein content of the effluent was less than 10 µg./ml. Mixing with Sephadex was necessary to give an adequate column flow rate (approx. 10 ml./hr. at 4°).

The capacity of each immunosorbent preparation for its homologous antigen was determined in a preliminary run on a small sample of the disulphide-linked protein preparation. An excess of the homologous antigen together with an equal quantity of the heterologous antigen was added to this column, which was then eluted with phosphate-buffered

saline (2). Fractions were collected and analysed for homologous antigen to indicate the capacity of the immunosorbent.

The quantity of homologous antigen indicated by the preliminary experiment, together with an equal quantity of heterologous antigen, was added to the main column, which was then eluted with phosphate-buffered saline (2). The protein profile of the effluent was followed by measuring E_{280} and individual fractions (5 ml.) were assayed for heterologous and homologous antigens. The presence of Sephadex G-200 in the column resulted in elution of the heterologous antigen and the residuum of uncombined homologous antigen from the immunosorbent columns in a manner reflecting their respective molecular weights. For this reason, the assessment of the contamination of heterologous antigen by homologous antigen and by extraneous column protein was made on the pool of all the fractions and not just those comprising the main protein peak (cf. Gallop *et al.* 1966). The pooled fractions were concentrated with polyethylene glycol. Occasionally small precipitates were formed at this stage and were removed by centrifugation. The recovered heterologous antigens in the concentrated fractions were subjected to electrophoretic and precipitin analyses as described by Gallop *et al.* (1966).

RESULTS

In preliminary experiments with 50–100 mg. of disulphide-linked protein (of which 12–20% was antibody) the extent of thiol substitution of the γ -globulin was studied in relation to the variation in the specificity of antigen uptake, the capacity for homologous antigen and the solubility of the disulphide-linked immunosorbents. The results of many experiments showed that immunosorbents prepared from globulins with thiol content in the range 5–20 thiol groups/mol. were all highly specific (95–100% of the heterologous antigen was recovered). However, marked differences were observed in their capacity for homologous antigen and in their solubility as judged by the equilibrium values of the protein content of the column effluent (see Table 1).

In these experiments the buffer was phosphate-

Table 1. *Variation in capacity for homologous antigens and in solubility of disulphide-linked immunosorbents with the number of thiol groups/mol. of globulin*

γ -Globulin concentrates were thiolated, cross-linked, mixed with Sephadex G-200 and converted into columns as described in the text. Column 2 indicates the amount of homologous antigen that was absorbed by such a column. Column 3 shows the amount of protein eluted from the column by phosphate-buffered saline (1), in the absence of added antigen solution.

Nature of γ -globulin concentrate	No. of thiol groups/mol. of globulin	Antigen/antibody molar ratio	Protein content (µg./ml. of column effluent)
Anti-(human serum albumin)	20	1:70	3
Anti-ovalbumin	20	1:140	3
Anti-(human serum albumin)	9	1:3	8–20
Anti-ovalbumin	9	1:4	8–20
Anti-(human serum albumin)	5	1:2	20
Anti-ovalbumin	5	1:2	20

Table 2. *Separation of fluorescent ovalbumin and ^{131}I -labelled human serum albumin*

This Table summarizes the results of two experiments (each representative of five similar experiments) with immunosorbents prepared from anti-ovalbumin and anti-(human serum albumin) respectively. The columns were prepared and used as described in the text. A mixture containing 13.6 mg. of fluorescent ovalbumin and 13.6 mg. of human serum albumin was used in both experiments.

	Immunosorbent	Anti-ovalbumin	Anti-(human serum albumin)
Nature of column			
Total protein (mg.)		1000	1000
Total antibody (mg.)		168	117
Heterologous antigen recovered			
Recovery (%)		97.0	100
Contamination with homologous antigen (%)		2.0	3.0
Contamination with extraneous column protein (%)		2.0	0.0
Homologous antigen adsorbed			
Weight in mg. (% based on antibody content of column)		13.3 (8)	13.2 (11)

Table 3. *Separation of fluorescent ovalbumin and ^{131}I -labelled human serum albumin from lysozyme or ribonuclease*

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

	Immunosorbent	Anti-ovalbumin	Anti-(human serum albumin)
Nature of column			
Total protein (mg.)		1000	1000
Total antibody (mg.)		280	160
Mixture of antigens added to column			
Fluorescent ovalbumin (mg.)		22.0	—
^{131}I -labelled human serum albumin (mg.)		—	17.6
Lysozyme or ribonuclease (mg.)		22.0	17.6
Heterologous antigen recovered		Lysozyme	Ribonuclease
Recovery (%)		90.0	94.0
Contamination with homologous antigen (%)		3.0	3.2
Contamination with extraneous column protein (%)		0.0	2.0
Homologous antigen adsorbed			
Weight in mg. (% based on antibody content of column)		21.3 (7.6)	20.7 (7.4)

buffered saline (1). By increasing the sodium chloride concentration of the buffer to that of phosphate-buffered saline (2) the solubility of the preparations derived from thiolated materials containing 9 thiol groups/mol. was decreased to 5–10 $\mu\text{g.}$ of protein/ml. The specificity of antigen uptake and the capacity for homologous antigen were unaffected by this increase in salt concentration. All subsequent work was carried out under these conditions and with materials prepared from globulins containing 9 thiol groups/mol. As with cellulose-linked immunosorbents (Gallop *et al.* 1966), the protein content of the effluent increased if elution was stopped, e.g. overnight, and then restarted. However, the equilibrium value was soon restored.

The results of typical experiments on the separation of fluorescent ovalbumin and ^{131}I -labelled human serum albumin by using immunosorbents prepared from anti-ovalbumin and anti-(human serum albumin) respectively are given in Table 2. The results of similar experiments in which lysozyme and ribonuclease were used as the heterologous antigens are given in Table 3.

In every case the low degree of contamination of the recovered heterologous antigen by homologous antigen and by extraneous column protein was checked by cellulose acetate electrophoresis and by precipitin analyses. As before (Gallop *et al.* 1966) the weights of antigen-antibody precipitates under optimum conditions were the same for equal weights of recovered and original antigen.

DISCUSSION

γ -Globulin concentrates containing anti-ovalbumin and anti-(human serum albumin) respectively were thiolated and rendered insoluble by conversion into disulphide-linked polymers. These have been used successfully for the removal of their homologous antigens from mixtures with other antigens.

The aim of the present work was to render antibodies insoluble while still retaining their biological activity and without recourse to insoluble matrices, which are always a potential source of non-specific adsorption. It is evident that, with respect to the specificity of uptake of those proteins that have been studied, there is little to choose between the disulphide-polymerized and cellulose-linked immunosorbents (cf. Gallop *et al.* 1966). However, the yield of γ -globulin obtained as insoluble disulphide-linked protein was higher than that coupled in reaction with diazotized cellulose. Further, the physical bulk of a disulphide-linked immunosorbent column for a given antibody capacity was considerably less than the equivalent cellulose-immunosorbent column and this resulted in higher flow rates.

Benesch & Benesch (1958) described a method for thiolating gelatin at pH 7.5 in the presence of Ag^+

ions. This method, which required strict pH control and a lengthy procedure to remove Ag^+ ions from the 'protein mercaptide', was not used in the present work. The simpler method used involved a short reaction time at pH 10.6 and 0° and did not impair the ability of thiolated antibody (containing not more than 9 thiol groups/mol.) to precipitate antigen at or near the region of optimum proportions for untreated antibody.

The fall in capacity for homologous antigen with increasing numbers of thiol groups/mol. of thiolated globulin could be explained in at least two ways. Heavy thiolation either predisposed towards a higher degree of three-dimensional cross-linking with concomitant burying of some of the binding sites or increased the probability of chemical interference with the binding site itself.

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