Separation of Antigens by Immunological Specificity

STUDIES ON THE DESORPTION OF HOMOLOGOUS ANTIGEN FROM DISULPHIDE-LINKED ANTIBODY IMMUNOSORBENTS

By J. W. CHIDLOW,* J. STEPHEN AND H. SMITH

Department of Microbiology, University of Birmingham, Edgbaston, Birmingham 15, U.K.

(Received 10 November 1969)

1. Glycine–hydrochloric acid buffer, pH 2.2, desorbed ^{131}I -labelled human serum albumin (100%), lysozyme (100%), ovalbumin (90%), fluorescent ovalbumin (50–60%) and fluorescent human γ -globulin (20%) from their respective homologous disulphide-linked antibody immunosorbents; reasons are suggested for the low recoveries of the fluorescently labelled proteins. 2. Approx. 40% of the recovered ^{131}I -labelled human serum albumin and fluorescent ovalbumin was desorbed above pH 6.0, but lysozyme was not eluted until the pH was 3.0 or below. 3. In all cases where high recoveries of antigen were obtained, the immunosorbents could be regenerated and recycled at least four times with full retention of specificity and minimal diminution of capacity. 4. The desorbed antigens were unchanged when compared with the original antigens by quantitative precipitin, specific-radioactivity, fluorescent and enzymic analyses and by cellulose acetate electrophoresis. 5. Desorption of antigen with a variety of reagents was investigated. These reagents were less satisfactory than glycine–hydrochloric acid.

Stephen, Gallop & Smith (1966) and Chidlow (1968) used disulphide-linked immunosorbents for fractionating mixtures of soluble antigens by the specific removal of unwanted antigens, leaving the required antigen in solution. Wood, Stephen & Smith (1968) have used disulphide-linked immunosorbents in a more direct manner for the purification of Semliki Forest virus, grown in chick-embryo fibroblasts. The virus was adsorbed at neutral pH by the immunosorbent (derived from rabbit antisera raised against the virus grown in mouse brain) and subsequently desorbed by treatment with alkaline buffers. The present paper describes similar attempts to purify soluble protein antigens, the ultimate objective being to define conditions under which an antigen can be desorbed from its homologous immunosorbent in a biologically active state with concomitant regeneration of the immunosorbent. Three groups of experiments were performed.

In the first group attempts were made to unravel an apparent anomaly encountered in previous experiments (J. Stephen, unpublished work). ^{131}I -labelled human serum albumin and fluorescent ovalbumin could not be desorbed from their respective disulphide-linked immunosorbents by NaCl–HCl, pH 3.2, despite the fact that antibodies

(including rabbit anti-ovalbumin) could be recovered from cellulose-linked antigen immunosorbents by using this solvent (Gurvich, Kapner & Nezlin, 1959). There were at least two reasons as to why these experiments were unsuccessful. First, an inherent instability of these antigens in the desorbing conditions could have led to denaturation and non-specific readsorption to the immunosorbent. Secondly, the lack of success may have been due to failure to disrupt the antigen–antibody bonds when antibody and not antigen was the solid-phase immunoreactant. Experiments were therefore carried out with an immunoglobulin (human γ -globulin, fluorescein-labelled to facilitate detection) as antigen in an attempt to discriminate between these two possibilities and thus reveal important aspects of this process.

In the second group of experiments various neutral (or near-neutral) solvents were used in attempts to achieve the objectives stated above.

The third and most successful group of experiments was carried out with a glycine–HCl buffer, pH 2.2, similar to that used by Avrameas & Ternynck (1967) in a batch process to desorb several proteins that they claimed were specifically adsorbed by various antibody polymers rendered insoluble by ethyl chloroformate. In our work desorptions were attempted from columns, since Galvez (1966) had reported that tobacco-mosaic

* Present address: Canada Department Agriculture Research Station, Vancouver, B.C., Canada.

virus was eluted before an acidic-pH front from a column of the virus adsorbed on cellulose-linked anti-(tobacco-mosaic virus).

A preliminary report of this work has been given (Chidlow, Stephen & Smith, 1968).

MATERIALS AND METHODS

All materials and methods were as described by Gallop, Tozer, Stephen & Smith (1966) and Stephen *et al.* (1966) with the following modifications.

Buffers and reagents

All chemicals were A.R. grade. NaCl (1%, w/v) was adjusted to pH 3.2 by the addition of 0.1 M-HCl and 0.1 M-sodium acetate (100 ml) was adjusted to pH 2.3 by the addition of 13.0 ml of 1 M-HCl. The following were made up as indicated: glycine-HCl buffer, pH 2.2 and 10.4, contained 21.5 g of glycine added to 1 litre of 0.2 M-HCl; glycine-NaOH buffer, pH 9.7, contained 58.2 g of glycine added to 1 litre of 0.2 M-NaOH; carbonate buffer, pH 10.0, contained 170 g of K_2CO_3 and 47.0 g of $NaHCO_3$ /l.

Phosphate buffer, pH 7.0 and 10.056, contained 4.76 g of $Na_2HPO_4 \cdot 12H_2O$ and 2.5 g of $NaH_2PO_4 \cdot 2H_2O$ /l, and phosphate buffer, pH 7.0 and 10.007, was obtained by dilution; the compositions of these buffers were correctly given by Stephen *et al.* (1966) but incorrectly described as 10.4 and 0.05 respectively.

In this paper phosphate-buffered saline (2) (Gallop *et al.* 1966) is referred to as phosphate-buffered saline.

Antigens

Fluorescent human γ -globulin. Human γ -globulin (lot no. Rd038; A.B. Kabi, Stockholm, Sweden) was conjugated with fluorescein isothiocyanate (BDH Chemicals Ltd., Poole, Dorset, U.K.) by the method of Nairn (1964). The substituted protein contained 7.4 mol of fluorescein/mol of human γ -globulin, was indistinguishable from human γ -globulin by immunodiffusion and was estimated as described for fluorescent ovalbumin by Gallop *et al.* (1966).

Antibodies

Rabbit anti-lysozyme and rabbit anti-(human γ -globulin). These antisera were produced in rabbits by using antigen-antibody complexes prepared according to the schedules described by Gallop *et al.* (1966) for anti-ovalbumin and anti-(human serum albumin). γ -Globulin concentrates were prepared as described by Smith, Tozer, Gallop & Scanes (1962).

Immunosorbents

Preparation of the immunosorbents. This was carried out as described by Stephen *et al.* (1966), with minor modifications to facilitate handling of the rubber-like polymer of disulphide-linked protein.

The paste (Stephen *et al.* 1966) was ground in a mortar and pestle and this was repeated several times during the cross-linking reaction. When necessary an Omnimixer (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) was used (chamber cooled in ice; time and speed as appropriate) in addition to the glass tissue-grinder to disperse the poly-

mer, but excessive homogenization (particularly with the Omnimixer) resulted in too fine suspensions and low flow rates of subsequent columns. Immunosorbents to be eluted with glycine-HCl buffer, pH 2.2, were washed twice with this buffer before the final washes with phosphate-buffered saline.

Use of immunosorbent columns. To each column, prepared as described by Stephen *et al.* (1966), was added a quantity of homologous antigen together with an equal quantity of heterologous antigen (e.g. fluorescent ovalbumin and ^{131}I -labelled human serum albumin for anti-ovalbumin immunosorbents), and elution with phosphate-buffered saline was continued until no protein was detectable (E_{280} and radioactivity) in the eluate. The column was then eluted with the desorbent under test and the eluate was continuously monitored at 280 nm with a Uvichord 11-Recorder 6520H-2 apparatus (LKB Produkter A.B., Stockholm, Sweden). In preliminary experiments with acidic buffers the pH of individual fractions (1.0 ml) was measured with a micro dual glass-reference electrode in conjunction with an EIL 23A pH-meter (Electronic Instruments Ltd., Richmond, Surrey, U.K.). In subsequent experiments the pH of column eluates was continuously monitored by using a pH-cell placed between the column and the Uvichord 11 instrument. The volume intervals between the column, the pH-cell, the Uvichord 11 and the fraction collector were kept to a minimum and were determined each time the apparatus was freshly assembled. In the early experiments the pH of each fraction was adjusted to neutrality immediately the pH had been determined. With continuous measurement of the pH, the eluate was collected (in fractions of volume not less than 1 ml) into a neutralizing buffer (2 ml); a borate buffer, pH 8.6 and 10.4, or 1.0 M- K_2HPO_4 was used for acidic eluates, and 1.0 M- NaH_2PO_4 was used for alkaline eluates.

When the desorbed homologous antigen was recovered in high yield, the columns were immediately re-equilibrated with phosphate-buffered saline, and a second adsorption-desorption cycle was carried out in which, unless otherwise indicated, the quantities of homologous and heterologous antigens and other conditions were identical with those of the first cycle. In most cases the columns were then re-equilibrated and a further two adsorption-desorption cycles carried out under conditions identical with the first except that no heterologous antigen was loaded with the homologous antigen. A fifth and final cycle was carried out under conditions identical with the first, including the heterologous antigen.

Several tests [quantitative precipitin analysis, micro-immunodiffusion, cellulose acetate electrophoresis and enzyme assays (where appropriate)] were carried out on desorbed antigens to determine whether or not their biological integrity had been impaired by the adsorption-desorption process.

RESULTS

Attempts to desorb fluorescent human γ -globulin from homologous immunosorbent with sodium chloride-hydrochloric acid, pH 3.2

Three immunosorbent columns (1600 mg of total protein; 55 mg of antibody) derived from anti-

(human γ -globulin) and to which fluorescent human γ -globulin (4.5mg) had been adsorbed were eluted with NaCl-HCl, pH 3.2. The total yield of fluorescent material detected in the neutralized eluate, the pH of which fell to 3.2 only after elution with approx. 400ml of solvent, was 0.5mg; the fluorescent material was spread throughout all the fractions. Further lowering of the pH to 2.3 (with NaCl-HCl, pH 2.3) did not increase the yield of fluorescent material.

Attempts to desorb ^{131}I -labelled human serum albumin from homologous immunosorbents with various neutral and near-neutral reagents

Six replicate columns, each containing 700mg of total protein/90mg of antibody, were prepared, and 9–10mg of ^{131}I -labelled human serum albumin was adsorbed. Each column was eluted with one of the following six reagents with the indicated result. Guanidine hydrochloride (2.0M in phosphate-buffered saline) and 2.0M-ammonium thiocyanate desorbed 27 and 30% respectively of the bound ^{131}I -labelled human serum albumin, but both dissolved some column protein and caused the flow rate to cease. Potassium iodide (5.5M in tris buffer, pH 8.2) dissolved the immunosorbent protein completely. Aqueous 10% (v/v) dioxan, 1.0M-glycine and 2.0M-glycine (in phosphate-buffered saline) desorbed 13, 28 and 0% respectively of the bound ^{131}I -labelled human serum albumin, but did not significantly dissolve the immunosorbent.

Desorption of antigens with glycine-hydrochloric acid buffer, pH 2.2

Disulphide-linked anti-(human serum albumin). Immunosorbent columns were prepared from several batches of anti-(human serum albumin) (see Table 1), loaded with ^{131}I -labelled human serum albumin and eluated with glycine-HCl buffer, pH 2.2. ^{131}I -labelled human serum albumin was desorbed in high yield in a single peak across which a pH gradient could be demonstrated; fractions were combined into pools A and B (Fig. 1). Pool A (approx. 40% of the desorbed ^{131}I -labelled human serum albumin) represented the near-neutral fractions collected between pH 7.0 and 6.3 and pool B the fractions collected between pH 3.5 (mostly) and 2.2. The recovered ^{131}I -labelled human serum albumin in pools A and B was indistinguishable from the original material by quantitative precipitin, specific-radioactivity, immunodiffusion and electrophoretic analyses. The exact position of the ^{131}I -labelled human serum albumin peak in relation to the pH gradient was determined in subsequent recycling runs (of which a further four were carried out) by continuously monitoring the pH of the eluate (Fig. 1). The results of these experiments are summarized in Table 1.

Desorption of antigen was also investigated under a variety of conditions. Columns on which ^{131}I -labelled human serum albumin was adsorbed were first eluted by alkaline buffers and then by glycine-HCl buffer, pH 2.2 (after the pH of the

Table 1. *Recovery of ^{131}I -labelled human serum albumin from anti-(human serum albumin) immunosorbents by desorption with glycine-hydrochloric acid buffer, pH 2.2, and subsequent regeneration and re-use of such immunosorbents*

This table summarizes the results of experiments in which ^{131}I -labelled human serum albumin was desorbed from anti-(human serum albumin) immunosorbent columns by elution with glycine-HCl buffer, pH 2.2. The immunosorbents were subsequently regenerated by re-equilibration with phosphate-buffered saline and re-used in another four adsorption-desorption cycles. The specificity of adsorption was tested during cycles 1, 2 and 5. The data are representative of five similar experiments. Total protein was 1680mg; antibody protein was 135mg. Abbreviations: HSA, human serum albumin; Flov, fluorescent ovalbumin.

	Cycle	...	1	2	3	4	5
¹³¹ I-labelled HSA applied (mg)			15.0	15.0	15.0	15.0	15.0
Flov applied (mg)			15.0	15.0	—	—	15.0
Flov recovered (% of load)			97	94	—	—	97
¹³¹ I-labelled HSA adsorbed (mg)			14.6	14.0	14.3	13.5	12.2
(% of initial capacity)			100	96	98	92	88
¹³¹ I-labelled HSA desorbed (% adsorbed):							
Fraction A, collected between pH 7.0 and	...	pH 6.3	pH 6.4	pH 6.1	pH 6.6	pH 6.3	
(i) by radioactivity assay		42	41	48	40	45	
(ii) by total protein assay		45	40	50	36	47	
Fraction B, collected between pH 2.2 and	...	pH 2.6	pH 5.2	pH 3.5	pH 4.0	pH 2.5	
(i) by radioactivity assay		48	56	50	51	45	
(ii) by total protein assay		50	59	46	55	44	

eluate had reached that of the alkaline buffer); three replicate columns were used. The first was treated with carbonate buffer, pH 10.6, then glycine-HCl buffer, pH 2.2, and 84% of the adsorbed ^{131}I -labelled human serum albumin was recovered as follows: 34%, pH 7.0–10.6; 30%, pH 10.6–7.5; 20%, pH 7.5–2.2. The second was treated with

carbonate buffer, pH 10.0, then glycine-HCl buffer, pH 2.2, and of a total yield of 70% of the adsorbed ^{131}I -labelled human serum albumin only 15% was recovered during the rising pH gradient. When the third column was eluted with glycine-NaOH buffer, pH 9.7, and then with glycine-HCl buffer, pH 2.2, 62% of the bound ^{131}I -labelled human serum albumin was recovered of which not more than 2% was recovered between pH 7.0 and 9.7.

Disulphide-linked anti-lysozyme. Five replicate anti-lysozyme immunosorbent columns were prepared (see Table 2), loaded with lysozyme, and four were desorbed with glycine-HCl buffer, pH 2.2. This was followed by the recycling programme described above for ^{131}I -labelled human serum albumin. High yields of the bound lysozyme were obtained. The recovered lysozyme was indistinguishable from the original material as judged by quantitative precipitin, enzymic, immunodiffusion and electrophoretic analyses. However, no significant near-neutral fraction (cf. ^{131}I -labelled human serum albumin) was obtained (Fig. 2). The results of these experiments are summarized in Table 2. The fifth column was desorbed with phosphate-HCl buffer, pH 2.2 and 1.0.4, containing no glycine, and 85% of the enzyme was recovered in a manner similar to that illustrated in Fig. 2.

Disulphide-linked anti-ovalbumin. Three replicate anti-ovalbumin columns were prepared (see Table 3), loaded with fluorescent ovalbumin and desorbed with glycine-HCl buffer, pH 2.2. The elution of fluorescent ovalbumin was similar to that described for ^{131}I -labelled human serum albumin (Fig. 1), but the total recovery of fluorescent ovalbumin was only 50–60% of the adsorbed material. The recovered fraction was identical with the original material as judged by quantitative precipitin, fluorimetric, immunodiffusion and electro-

Fig. 1. Desorption of ^{131}I -labelled human serum albumin from an anti-(human serum albumin) immunosorbent column. ^{131}I -labelled human serum albumin (14.6 mg) was adsorbed at pH 7.0 by the immunosorbent (1680 mg of total protein; 135 mg of antibody) from a mixture containing ^{131}I -labelled human serum albumin (15.0 mg) and fluorescent ovalbumin (15.0 mg); 97% of the fluorescent ovalbumin was recovered. The amounts of adsorbed ^{131}I -labelled human serum albumin eluted by glycine-HCl buffer, pH 2.2, were: 42%, fraction A (pH 7.0–6.3); 48%, fraction B (pH 6.4–2.2). O, pH; ●, ^{131}I -labelled human serum albumin (HSA).

Table 2. Recovery of lysozyme from anti-lysozyme immunosorbents by desorption with glycine-hydrochloric acid buffer, pH 2.2, and subsequent regeneration and re-use of such immunosorbents

This table summarizes the results of experiments in which lysozyme was desorbed from anti-lysozyme immunosorbent columns by elution with glycine-HCl buffer, pH 2.2. The immunosorbents were subsequently regenerated, by re-equilibration with phosphate-buffered saline, and re-used in another four adsorption-desorption cycles. The specificity of adsorption was tested during cycles 1, 2 and 5. The data are representative of four similar experiments. Total protein was 1250 mg; antibody protein was 155 mg. Abbreviations: Ly, lysozyme; Flov, fluorescent ovalbumin.

	Cycle ...	1	2	3	4	5
Ly applied (mg)		40.0	40.0	40.0	40.0	40.0
Flov applied (mg)		40.0	40.0	—	—	40.0
Flov recovered (% of load)		97	94	—	—	98
Ly adsorbed (mg)		20.2	19.8	19.3	19.0	18.0
(% of initial capacity)		100	98	96	94	89
Ly desorbed (% adsorbed):						
(i) by enzyme assay		100	93	79	72	68
(ii) by total protein assay		97	100	74	71	72

phoretic analyses. Two columns were recycled another four times as described for anti-(human serum albumin); the results of these experiments are summarized in Table 3. The third column was used in one of two experiments to investigate the possible reasons for the low recoveries of fluorescent ovalbumin. Unlabelled ovalbumin (11.0mg) was applied to this column; 10.2mg was adsorbed.

Fig. 2. Desorption of lysozyme from an anti-lysozyme immunosorbent column. Lysozyme (20.0mg) was adsorbed at pH 7.0 by the immunosorbent (1250mg of total protein; 155mg of antibody protein) from a mixture containing lysozyme (40.0mg) and fluorescent ovalbumin (40.0mg); 97% of the fluorescent ovalbumin was recovered. The amount of the adsorbed lysozyme eluted by glycine-HCl buffer, pH 2.2, was 100% (pH 3.0-2.2). ○, pH; ●, lysozyme.

Desorption with glycine-HCl buffer, pH 2.2, recovered approx. 90% of the adsorbed protein nitrogen.

In the second experiment two samples of fluorescent ovalbumin and two of ovalbumin (each 500 µg/ml of phosphate-buffered saline) were dialysed against three separate changes of glycine-HCl buffer, pH 2.2. The dialysis bags, now containing various quantities of precipitated material, were placed in five successive changes of phosphate-buffered saline. Finally the contents were clarified by centrifuging; 40-45% of fluorescent ovalbumin and 60-63% of the ovalbumin remained in solution.

Disulphide-linked anti-(human γ-globulin). For these experiments, four immunosorbent columns were used each containing 385mg of total protein of which 73mg was antibody. The columns were exposed to 14.0, 6.0, 3.4 and 3.4mg of fluorescent human γ-globulin, resulting in the adsorption of 6.0, 3.6, 3.0 and 3.2mg of antigen respectively. Three of the columns were eluted with glycine-HCl buffer, pH 2.2, and one (containing 3.0mg of antigen) with guanidine hydrochloride, pH 2.2. Although the yields of fluorescent human γ-globulin were low (10-20%) the material was partitioned into a near-neutral and an acidic fraction. One of the three columns (which had been treated with glycine-HCl buffer, pH 2.2) was recycled after re-equilibration with phosphate-buffered saline under conditions identical with those of the first cycle; 89% of the initial capacity was realized.

A dialysis-stability experiment was carried out similar to that described above for fluorescent oval-

Table 3. *Recovery of fluorescent ovalbumin from anti-ovalbumin immunosorbents by desorption with glycine-hydrochloric acid buffer, pH 2.2, and subsequent regeneration and re-use of such immunosorbents*

This table summarizes the results of experiments in which fluorescent ovalbumin was desorbed from anti-ovalbumin immunosorbent columns by elution with glycine-HCl buffer, pH 2.2. The immunosorbents were subsequently regenerated, by re-equilibration with phosphate-buffered saline, and re-used in another four adsorption-desorption cycles. The specificity of adsorption was tested during cycles 1, 2 and 5. The data are representative of two similar experiments. Total protein was 630 mg; antibody protein was 113 mg. Abbreviations: Flov, fluorescent ovalbumin; HSA, human serum albumin.

	Cycle	...	1	2	3	4	5
Flov applied (mg)			11.0	11.0	11.0	11.0	11.0
¹³¹ I-labelled HSA applied (mg)			11.0	11.0	—	—	11.0
¹³¹ I-labelled HSA recovered (% of load)			96	102	—	—	97
Flov adsorbed (mg)			9.8	9.7	9.0	8.7	8.1
(% of initial capacity)			100	99	92	89	82
Flov desorbed (% adsorbed):							
Fraction A, collected between pH 7.0 and	...		pH 6.4	pH 6.3	pH 6.1	pH 6.6	pH 6.3
(i) by fluorimetry			20	19	26	17	21
(ii) by total protein assay			21	22	25	15	23
Fraction B, collected between pH 2.2 and	...		pH 3.4	pH 4.0	pH 3.1	pH 2.5	pH 2.5
(i) by fluorimetry			35	38	37	33	33
(ii) by total protein assay			33	39	36	36	33

bumin. The final supernatants contained 19% of the original fluorescence and 43% of the total protein with fluorescent human γ -globulin, and 95% of the native human γ -globulin.

DISCUSSION

The aim of these studies was to develop techniques for the desorption of bound antigens from homologous disulphide-linked immunosorbents, avoiding, if possible, the use of harsh reagents and extreme conditions of pH, ionic strength, temperature etc. The desorption of antigens with neutral or near-neutral reagents has not yet been achieved. However, given moderate stability to low pH for relatively short periods, some antigens can be desorbed from disulphide-linked immunosorbents with acidic glycine-containing buffers, and in some cases up to 40% of antigen was recovered above pH 6.0. Where high yields of antigen were recovered, columns could be recycled at least four times without loss of specificity and only a slight fall in antigen-binding capacity. The studies by Wood *et al.* (1968) on the purification of Semliki Forest virus with disulphide-linked immunosorbents indicated similar possibilities for recycling.

Elution of immunosorbent columns containing ^{131}I -labelled human serum albumin with glycine-HCl buffer, pH 2.2, desorbed this antigen in high yield and in a sharp peak straddling the pH gradient that developed during the elution process. There was no clear separation between the eluted protein and the pH gradient (cf. desorption of tobacco-mosaic virus; Galvez, 1966). This could be explained by assuming that all the antigen molecules are dissociated from antibody sites under the same set of conditions of pH, ionic strength and glycine concentration (these being achieved as the buffer travels down the column), and that the form of the protein profile is caused by diffusion of the antigen. Alternatively, immunosorbents may have been derived from antibodies exhibiting a spectrum of binding affinities. If this were so, antigen molecules would be expected to desorb under different conditions, those molecules that were bound by the least avid antibody sites being most easily desorbed. The observed result may be a combination of both effects. By the criteria used, the desorbed ^{131}I -labelled human serum albumin in both the neutral and acidic pools was not denatured.

The attempts to increase the yield of ^{131}I -labelled human serum albumin desorbed at near-neutral pH by first pre-equilibrating the columns with alkaline buffers were disappointing. However, the experiments suggested the possibility of manipulating the pH conditions such that other antigens of differing pH-stability might be recovered in appropriately suitable conditions.

Lysozyme was desorbed in good yields by elution with glycine-HCl buffer, pH 2.2, but, in contrast with the ^{131}I -labelled human serum albumin system, significant quantities were not recovered before the pH of the eluate was at or below 3.0. The reasons for this might be several. First, sufficient conformational distortion of the antigenic sites of lysozyme may not take place to allow dissociation until the environmental pH approaches 2.2. Secondly, reversible readsorption of the desorbed lysozyme on the immunosorbent protein or the Sephadex G-200 (present as a solid diluent in these columns) may have occurred under the desorbing conditions. Thirdly, the lysozyme may have been retarded (relative to ^{131}I -labelled human serum albumin and fluorescent ovalbumin) by the molecular-exclusion properties of the Sephadex G-200. Unfortunately, this last idea could not be tested experimentally, since stable homogeneous suspensions of disulphide-linked protein could not be made with any other grade of Sephadex (in particular Sephadex G-25) as the densities of the materials are too disparate.

Fluorescent ovalbumin was not obtained in good yields when desorbed with glycine-HCl buffer, pH 2.2. Either fluorescent ovalbumin was not completely dissociated from all the specific antibody sites, or it was dissociated only to be readsorbed non-specifically. The first explanation is improbable in that during the fifth cycle 80% of the antigen-binding capacity experienced during the first cycle was realized. It is more likely in view of the dialysis-stability experiments that the labelled antigen was dissociated and that a fraction was denatured and trapped as precipitated material or readsorbed non-specifically to the column protein.

The less extensive experiments with the fluorescent human γ -globulin system and glycine-HCl buffer, pH 2.2, produced similar results, which may be explained on a similar basis as that offered above for the fluorescent ovalbumin system.

All successful experiments reported here were achieved by the production of sharp pH gradients and not merely the application of acidic solutions. This may partly explain the failure to desorb fluorescent human γ -globulin with acidic saline solutions when, as a result of the non-buffering capacity of NaCl-HCl, combined with the considerable buffering capacity of the immunosorbent protein, no dramatic pH gradients were produced.

A final observation (which might have wider implications) concerns the use of chemically modified proteins. Both fluorescein-labelled ovalbumin (J. W. Chidlow, unpublished work) and human γ -globulin give perfect reactions of identity with their native counterparts in immunodiffusion tests at pH 7.0, but clearly their behaviour in acidic media is markedly different. This experience

underlines a need for caution in the use of chemically altered proteins in quantitative studies such as are described in this paper.

We thank the Director, Dr C. E. Gordon Smith, and Dr J. Keppie and his colleagues of the Microbiological Research Establishment, Porton, nr. Salisbury, Wilts., U.K., for providing the antisera. J.W.C. was in receipt of a Science Research Council Research Scholarship.

REFERENCES

- Avrameas, S. & Ternynck, T. (1967). *J. biol. Chem.* **242**, 1651.
- Chidlow, J. W. (1968). Ph.D. Thesis: University of Birmingham.
- Chidlow, J. W., Stephen, J. & Smith, H. (1968). *Nature, Lond.*, **218**, 773.
- Gallop, R. G. C., Tozer, B. T., Stephen, J. & Smith, H. (1966). *Biochem. J.* **101**, 711.
- Galvez, G. E. (1966). *Virology*, **28**, 171.
- Gurvich, A. E., Kapner, R. B. & Nezlin, R. S. (1959). *Biokhimiya*, **24**, 144.
- Nairn, R. C. (1964). In *Fluorescent Protein Tracing*, 2nd ed., p. 14 *et seq.* Ed. by Nairn, R. C. Edinburgh and London: E. and S. Livingstone Ltd.
- Smith, H., Tozer, B. T., Gallop, R. C. & Scanes, F. S. (1962). *Biochem. J.* **84**, 74.
- Stephen, J., Gallop, R. G. C. & Smith, H. (1966). *Biochem. J.* **101**, 717.
- Wood, K. R., Stephen, J. & Smith, H. (1968). *J. gen. Virol.* **2**, 313.