SUMMARY

1. [^{14}C]Glucose cycloacetoacetate was injected into chloretone-treated rats and the ascorbic acid isolated from the urine was degraded to determine the activities of carbon atoms 1, 2 and 6.

2. Intraperitoneal injection of [^{14}C]glucose cycloacetoacetate resulted in excretion of ascorbic acid with 64–71% of the activity in C-6 and 8–10% in C-1.

3. Ascorbic acid recovered from the urine of rats injected with [^{14}C]glucose cycloacetoacetate contained 66–68% of the activity in C-1 and 8–11% in C-6.

4. The results indicate that the glucose moiety of glucose cycloacetoacetate remains intact and simultaneously it undergoes inversion.

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Biochem. J. (1962) **84**, 74

Separation of Antigens by Immunological Specificity

1. METHOD FOR SEPARATING INDIVIDUAL ANTIGEN-ANTIBODY COMPLEXES FROM MIXED ANTIGENS AND ANTIBODIES

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This paper describes the initial stage of attempts to use serological specificity for the fractionation of protein and other antigens. The potential of such a method for resolving mixtures of antigens with similar chemical properties is indicated by the serological differentiation of bacterial strains and more recently by the results of immunological analysis of mixed antigens by gel diffusion and by immunoelectrophoresis. The success of such a method of fractionation would depend on three

main conditions. (a) A method should be found for isolating individual antigen-antibody complexes from the interaction of mixtures of unknown antigens with mixtures of corresponding antibodies. The authors are not aware of any previous method for obtaining monospecific complexes from such mixtures. Monospecific antibody has been prepared by immunochemical methods [see review by Isliker (1957)], but only when the appropriate single antigen was available either for preparing the

antiserum or for forming the immune complex. (b) The formation of the complex with antibody should not denature the antigen or irreversibly destroy its biological activity. We cannot be sure that the antigen will retain its character during complexing in all cases but there are some examples where this appears to be so. The restoration of toxicity by diluting a neutral mixture of toxin and an antiserum of low avidity (Wilson & Miles, 1955) indicates this, as does the reactivation of some neutral complexes of toxins, antigens and pathogenic bacteria and viruses by digesting the antibody with papain (Kalmanson & Bronfenbrenner, 1943). Further, antigen-antibody complexes of some biologically active antigens (e.g. catalase) retain some of the latter's biological activity (Cinader, 1957). (c) The antibody-antigen linkages should be broken and all antibody removed by methods which do not denature the antigen or destroy its biological activity. Specific antibody has been prepared by removing some antibody from antigen-antibody complexes [for review of methods see Tozer, Cammack & Smith (1962)]. The remainder of the antibody seems more firmly held (Islaker, 1957), and the final removal of all antibody from the antigen has not been accomplished except for polysaccharide antigens, when antibody can be preferentially destroyed.

We shall attempt to solve these problems, using, at first, mixtures of antigens (and their corresponding antibodies) which are known and labelled to facilitate analysis. Work described in this paper provides a solution to the first of the three problems.

MATERIALS AND METHODS

Antigens

Ovalbumin. The ovalbumin was a thrice-recrystallized sample prepared by the method of Kekwick & Cannan (1936). After removal of sodium sulphate by dialysis the sample was freeze-dried.

Fluorescent ovalbumin. Ovalbumin was treated with fluorescein II isothiocyanate. The latter was prepared from aminofluorescein II by the method of Chadwick & Slade (1960). Their method of conjugation of this reagent to protein was modified to give a decreased fluorescein content and hence to yield a product with antigenic properties practically identical with those of the original ovalbumin.

Ovalbumin (300 mg.) in 'phosphate-buffered saline', pH 7.8 (7 ml. of a solution containing 25.5 g. of Na_2HPO_4 , $12\text{H}_2\text{O}$, 0.5 g. of KH_2PO_4 and 10.0 g. of NaCl/l.), was allowed to react with fluorescein II isothiocyanate [12 mg. in 7 ml. of 2% (w/v) NaHCO_3] at 20° for 30 min. The solution was freed from excess of fluorescent material as rapidly as possible by dialysis at 3° against phosphate-buffered saline, pH 7.8 (4-5 days); it was then dialysed against water and freeze-dried.

The yield, based on original ovalbumin, varied in different preparations from 90 to 100%. The fluorescein content, as determined by comparing the light-absorption

values in 0.1 M-phosphate buffer, pH 11.5, at λ_{max} (497 m μ) with that of standard solutions of fluorescein II isothiocyanate at λ_{max} (495 m μ ; there is a slight shift in absorption peak on conjugation), varied from 0.6 to 1.2%. The intensity of fluorescence of the fluorescent-ovalbumin solutions was approximately one-quarter of that from an equivalent concentration of uncombined fluorescein.

Haemoglobin. Red cells were removed from citrated horse blood and washed six times with 0.9% NaCl (volume equal to the original volume of blood). The cells (250 ml.) were suspended in water (250 ml.) and dialysed at 0° against water (14 l., changed daily) for 5 days. Cell debris was removed by centrifuging and the supernatant was freeze-dried and stored at 0°.

Human serum albumin. This was obtained from the Blood Products Laboratory, Lister Institute of Preventive Medicine, Elstree, Herts. Batch E.P.A. 133, AP3 was used; by moving-boundary electrophoresis it appeared to contain not less than 99% of albumin but it showed a band of β -globulin when examined by electrophoresis on an Oxoid cellulose acetate membrane (W. d'A. Maycock, personal communication).

^{125}I -labelled human serum albumin. This was supplied by The Radiochemical Centre, Amersham, Bucks. (code no. 1B17), and was made from albumin (provided by the Blood Products Laboratory, Lister Institute) which had been heated to 60° for 10 hr. in the presence of a small amount of sodium caprylate (J. S. Glover, personal communication). A solution of the product (approx. 2%, w/v) in 0.9% NaCl contained not less than 100 mc/ml. The product contained less than 0.2% of I and not more than 2% of the total radioactivity was in the form of free iodide. When the radioactivity of the sample was too large for the experiment, the sample was diluted with the uniodinated human serum albumin described above.

Antibodies

Rabbit antiovalbumin I. To a solution of ovalbumin (0.5%) and ammonium alum (0.05%) in 0.9% NaCl was added N-NaOH until maximum precipitation of ovalbumin occurred (at approx. pH 7.6). Rabbits (Copenhagen white) were given intravenously 0.5 ml. of this suspension on the 1st, 3rd and 5th days, 1.0 ml. on the 8th, 10th and 12th days, 2.0 ml. on the 15th, 17th and 19th days, and 4.0 ml. on the 22nd, 25th and 27th days. On the 33rd day, the rabbits were bled out; serum was prepared and kept at 0-2° with Merthiolate (0.01%). γ -Globulin was separated from this antiserum as described below.

Rabbit antihaemoglobin I. Rabbits (Copenhagen white) were given: (a) intracutaneously, 1 ml. of Freund's adjuvant (Freund & McDermott, 1942) mixed with 4, 8, 16 and 16 mg. of haemoglobin [as a 20% (w/v) solution in 0.9% NaCl] on the 1st, 5th, 8th and 12th days respectively; (b) intraperitoneally, 60 mg. of haemoglobin on the 34th day; (c) intravenously, 20 mg. of haemoglobin on the 35th, 37th and 39th days; (d) intraperitoneally, 60 mg. of haemoglobin on the 55th day; (e) intravenously, 20 mg. of haemoglobin on the 56th, 58th and 60th days. Ten days after the final injection, the animals were bled from the ear; serum was prepared and kept at 0-2° with Merthiolate (0.01%). At intervals, the same animals were used for preparing more antiserum by repeating operations (b)-(e). γ -Globulin was prepared from this antiserum as described below.

Rabbit anti-(human serum albumin). Rabbits (Agouti type bred by Dr Paterson of Allington Farm, Porton) were given, intravenously, 5 mg. of human serum albumin [as a 5% (w/v) solution in 0.9% NaCl] on the 1st, 3rd and 5th days, 10 mg. on the 15th, 17th and 18th days, and 20 mg. on the 48th, 50th and 52nd days. On the 57th day the sera of individual rabbits were examined and those animals showing a poor antibody response were discarded. The remaining animals were left for 3 weeks and then were given three intravenous injections of 20 mg. of human serum albumin at 2-day intervals. After 5 days the animals were bled. Serum was prepared and kept at 0–2° with Merthiolate (0.01%). γ -Globulin was prepared from this antiserum as described below.

Separation of γ -globulin from rabbit antisera. The globulin fraction was isolated from antisera by a modification (Marrack, Hoch & Johns, 1951) of Kekwick's (1940) sodium sulphate fractionation of rabbit serum. The average recoveries of antibody, as determined by quantitative precipitin analyses, of rabbit antiovalbumin I, rabbit anti-haemoglobin I and rabbit anti-(human serum albumin) were respectively 66% (mean of 13 preparations), 82% (mean of 4 preparations) and 57% (mean of 8 preparations). Antibody isolated by this method is free of complement (J. R. Marrack, personal communication).

Method for isolating individual antigen-antibody complexes after their separation by gel diffusion

Gel diffusion of mixed antigens and mixed antibodies occurred over a large cross-sectional area in a specially designed diffusion cell. Appreciable quantities of individual antigen-antibody complexes which formed as parallel planes in the gel were separated by freezing the gel and then cutting sections of it with a freezing microtome.

Diffusion cell. The cell was machined from Perspex in three parts (two ends and a centre piece) which were held together by five brass bolts with wing-nuts to form a unit which was easily dismantled (Fig. 1). The gel ($\frac{1}{2}$ in. wide, cross-sectional area $1\frac{1}{2}$ in. $\times$ $1\frac{1}{2}$ in.) was in a space (A) enclosed between two parallel stainless-steel gauzes (B) held in frames set in recesses in the ends of the cell. The frames stopped leakage along the bottom and sides of the gel. Between the gauzes and the ends of the cell were narrow spaces (C; width 0.04 in., approx. capacity 1.5 ml.) for the reagents; the volume of these spaces could be varied by placing in them stainless-steel plates (D) of various thicknesses (0.036, 0.03 and 0.007 in.). At the bottom corners of these spaces for the reagents were two small air-access holes (E) which were usually closed with Plasticine.

The cell was made in various sizes; with sides of 0.5, 1, 1.5 and 2 in. The 1.5 in. $\times$ 1.5 in. cell was of optimum size and is described above. Relatively little product was obtained from the smaller cells, and the larger the cell the more difficult it was subsequently to arrange the gel sufficiently square on the microtome to allow clean isolation of two zones of precipitation.

Gel. To avoid denaturation of antigen-antibody precipitates a gel was required which could be removed from these precipitates at low temperatures. For this reason, agar, the gel used in most serological diffusion work, was replaced by gelatin. A solution of gelatin (15 g.) in warm (60–70°) phosphate-buffered saline, pH 7.3 (containing 4.25 g. of NaCl, 8.096 g. of Na_2HPO_4 and 2.438 g. of KH_2PO_4 /l.), was filtered through Millipore filters, and sodium azide (0.1%)

was added as a preservative. When cooled to 0–5°, this solution formed a solid gel which liquified at room temperature. Batches of gelatin differed to some extent in gel-forming ability and the concentration used was varied slightly from batch to batch.

Warm gelatin solution was poured into the space between the gauzes of the diffusion cell, the reagent spaces having been filled completely by inserting the thickest stainless-steel plates. The cells were placed in a sealed container at 0–5° for 16 hr. to allow the gel to set. The Plasticine in the air-access holes was pierced and, while the stainless-steel gauzes were held firmly in position, the steel plates were withdrawn, leaving free the spaces for the immunological reagents. If the air-access holes were not opened and the gauzes were not held firmly in position when the steel plates were withdrawn, the gel tended to pull away from the sides and bottom of the cell, leading to subsequent leakage of reagents. When the air-access holes were replugged with Plasticine the cell was ready for use.

Serological precipitation in the diffusion cell. One end of the cell was clearly marked 'antigen' and the other 'antibody', and the requisite quantities of antigen and antibody solutions (usually 0.75–1.5 ml.) were introduced into the reagent spaces. Thin stainless-steel plates were then inserted either fully or partially into the spaces until the solution of the reagent completely filled the available space and could diffuse through the whole area of the gel. To prevent evaporation and cracking of the gel, the cells were placed in a sealed container at 0–5° and left until the parallel zones of precipitation, which formed in a few days, appeared maximal (1½–3 weeks). The parallel zones of precipitation had to be not less than 1.5 mm. apart (by macroscopic examination) to allow the individual antibody-antigen precipitates to be removed cleanly by the

Fig. 1. Scale drawing of diffusion cell. A, Gel space; B, gauze (stainless steel); C, reagent space for antigens or antibodies; D, spacing plate (stainless steel); E, air-access holes; F, clamping plate (brass); G, bolts and wing-nuts (brass); H, cell body (Perspex).

freezing microtome. This was arranged by varying the concentration of the serological reactants and sometimes by allowing the antigens or antibodies to diffuse into the cell some days before the other reactant was added.

Use of a freezing microtome to remove individual antigen-antibody precipitates. The diffusion cell was surrounded with solid CO_2 and in 3-4 hr. the gel had completely frozen. The cell was then dismantled and the frozen block of gel set squarely on the stage of a freezing microtome (M.S.E. Base Sledge microtome, freezing attachment with stage of 90 mm. diam., wedge-shaped 240 mm. microtome knife) in the same plane as the knife. The block was fixed by freezing water around the base. Sections (120μ thick) were cut from the block and collected individually with a hair brush in 0.9% NaCl (0.1 ml.) contained in the wells of a Perspex haemagglutination tray. After the frozen gel had been allowed to melt at room temperature, the zones of precipitation were easily seen (Fig. 2), and the contents of the wells corresponding to individual zones of precipitation were removed and combined. Usually the wells corresponding to the zones of precipitation were separated by 15-20 wells containing either no precipitate or a negligible quantity.

Removal of the gel. In each experiment 10-30 diffusion cells were used. After the gels from all the cells had been sectioned as described above, the contents of all the wells corresponding to the same antigen-antibody precipitate were combined in the same centrifuge tube, diluted with phosphate-buffered saline (see preparation of gel; volume equal to that of material from wells) and left at room temperature overnight. The precipitate was removed by centrifuging (1200g for $\frac{1}{2}$ hr. at room temperature) and washed six times with phosphate-buffered saline (40, 40, 40, 40, 5 and 5 ml.). The final washing was retained for analysis to confirm that all gelatin had been removed.

Analytical methods

Antibody. The antibody contents of sera and γ -globulin concentrates were estimated by the quantitative precipitin technique as described by Kabat & Mayer (1948) except

Fig. 2. Precipitates in solutions of sections cut from frozen gel. The well (section) order starts in the bottom left-hand corner and reads from left to right in each row.

that protein N (50-100 $\mu\text{g.}$ of N) was determined by direct nesslerization after acid digestion.

Solution of serological complexes for protein, fluorescent ovalbumin, haemoglobin and ^{125}I -labelled human serum albumin analysis. Serological complexes were dissolved in 0.1M-phosphate buffer, pH 11.5 (containing 14.2 g. of Na_2HPO_4 and 23.5 ml. of 2N-NaOH/l.). Sometimes the solutions were clarified by removal of a small amount of insoluble material by centrifuging at 15 000g.

Protein. Occasionally this was measured by N (Kjeldahl) determinations. More usually, however, to conserve material for subsequent antigen analysis, protein was measured by comparing the absorption of light at $280\text{ m}\mu$ (1 cm. cell, Unicam spectrophotometer SP. 500) of a solution of the material with that of a standard solution of normal rabbit γ -globulin in 0.1M-phosphate buffer, pH 11.5. In the estimation of total protein in fluorescent-ovalbumin complexes the errors introduced by neglecting the differences in extinction coefficients between the antigen and the antibody were small and could be ignored. The extinction coefficients of haemoglobin and human serum albumin at $280\text{ m}\mu$ were, however, significantly different from that of antibody, and hence in protein estimations on their complexes a correction was made for the antigen content (determined as described below). Protein contents of the same sample determined by N estimations and by absorption of light of $280\text{ m}\mu$ agreed well.

Fluorescent ovalbumin. The fluorescence of samples (minimum vol. 10 ml.) was measured and compared with that of the appropriate blank and standard solutions as described below.

Fluorescence was measured with a B-S Light-Scattering photometer (Phoenix Precision Instrument Co., Philadelphia, Pa., U.S.A.). The fluorescent solution was excited by light of wavelength $436\text{ m}\mu$ (light from a high-pressure mercury lamp filtered through Corning filters 3389 and 5113), and the fluorescence emerging at 135° to the direction of excitation was measured, a yellow (Corning 3381) filter being used to prevent light of wavelength below $450\text{ m}\mu$ from reaching the photomultiplier.

For fluorescent ovalbumin in fluorescent ovalbumin-antibody complexes, samples (10 ml.) containing approx. $20\mu\text{g.}$ of protein/ml. were prepared and the fluorescence was compared with that of standards containing 0-6 $\mu\text{g.}$ of fluorescent ovalbumin/ml. in a solution of $20\mu\text{g.}$ of normal rabbit γ -globulin/ml.

For fluorescent-ovalbumin contamination in haemoglobin-antibody complexes, determinations of fluorescent ovalbumin were limited to samples containing 300 $\mu\text{g.}$ or less of protein/ml., to avoid unduly high blanks and excessive interference with the fluorescence. Even at the low protein concentration it was necessary to correct for some interference with fluorescence, and standard solutions (0-6 $\mu\text{g.}$ of fluorescent ovalbumin/ml.) were prepared containing uncontaminated haemoglobin complex at the same concentration as the sample. Inclusion of the complex in the standard solutions, rather than a synthetic mixture of haemoglobin and normal γ -globulin, was essential for adequate control on the interference. The validity of the determination was confirmed by assaying a known amount of fluorescent ovalbumin which had been added to the experimental sample.

For fluorescent-ovalbumin contamination in ^{125}I -labelled human serum albumin-antibody complexes, the samples

and controls were prepared and analysed as described for the haemoglobin complex, except that the upper limit of protein concentration used could be much higher before serious interference with fluorescence analysis was experienced.

Concentrations of 0.5 $\mu\text{g.}$ of fluorescent ovalbumin/ml. could be determined accurately. Hence contaminations of antigen-antibody complexes with 0.17% of fluorescent ovalbumin when haemoglobin was the antigen, and with less than 0.07% when ^{131}I -labelled human serum albumin was the antigen, were accurately measured, provided that the controls described above were available.

Haemoglobin. This was measured by spectrophotometric determinations at 410 $\mu\text{m.}$

For haemoglobin in haemoglobin-antibody complexes, the haemoglobin content of a solution of specific precipitate (concn. of protein approx. 0.5 mg./ml.) was measured by comparing the absorption of the solution at 410 $\mu\text{m.}$ (Unicam spectrophotometer SP. 500) with the absorption of suitable standard solutions of haemoglobin (0–100 $\mu\text{g.}$) in 0.1 M-phosphate buffer, pH 11.5. At this pH the extinction of haemoglobin changed gradually, and hence the interval between preparation of the solutions and measurement of the absorption at 410 $\mu\text{m.}$ was fixed at 1 hr. The validity of the determination was confirmed by assaying a known amount of haemoglobin which had been added to the experimental sample.

For haemoglobin contamination in fluorescent-ovalbumin-antibody complexes (see Table 1), solutions of the complexes (concn. of protein approx. 3 mg./ml.) were prepared and their haemoglobin content was estimated as described above. This value was then corrected for the absorption at 410 $\mu\text{m.}$ due to fluorescent ovalbumin and to traces of substances (possibly haemoglobin) present in the preparations of rabbit γ -globulin (in 13 batches of rabbit anti-ovalbumin I absorptions equivalent to 0.94–2.5 $\mu\text{g.}$ of haemoglobin/mg. of protein were observed) which were co-precipitated with the antibody on the complex. Control solutions were prepared from serological complexes formed under identical conditions to the experimental samples, but, in the absence of horse haemoglobin (e.g. in experiments described in Table 1), the control solutions were prepared from fluorescent-ovalbumin precipitates obtained by reaction of fluorescent ovalbumin and the homologous antibody in diffusion cells under identical conditions to the experimental precipitates. Measurements at 410 $\mu\text{m.}$ were made on these control solutions and a haemoglobin correction factor (suitably adjusted for total protein content of solution) was subtracted from the uncorrected haemoglobin content of the experimental sample.

The method would detect 0.1% contamination of a serological complex with haemoglobin.

^{131}I -labelled human serum albumin. A solution of the material under test was prepared as described above and its radioactivity was compared in a liquid counter (Twentieth Century Electronics Ltd. type MGH; decay and dead-time corrections were made when necessary) with that of the original solution (usually 0.025%) of ^{131}I -labelled human serum albumin. The radioactivity of the latter was such that, after diffusion and recovery of antigen-antibody precipitates, 0.05–0.1% of ^{131}I -labelled human serum albumin could be detected in 5–10 mg. of final product.

Gelatin. Gelatin is not precipitated by 4% (w/v) trichloroacetic acid (Jacobs, 1949; Kakimoto & Kanazawa,

1953), and it was estimated in solutions of the products described here as follows: an equal volume of 8% (w/v) trichloroacetic acid was added, the mixture was centrifuged after being left overnight at room temperature, and the N content (determined by Kjeldahl method with SeO_2 and nesslerization) of the supernatant was compared with those of supernatants from similarly treated solutions of gelatin.

The final wash liquors of all the antigen-antibody precipitates (5–10 mg.) were analysed for gelatin and in no case did the gelatin content exceed 10 $\mu\text{g.}$ Occasionally a washed antigen-antibody precipitate (5 mg.) was examined; it was dissolved in water (2 ml.) with 1 drop of N-NaOH before precipitation with trichloroacetic acid. In all cases the nitrogen content of the supernatant indicated less than 1% contamination of the precipitate with gelatin; some of the nitrogen in the supernatant was due to a small solubility of the precipitate in trichloroacetic acid since precipitates formed in the absence of gelatin and treated as described above also left some nitrogen (equivalent to 0.2–0.4% contamination with gelatin) in the supernatant.

RESULTS

Separation of individual antigen-antibody precipitates from reaction of a mixture of fluorescent ovalbumin and haemoglobin with their mixed antibodies

A solution (1.5 ml.) of rabbit γ -globulin containing rabbit antiovalbumin I (7.5 mg.) and rabbit antihemoglobin I (2.9 mg.) was introduced into the 'antibody space' of each of 10–20 diffusion cells. After 1 week at 0–3° a solution (0.75 ml.) containing fluorescent ovalbumin (0.188 mg.) and haemoglobin (0.94 mg.) was introduced into each 'antigen space'. Preliminary experiments indicated that these proportions of reactants and the pre-diffusion into the gel of antibody produced an adequate separation of the zones of precipitation. Appropriate control experiments were done (see haemoglobin analysis).

Two zones of precipitation formed and the precipitate from each was removed as described above and analysed for total yield of protein, the principal antigen and contamination with the other antigen. Table 1 shows the results of five experiments in which diffusion occurred for different times and different batches of antibodies were used. These results indicate that the two individual antigen-antibody precipitates were separated cleanly by the method described. The yields of precipitates varied with the batches of antibodies used and increased when the diffusion time was increased: 5–15 mg. of precipitates was separated from 10 to 20 cells. In Expt. 5, the recoveries in the precipitates of fluorescent ovalbumin, rabbit antiovalbumin I, haemoglobin and rabbit antihemoglobin I were 37, 10, 15 and 23% respectively of the original quantities of reactants.

Table 1. *Separation of individual antigen-antibody precipitates from reaction of a mixture of fluorescent ovalbumin and haemoglobin and their mixed antibodies*

The solution (0.75 ml.) of mixed antigens contained fluorescent ovalbumin (0.188 mg.) and haemoglobin (0.94 mg.); the solution (1.5 ml.) of mixed antibodies contained rabbit antiovalbumin (7.5 mg.) and rabbit anti-haemoglobin I (2.9 mg.). The batches of antigens remained the same for all experiments but the batches of antibody varied; in Expts. 1, 2, 3, 4 and 5 rabbit antiovalbumin I batches 20, 22, 23, 23 and rabbit antihaemoglobin I batches F4, F4a, F4b, F4b and F4b were used respectively. FIOv, fluorescent ovalbumin; Hb, haemoglobin.

Expt.	Diffusion time after adding antigens (days)	FIOv precipitate			Hb precipitate		
		Yield/cell (mg.)	FIOv (%)	Hb (%)	Yield/cell (mg.)	Hb (%)	FIOv (%)
1	15	0.75	9.0	0.4	0.80	11.7	<0.1
2	15	0.41	8.7	0.1	0.61	12.4	<0.1
3	12	0.53	9.4	—*	0.57	16.3	<0.1
4	10	0.30	8.0	0.1	0.43	16.7	<0.1
5	21	0.85	8.2	0.4	0.82	17.1	<0.1

* Not determined: turbidity in the control sample made it impossible to determine a correction factor for the haemoglobin content of the fluorescent-ovalbumin precipitate; the uncorrected haemoglobin content was within the range observed in the other four experiments.

Table 2. *Separation of individual antigen-antibody precipitates from reaction of a mixture of ¹³¹I-labelled human serum albumin and fluorescent ovalbumin with their mixed antibodies*

The solution (0.75 ml.) of mixed antigens contained fluorescent ovalbumin (0.28 mg.) and ¹³¹I-labelled human serum albumin (0.09 mg.); the solution (1.5 ml.) of mixed antibodies contained rabbit antiovalbumin I (0.94 mg.) and rabbit anti-(human serum albumin) (7.5 mg.). The same batches of antigens and antibodies were used in all experiments and the antigens and antibody were placed in the diffusion cell at the same time. FIOv, fluorescent ovalbumin; ¹³¹IHSA, ¹³¹I-labelled human serum albumin.

Expt.	Diffusion time (days)	FIOv precipitate			¹³¹ IHSA precipitate		
		Yield/cell (mg.)	FIOv (%)	¹³¹ IHSA (%)	Yield/cell (mg.)	¹³¹ IHSA (%)	FIOv (%)
1	14	0.22	7.8	0.20	0.26	8.5	0.27
2	25	0.19	6.5	0.16	0.33	4.5	0.18
3	21	0.19	6.5	Negligible	0.29	6.8	0.18

Separation of individual antigen-antibody precipitates from reaction of a mixture of ¹³¹I-labelled human serum albumin and fluorescent ovalbumin with their mixed antibodies

Table 2 shows the results of experiments similar to those carried out with mixtures of fluorescent ovalbumin and haemoglobin but substituting ¹³¹I-labelled human serum albumin for haemoglobin. Preliminary experiments showed that the zones of precipitation of the two antigen-antibody complexes were almost superimposed unless large excesses of rabbit anti-(human serum albumin) and fluorescent ovalbumin were used. The quantities of reactants described in Table 2 produced an adequate separation of the zones so that again individual antigen-antibody precipitates (8–12 mg. from 40 cells) could be isolated with less than 5% contamination with the extraneous system. Whereas the recoveries of ¹³¹I-labelled human serum albumin and rabbit antiovalbumin I were satisfactory (e.g. 25 and 22% respectively in Expt. 1), the recoveries of rabbit anti-(human serum albumin) and fluorescent ovalbumin were low (e.g. 3 and 6%

respectively in Expt. 1). This was due to the necessary use of large quantities of the latter reactants for reasons described above; the large quantity of rabbit anti-(human serum albumin) resulted in the formation of precipitates containing antibody in excess of the equivalence ratio if the diffusion was prolonged (cf. analysis of precipitates from Expts. 1 and 2; in the latter experiment the zone of precipitation was diffuse).

SUMMARY

1. A method is described for isolating individual antibody-antigen complexes from reaction of mixtures of antigens and mixtures of their corresponding antibodies.

2. Diffusion of mixed antigens and mixed antibodies has been done in a gelatin gel over a large cross-sectional area in a specially designed diffusion cell. Appreciable quantities of individual antigen-antibody precipitates, which form as parallel planes in the gel, have been separated by freezing the gel, cutting sections of it with a freezing microtome, and subsequently removing the gel from appropriate sections. Quantities of 10–20 mg.

of serological precipitates can be obtained by using a number of cells.

3. In this manner individual antigen-antibody complexes have been isolated with 5% or less contamination with the extraneous complex from the reaction of: (a) mixtures of fluorescent ovalbumin and haemoglobin and their mixed antibodies; (b) mixtures of fluorescent ovalbumin and ¹³¹I-labelled human serum albumin and their mixed antibodies.

In the fluorescent ovalbumin-haemoglobin system the recoveries in the two precipitates of fluorescent ovalbumin, its antibody, haemoglobin and its antibody were satisfactory, namely 37, 10, 15 and 23% respectively of the original reactants. In the second system, the recoveries of ¹³¹I-labelled human serum albumin and ovalbumin antibody were also satisfactory (approx. 25 and 22% respectively), but the recoveries of human serum albumin antibody and fluorescent ovalbumin were low (3 and 6% respectively).

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Separation of Antigens by Immunological Specificity

2. RELEASE OF ANTIGEN AND ANTIBODY FROM THEIR COMPLEXES BY AQUEOUS CARBON DIOXIDE

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The isolation of individual antigen-antibody precipitates from mixtures of antigens and antibodies by a gel-diffusion method was described by Smith, Tozer, Gallop & Scanes (1962). Ideally the next step in the purification of protein and other antigens by specific immunochemical methods would be the complete reversal of the serological reaction and the separation of antigen from antibody. Unfortunately there is no general method for completely liberating antigens although there are isolated reports (Pope, 1957; Hummeler & Ketler, 1958) that it can be done in some cases without obvious denaturation. On the other hand, partial dissociation of antibody from complexes has been achieved (Isliker, 1957) by methods involving acid or alkaline conditions (either alone or in combination with heat, denaturing agents, enzymes etc.) which had to be relatively drastic to obtain a satisfactory yield of antibody (cf. Klein-

schmidt & Boyer, 1952; Singer & Campbell, 1955). Antibody has been removed at neutral pH from pneumococcal polysaccharide complexes, by using strong salt solutions, in sufficient quantity to enable a fairly extensive study of its physico-chemical properties (Heidelberger & Kabat, 1938; Kabat, 1939) to be made; but, although some protein complexes have yielded to similar treatment (Oudin & Grabar, 1944), it does not seem to be generally applicable (Campbell & Lanni, 1951).

In exhaustive attempts to find a general method for separating antigen completely from serological complexes the following procedures were investigated with the complex of ovalbumin and rabbit antibody as test material: solution in pure and mixed non-aqueous solvents [e.g. liquid ammonia, sulphur dioxide etc. (cf. Katz, 1954; Rees & Singer, 1956)]; treatment with ultrasonic waves; irradiation