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 ^{131}I-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 ^{131}I-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).

We are indebted to Mr J. F. H. Peel for help in designing the diffusion cell and to Mr V. M. O. Longyear, Mrs M. Restall and Mr W. Brent for excellent technical assistance.

REFERENCES

Chadwick, P. & Slade, J. H. R. (1960). *J. Hyg., Camb.*, **58**, 147.
Cinader, B. (1957). *Annu. Rev. Microbiol.* **11**, 371.
Freund, J. & McDermott, K. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 548.
Isliker, H. C. (1957). *Advanc. Protein Chem.* **12**, 387.
Jacobs, J. L. (1949). *J. Amer. Leath. Chem. Ass.* **44**, 722.
Kabat, E. A. & Mayer, M. M. (1948). *Experimental Immunochemistry*, p. 59. Springfield: C. C. Thomas.
Kakimoto, D. & Kanazawa, A. (1953). *Bull. Jap. Soc. sci. Fish.* **18**, 1133.
Kalmanson, G. M. & Bronfenbrenner, J. (1943). *J. Immunol.* **47**, 387.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Kekwick, R. A. & Cannan, R. K. (1936). *Biochem. J.* **30**, 227.
Marrack, J. R., Hoch, H. & Johns, R. G. S. (1951). *Brit. J. exp. Path.* **32**, 212.
Tozer, B. T., Cammack, K. & Smith, H. (1962). *Biochem. J.* **84**, 80.
Wilson, G. S. & Miles, A. A. (1955). *Topley & Wilson's Principles of Bacteriology and Immunity*, p. 277. London: Edward Arnold Ltd.

Biochem. J. (1962) **84**, 80

Separation of Antigens by Immunological Specificity

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

BY B. T. TOZER, K. A. CAMMACK AND H. SMITH
Microbiological Research Establishment, Porton, Wilts.

(*Received* 4 *October* 1961)

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. Kleinschmidt & 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 physicochemical 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; irradia-

tion with an intense ^{60}Co source; treatment with reagents which break hydrogen bonds (thiocyanates, iodides, salicylates, urea), with concentrated salt solutions, with bisulphite (cf. Pope, 1957), and with dioxan (cf. Grant, 1959); selection of a variant from *Bacillus subtilis* and *Escherichia coli* which would hydrolyse rabbit γ-globulin but not other proteins. All were without success. However, these investigations led to a method of preparing antibody from monospecific complexes by a milder procedure than that used before. This liberated antibody could then be covalently coupled to an insoluble matrix [see Isliker (1957) for corresponding work with antigens] to yield a specific absorbent for antigens more likely, on subsequent treatment, to yield pure antigen than the original serological complex.

Aqueous carbon dioxide in the absence of salt has proved to be of value as a reagent for removing antibody from many different serological complexes at a more nearly neutral pH than that used hitherto. This system has been shown (Mitz, 1957) to increase the solubility of some proteins and to elute them from chromatographic columns. The first part of the paper describes factors affecting the dissociation by aqueous carbon dioxide of two model systems (the specific precipitates of rabbit antiovalbumin and antihaemoglobin), and the properties of the two antibodies isolated by this method. The second part describes the successful application of the carbon dioxide method to other systems. Finally the paper describes a brief examination of the dissociation of serological complexes by other systems at low ionic strength.

A preliminary account of some of this work has been published (Tozer, Cammack & Smith, 1958).

EXPERIMENTAL

Antigens. Ovalbumin, fluorescent ovalbumin, horse haemoglobin, human serum albumin and ^{131}I-labelled human serum albumin were as described by Smith *et al.* (1962). Other antigens used were as follows (source in parentheses): bovine serum albumin, lysozyme, sperm-whale myoglobin and *Pneumococcus* polysaccharide SI (Professor J. R. Marrack); sperm-whale myoglobin (Dr K. A. Munday); horse serum albumin (Dr R. A. Kekwick); ribonuclease (Armour Laboratories, Eastbourne); *Pneumococcus* undegraded polysaccharide SIII (Dr J. H. Humphrey); degraded and undegraded polysaccharides of *Shigella dysenteriae* and antigen 3 of *Pasteurella pestis* (Dr D. A. L. Davies); and crystalline diphtheria toxin (Dr C. G. Pope).

Antisera

After preparation as described below Merthiolate (0·01%) was added and the antisera were kept at 0–3°.

Antiovalbumin I. This was prepared as described by Smith *et al.* (1962).

Antiovalbumin II. Rabbits (Copenhagen White) received intravenously three injections of alum-precipitated ovalbumin in the first week of each month for 7 months as follows: injections 1–3, 5 mg. of ovalbumin; injections 4–6, 10 mg.; injections 7–9, 20 mg.; injections 10–21, 50 mg. The rabbits were bled out 3 days after the last injection.

Antiovalbumin III. This antiserum (R50/56) was supplied by Dr J. H. Humphrey. Rabbits were given, intramuscularly, ovalbumin ($2 \times 12{\cdot}5$ mg.) in Freund's adjuvant. After 1 month two intravenous injections of alum-precipitated ovalbumin (4 mg.) were given at weekly intervals and after a further week the rabbits were bled out.

Antiovalbumin IV. Rabbits were given alum-precipitated ovalbumin (25 mg., intravenously) on the 1st and 30th days and were bled out on the 37th day.

Antihaemoglobin I. This was prepared as described by Smith *et al.* (1962).

Antihaemoglobin II. This rabbit antiserum was prepared with *Monilia* as adjuvant (Boyd & Malkiel, 1944).

Antihaemoglobin III. Rabbits (Copenhagen White) were injected with 10% (w/v) haemoglobin mixed with a 0·2% suspension of heat-killed human tubercle bacilli as follows: intravenously, 1 ml. on the 1st, 3rd and 5th days; intraperitoneally, 3 ml. on the 8th day; and, intravenously, 1 ml. on the 10th and 12th days. The injection course of the 2nd week was repeated in the 3rd, 4th, 5th and 6th weeks. Animals were bled 7 days after the last injection.

Antihaemoglobin IV. This antiserum was prepared as described for antihaemoglobin III, by substituting 0·5% haemoglobin for the 10% (w/v) solution.

Anti-(horse serum). This rabbit antiserum to horse serum was supplied by Dr M. C. Lancaster.

Rabbit anti-(bovine serum albumin), antilysozyme and anti-(sperm-whale myoglobin) I. These were supplied by Professor J. R. Marrack as globulin concentrates from hyperimmune sera.

Rabbit anti-(sperm-whale myoglobin) II and III. These antisera were produced by the methods used for antihaemoglobin I and III [with 5% (w/v) myoglobin] respectively.

Rabbit antiribonuclease. This hyperimmune serum ($2\frac{1}{2}$-years immunization course) was supplied by Dr J. H. Pearce.

Rabbit antiserum to antigens of Pasteurella pestis. This antiserum, supplied by Dr D. A. L. Davies, was prepared after 12 injections of living *P. pestis* (50 μg. increasing to 1 mg., over 3 months).

Rabbit antiserum to the specific polysaccharide of Shigella dysenteriae. This antiserum, supplied by Dr D. A. L. Davies, was prepared after 13 injections of homologous 'O' somatic antigen (4 μg. increasing to 1 mg., over 3 months).

Rabbit anti-(Pneumococcus *polysaccharide SIII*). This was supplied by Dr J. H. Humphrey.

Horse anti-(Pneumococcus *polysaccharide SI*). This antiserum, supplied by Professor J. R. Marrack, had 'tricresol' (mixed isomers of cresol) as preservative.

Diphtheria antitoxin (horse). Batch DP. 1252 was supplied by Dr C. G. Pope and Batch Ex1930 (produced by a short-term immunization) by Dr M. Barr.

Rabbit serum γ-globulin. Antisera were fractionated by the sodium sulphate method of Marrack, Hoch & Johns (1951) which removed complement (J. R. Marrack, personal communication).

Methods

Preparation of specific precipitates for dissociation experiments. Antiserum (3 vol., heated at 56° for 30 min. to inactivate complement) or concentrated γ-globulin (1 vol.) was diluted to 10 vol. with 'phosphate-buffered saline' [pH 7·0; sodium phosphates (I 0·045) plus NaCl (I 0·125); 1·10 g. of $NaH_2PO_4,2H_2O$, 4·53 g. of $Na_2HPO_4,12H_2O$ and 7·31 g. of NaCl/l.]. The calculated amount of antigen (0·1% in phosphate-buffered saline) was added, and the mixture was diluted to 15 vol. and kept for 1 hr. at 20° and for 16 hr. at 2–3°; the precipitate was then centrifuged down, washed twice with phosphate-buffered saline (2 × 15 vol., at 1100g for 20–30 min.) and three times with de-ionized water (2 × 15 vol. and 1 × 4 vol., at 15 000g for 20 min.). The final sludge contained approx. 1 g. of specific precipitate/7 g. of water.

Aqueous carbon dioxide solutions of specific precipitates. The precipitate prepared as described above was mixed with the required amount of aqueous CO_2 and transferred to the apparatus, described below, for saturating the solution with CO_2. A vertical stoppered tube (8 or 27 ml. total capacity to take volumes up to 4 and 20 ml. respectively), with a side arm for entry of water-washed CO_2 and an escape hole (diam. 1 mm.) in the stopper, was fixed at the centre of a platform which moved (110 cyc./min.) so that the tube underwent a vertical lift of 4 mm. and angular displacement of 5°; this gentle agitation dissolved CO_2 and avoided surface denaturation. The gas (50–100 ml./min.) was passed for 1–2 hr. to effect maximum dissolution.

Operations with CO_2 solutions, e.g. the filling of ultracentrifuge cells, were carried out in a flat-bottomed glass cylinder (6 in. diam. × 6 in. high) through which CO_2 (2 l./min.) was passed to maintain an atmosphere of at least 95% CO_2.

Unless stated otherwise, aqueous CO_2 refers to a solution saturated with CO_2 at room temperature (20±1°). The chromatographic separations of antibody were carried out in a cold room with aqueous CO_2 saturated at 2–3° as eluent.

Analytical methods

Proteins. Antibody, fluoresent ovalbumin, haemoglobin, ^{131}I-labelled human serum albumin and protein were determined as described by Smith *et al.* (1962).

Carbon dioxide. The sample (approx. 1 ml.) was withdrawn slowly into a well-fitting hypodermic syringe and injected into 0·1 N-$Ba(OH)_2$ (1·0 ml.) through a no. 25 Suba-seal (Williams Freeman Ltd., Barnsley) stoppered conical flask (25 ml., B 14 joint) which had been filled with N_2 before adding the alkali. The increase in weight of the flask gave the sample size, and back-titration—with 0·1 N-HCl and phenolphthalein–thymolphthalein as indicator—the CO_2 content. If necessary the protein in the sample could be protected from the alkali by using for the assay two compartments of a modified Warburg flask.

Minimum and maximum pH *values producing complete solubility of specific precipitates.* A series of acetate buffers of pH 3·8–5·0, prepared by titration of sodium acetate (I 0·1) with HCl, and a series of glycine–NaOH buffers (I 0·2) of pH 10·5–11·5, both in steps of 0·2 pH unit, were used. The specific precipitate, washed in 0·9% NaCl, was gently stirred with buffer at 2° at a protein concentration of at least 2 mg./ml. If complete solubility were not observed within 1 hr., the experiment was repeated with another sample of precipitate and a buffer of more extreme pH.

Physicochemical methods

Sedimentation. An analytical Spinco Model E ultracentrifuge was used to determine the degree of dissociation of complexes in various solvents, and for measuring sedimentation coefficients. The diagonal-schlieren optical system was modified by the use of a half-wave phase plate instead of the normal diagonal-bar analyser. Sedimentation diagrams were measured with a Cambridge two-dimensional measuring machine. The refractive increment, $(\Delta n)_{uc}$, across the velocity boundary was obtained from the area below the gradient curve after correction for sectorial dilution. The effect of adiabatic expansion and contraction of the rotor on temperature (Waugh & Yphantis, 1952) was taken into account only in runs on purified γ-globulin and antibody. A partial specific volume, $\bar{v}$, for human γ-globulin of 0·745 (Cann, 1953) has been used in the buoyancy corrections and all sedimentation-coefficient values were reduced to water at 20°.

Sedimentation coefficients for dissociated complexes in salt-free aqueous CO_2, denoted here by $S^*_{20,w}$, were subject to a large diminution due to the 'primary charge effect' (Svedberg & Pederson, 1940), which for rabbit γ-globulins led to $S_{20,w}$ values 30% lower than normal. Further, one could not neglect the possibility of rapidly reversible equilibria between several species of complex which would lead not only to very complicated sedimentation diagrams (Gilbert & Jenkins, 1956) but also to sedimentation coefficients which would bear no obvious relationship with the molecular properties of a complex species. For this reason and because of the Johnston–Ogston effect [see Schachman (1959)] no accurate measurement of degrees of dissociation of complexes was possible; approximate values are quoted and these were obtained by comparing the concentration indicated by the area under a peak with the total protein concentration.

Preparative ultracentrifuging was done in the Spinco Model L ultracentrifuge with either a bucket (SW 25 and SW 39) or an angle-head rotor (40).

Refractive increment. The refractive increment, $(\Delta n)_{dr}$, was measured in a differential refractometer (Brice & Halwer, 1951) at 5461 Å with dialysed protein and the dialysis buffer.

Moving-boundary electrophoresis. A Spinco Model H electrophoresis-diffusion apparatus was used in conjunction with the 11 ml. long-limbed cell and an electrode assembly closed on one side. Currents of 12 or 16 mA were used; current leakage was absent in the majority of runs. Boundary displacement due either to gassing at a faulty Ag–AgCl electrode or to mechanical leakage was checked by observations on the stationary δ- and ϵ-boundaries before and after the current was switched off. Mobility calculations were based on the velocity of the descending-boundary maximum and the specific conductivity of the dialysed protein solution, measured at 0°. The conductivity cell (cell constant 3·054) was similar to that described by Jones & Bollinger (1931), and the bridge circuit contained many refinements including a Wagner earth (Shedlovsky, 1930). In the range 0·002–0·008 Ω/cm. specific-conductivity measurements, referred to standard KCl solutions (Harned & Owen, 1950), were accurate to ±0·2%.

To cover the pH range 5·4–8·6 it was necessary to use different buffer salts, but to minimize the influence of specific-ion interaction on mobility, low concentrations of uni-univalent salts [sodium cacodylate (I 0·02) for pH 5·45, 6·08 and 6·98; sodium veronal (I 0·02) for pH 7·88 and 8·60] were used. By increasing the total ionic strength to I 0·10 with NaCl (I 0·08) and keeping the protein concentration below 0·35%, boundary anomalies due to pH and conductivity gradients were suppressed (Alberty, 1949).

Diffusion measurements. The Gouy interferometric method used has been reviewed by Gosting (1956). Details of the Perspex cell (optical path 1·774 cm.) and boundary former were described by Coulson, Cox, Ogston & Philpot (1948) and Creeth (1952), but the optical lay-out of Gosting, Hanson, Kegeles & Morris (1949) was used. Measurements were made at 25·00±0·02° but corrected to the value in water at 20°. Experiments with sucrose and bovine plasma albumin (Armour, batch 1670) under conditions specified by Gosting & Morris (1949) and by Wagner & Scheraga (1956) gave $D_{20,w}$ values within 1% of their published figures. All measurements were carried out in the same buffer [pH 7·0; sodium phosphates (I 0·045) plus NaCl (I 0·125)] as that used for the determination of $S_{20,w}$. As calculation of the $D_{20,w}$ was based on the deviation of the outermost interference minimum from the optical axis (Kegeles & Gosting, 1947; Gosting & Morris, 1949), it was approximately equivalent to the height–area diffusion coefficient.

Solubility of rabbit globulin in water. Solutions of globulin in 0·9% NaCl containing known amounts of protein were dialysed, with stirring, against daily changes of water at 3° for 5 days. The contents of the dialysis sac were quantitatively removed, centrifuged at 15 000g at 3° and analysed for protein content by N determination.

Solubility of specific precipitates. The precipitate, washed with 0·9% NaCl, was mixed with phosphate-buffered saline, pH 7·0 (see above), to give approx. 3 mg. of protein/ml. and stirred for 16 hr. at 2–4°. The precipitate was deposited by centrifuging at 15 000g for 20 min. at 2–4° and the protein content of the supernatant determined.

Filter-paper electrophoresis. This was carried out on 37 cm. × 2·5 cm. strips of Whatman no. 1 paper in a horizontal-type EEL electrophoresis apparatus (Evans Electroselenium Ltd., Harlow) with veronal buffer (pH 8·6; I 0·05) unless otherwise stated.

Measurements of pH. These were made with a glass electrode and a direct-reading pH meter (Model 23A, Electronic Instruments Ltd.). Samples were discarded to avoid contamination of the bulk solution with KCl.

RESULTS

Dissociation of ovalbumin–rabbit-antiovalbumin complexes by aqueous carbon dioxide at pH 5

Fig. 1 shows a typical ultracentrifuge diagram of a solution of water-washed specific precipitate in saturated (at 20°) aqueous carbon dioxide (35 mM). The slower-moving boundary ($S^*_{20,w}$, 5S) corresponded to immune γ-globulin (by comparison with normal rabbit γ_2-globulin sedimented under identical conditions) and the faster ($S^*_{20,w}$, 13S) to an antigen–antibody complex. Though no evidence for the release of free ovalbumin ($S^*_{20,w}$, 3·2S) was obtained, the sensitivity of the sedimentation analysis was such that a small release of antigen would not have been detected. Absence of free antigen in this system was confirmed by preparative ultracentrifuging (see below) of an aqueous carbon dioxide solution of complex containing fluorescent ovalbumin as antigen. The antibody isolated from the supernatant (25% of the original antibody, 80% precipitable) was shown to contain less than 0·01% of fluorescent ovalbumin.

Although different batches of precipitates produced sedimentation diagrams showing the same general pattern of dissociation in aqueous carbon dioxide, the amount of antibody released and the nature of the remaining complex varied. Hence the factors affecting the dissociation were investigated.

Factors affecting the dissociation with aqueous carbon dioxide

Concentration of carbon dioxide. The minimum concentration of carbon dioxide required to dissolve a precipitate in the absence of salt varied directly with the protein concentration (e.g. 2 mM for 0·2% protein, 19 mM for 1·2% protein), and for a given protein concentration the degree of dissociation increased and $S^*_{20,w}$ of the complex decreased with increasing carbon dioxide concentration. Thus Figs. 2 (*a*), (*b*) and (*c*) show sedimentation diagrams of solutions containing 9 mM-, 32 mM- and 71 mM-carbon dioxide respectively. Solutions of low carbon dioxide concentration had a blue haze characteristic of Rayleigh scattering from molecular aggregates. Saturated (at 20°) aqueous carbon dioxide (approx. 39 mM) was used for further work.

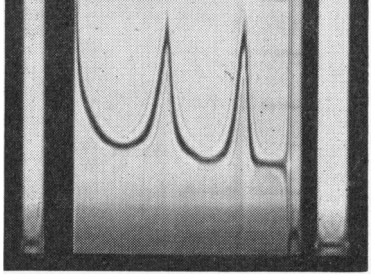

Fig. 1. Sedimentation diagram showing partial dissociation of ovalbumin–rabbit-antiovalbumin I complex in aqueous carbon dioxide (35 mM; pH 5). The initial protein concentration was 1·0% (w/v). Sedimentation was from right to left. The photograph was taken after 34 min. at 59 780 rev./min.

Removal of carbon dioxide (1–2 hr. at 10 cm. Hg pressure over potassium hydroxide) resulted in reprecipitation of protein, but occasionally a metastable solution was formed. Since small amounts of carbon dioxide considerably increased the solubility of complexes even in 0·9 % NaCl, it was necessary to remove completely the carbon dioxide to ensure maximal precipitation of the complex during preparation of antibody.

Ionic strength. Low ionic strength was imperative to obtain solution and dissociation of complexes in aqueous carbon dioxide. Table 1 shows the effect of increasing the concentration of sodium chloride on the solubility of ovalbumin specific precipitate in saturated aqueous carbon dioxide. The sudden drop in solubility, observed here between I $8{\cdot}5 \times 10^{-3}$ and I $9{\cdot}0 \times 10^{-3}$ (sodium chloride), also occurred with precipitates prepared from other antisera but at different ionic strengths [e.g. a precipitate from a more 'avid' antiserum showed a sudden decrease in solubility on raising the ionic strength to I $2{\cdot}1 \times 10^{-3}$ (sodium chloride)]. The 'salting out' effect of other sodium salts appeared to follow the Hofmeister series, i.e. $SCN^- > I^- > Cl^- > F^-$ ions.

Sedimentation diagrams of solutions of complex in saturated aqueous carbon dioxide at increasing ionic strengths showed a corresponding increase of $S^*_{20,w}$ of the complex and reduction in amount of released antibody.

Age of precipitate. No change in the normal pattern of dissociation was observed with precipitates collected and washed after antigen and antibody had been in contact for varying periods of 1 hr.–3 days (see Methods section for usual preparation of precipitates).

Age of solution. Solutions of complex in saturated aqueous carbon dioxide which were kept at 2° for 2, 20 and 168 hr., and re-equilibrated with carbon dioxide at 20° before ultracentrifuging, showed no significant changes either in $S^*_{20,w}$ of the globulin (4·7, 4·6 and 5·0s respectively) and complex peaks (10·9, 10·5 and 12·1s respectively) or in the areas under the peaks.

Temperature. Sedimentation diagrams of the same solution of specific precipitate prepared at 20° in aqueous carbon dioxide and ultracentrifuged at 4°, 20° and 35° indicated that no major change in globulin release occurred with increase in temperature, but there was an increase in $S^*_{20,w}$ of the complex, especially in the range 3–20°.

Protein concentration. On lowering the protein concentration from about 1 % no significant increase in dissociation was observed, but on raising the concentration to 2 % there was reduced dissoci-

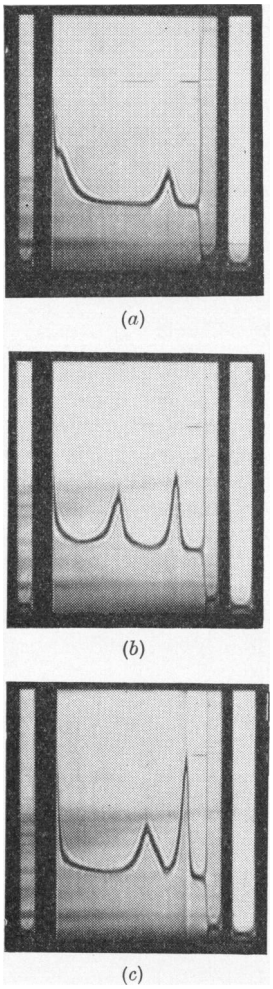

Fig. 2. Effect of carbon dioxide concentration on the dissociation of ovalbumin–rabbit-antibody complexes. Concentrations of aqueous carbon dioxide (mM) were: (*a*) 9, (*b*) 32, (*c*) 71. The initial protein concentration was 0·70 %. The sedimentation diagrams were photographed after 26 min. at 59 780 rev./min.

Table 1. *Effect of ionic strength (sodium chloride) on the solubility of ovalbumin specific precipitates in saturated aqueous carbon dioxide* (39 mM)

Experimental details are given in the text.

Ionic strength of NaCl (10^3 I)	Solubility of ovalbumin specific precipitate (%)
0	~3*
0·7	>1
8·5	0·93
9·0	0·36
170	0·12
850	0·11

* Gels were formed at concentrations greater than 3 %.

ation with a slight increase in $S^*_{20,w}$ of the residual complex. As the Johnston–Ogston boundary anomaly would tend to produce an 'apparent' increase in degree of dissociation with increasing protein concentration, the significance of the latter observation is further enhanced.

Antigen:antibody ratio in the specific precipitate. Sedimentation analysis of saturated aqueous carbon dioxide solutions of precipitates, formed at equivalence and in excess of antigen and antibody, showed that with increasing antibody:antigen ratio there was a proportionate increase both in $S^*_{20,w}$ of the residual complex and in the release of antibody.

Strength of antigen–antibody linkage. The strength of this linkage, i.e. the avidity of antibody for antigen (Cinader, 1957), is related to the course of immunization. The ease of solution and dissociation of precipitates in the aqueous carbon dioxide as well as the acid or alkaline pH needed to dissolve precipitates in a buffer would appear to reflect the avidity of the various batches of antibody from which precipitates were prepared (Table 2).

Isolation of rabbit antiovalbumin

Calculations based on sedimentation analysis of precipitates dissociated in aqueous carbon dioxide showed that by single-stage preparative ultracentrifuging it was possible to separate from residual complex about one-half of the antibody set free. This was accomplished experimentally by the scheme given in Fig. 3 where the yield was increased by a second-stage which used the redissolved deposit. Table 3 shows the results of this experiment and of three others in which 13–25 % of the original antibody was isolated. In an additional experiment, a high carbon dioxide concentration (70 mM) was used, but the overall yield of antibody was still approx. 25 %, i.e. any gain in antibody release was offset by the increased difficulty in separating it from a complex of decreased $S^*_{20,w}$.

Properties of rabbit antiovalbumin separated from specific precipitate after dissociation with aqueous carbon dioxide

Precipitability. Fig. 4 shows that the precipitin curve of freshly dissociated antiovalbumin was similar to that of the original antiserum. At optimum proportions under standard conditions of precipitin analysis eight different batches of antiovalbumin prepared during 1957–1959 from antiovalbumin I (i.e. the least 'avid' antiserum) were 65–85 % precipitable, and there was no significant difference between the precipitability of antibody prepared from the first extraction of the complex (S1C, see Fig. 3) and that from the second extraction (S2C, see Fig. 3). However, three further batches of material have since been prepared, and, although the antibody from S2C was normal (72–90 % precipitable), that from S1C was only 37–55 % precipitable; the reason for this is unknown. The supernatant from a typical precipitation of S1C contained antigen (28 % of the original fluorescent ovalbumin), together with 55 % of the original protein of the system. In an attempt to determine the amount of antibody combined with antigen as soluble complexes and also that remaining uncombined (and hence possibly denatured), the following experiments were carried out on the supernatant. Sodium sulphate (10 %, w/v) did not precipitate any of either the original antigen or the original antibody (examined at about the same concentrations as those occurring in the supernatant), but it did precipitate 60 % of the protein remaining in the supernatant and 50 % of a soluble complex (or complexes) made for comparison by dissolving an antigen–antibody precipitate in

Table 2. *Behaviour of ovalbumin specific precipitates prepared from different types of antibody in saturated aqueous carbon dioxide and in acid and alkaline buffers*

Details of the immunization courses and tests of solubility in buffer are described in the Experimental section. The amount (%) of antibody liberated was determined by sedimentation analysis.

		Behaviour of specific precipitate					
		Dissociation in aqueous CO_2			pH for complete solubility in buffer		
Antibody	Immunization course	Solubility	Antibody liberated (%)	$S^*_{20,w}$ of residual complex (s)	Acid	Alkaline	Indicated order of avidity of antibody
Antiovalbumin I	5–6 weeks	+ + + +	25–30	13	3·5	10·5	4
Antiovalbumin II	7 months	+ +	10–15	20–40	3·1	11·5	2
Antiovalbumin III	Freund's adjuvant	±	0	Very great†	2·5	11·7	1
Antiovalbumin IV	Large antigen doses, short course	+ + +	20–30	15	—	—	3

† Sedimented before the ultracentrifuge had reached full speed.

excess of antigen (cf. supernatant). These experiments indicated that most of the antibody in the supernatant was combined with antigen and not denatured.

The fact that soluble complexes, probably of different solubilities, were formed meant that the 'precipitability' of a particular sample of antibody depended on the concentration at which the re-

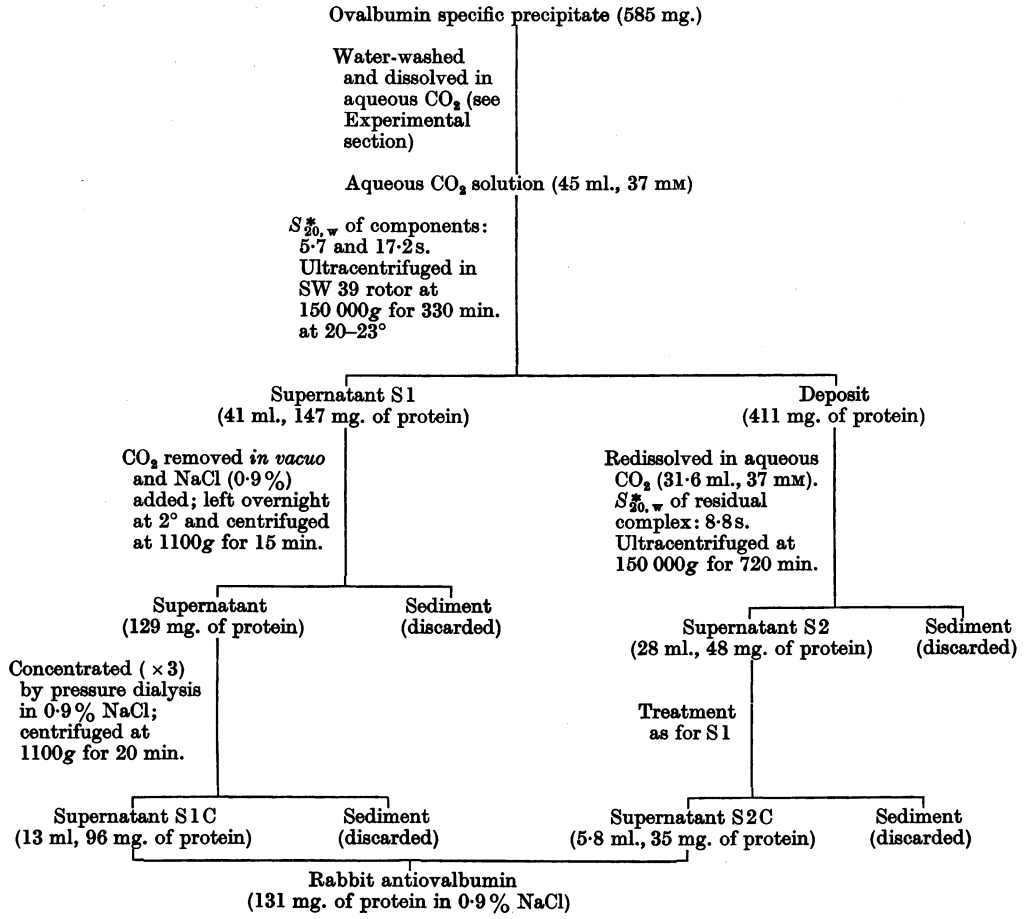

Fig. 3. Preparation of rabbit antiovalbumin by differential ultracentrifuging of an aqueous carbon dioxide solution of ovalbumin specific precipitate. Details of sedimentation analysis and preparative ultracentrifuging are given in the Experimental section.

Table 3. *Summary of the preparative isolation of dissociated antiovalbumin globulin*

Isolation of antiovalbumin from aqueous carbon dioxide solutions of specific precipitates was carried out by sedimentation in the Spinco Model L ultracentrifuge (see Fig. 3).

		Starting material		Yield of antibody			
Run no.	Spinco rotor head	Specific precipitate (mg.)	Antibody content (mg.)	S1C (mg.)	S2C (mg.)	Total (mg.)	Antibody recovered (% of original)
1	SW39	585	520	96	35	131	25·2
2	40	1110	1000	137	117	254	25·4
3	40	1560	1380	119	64	183	13·2
4	SW39 and 40	1260	1130	133	52	185	16·4
		Total 4515	4030	485	268	753	18·7

actants were mixed and the number and nature of the washings of the precipitate. Thus, by alteration of the concentration of antigen and antibody and of the washing procedure, apparent precipitabilities varying from 62 (Allison & Humphrey, 1960) to 80% were observed on the antiovalbumin sample obtained by combining the preparations described in Table 3.

Solubility of antiovalbumin in water. Carbon dioxide-dissociated antiovalbumin was about 96% soluble in salt-free water at 3°.

Recombination of dissociated antibody with ovalbumin. The dissociated antibody formed a complex with ovalbumin which did not dissociate completely in aqueous carbon dioxide; in the ultracentrifuge it showed a dissociation pattern similar to that of the parent complex.

Dissociation of haemoglobin–rabbit-antihaemoglobin precipitates by saturated aqueous carbon dioxide at pH 5

These specific precipitates were partly or completely dissociated in aqueous carbon dioxide (39 mM at 20°) in the absence of salt. Although the factors affecting dissociation were not investigated in detail it was clear that the immunization procedure greatly influenced the degree of dissociation of the complexes.

Complete dissociation of haemoglobin from antibody by aqueous carbon dioxide with precipitates obtained from antihaemoglobin II and III. Fig. 5 (*a*) shows the sedimentation diagram of a precipitate prepared from antihaemoglobin III which had been dissolved in saturated aqueous carbon dioxide. The faster component ($S^*_{20,w}$, approx. 5s) was antibody and the slower component ($S^*_{20,w}$, approx. 2s) haemoglobin, from comparison with rabbit γ-globulin and haemoglobin sedimented separately under identical conditions. The absorption of light was clearly associated with the minor boundary, and the uniformity of absorption over the major boundary suggested that complete dissociation of the complex had been achieved. Further, the area below the slower-boundary diagram corresponded to the total amount of haemoglobin in the complex. A precipitate from antihaemoglobin II behaved similarly.

Partial dissociation by aqueous carbon dioxide of precipitates obtained from antihaemoglobin I and IV. Fig. 5 (*b*) shows the sedimentation diagram of a precipitate prepared from antihaemoglobin IV

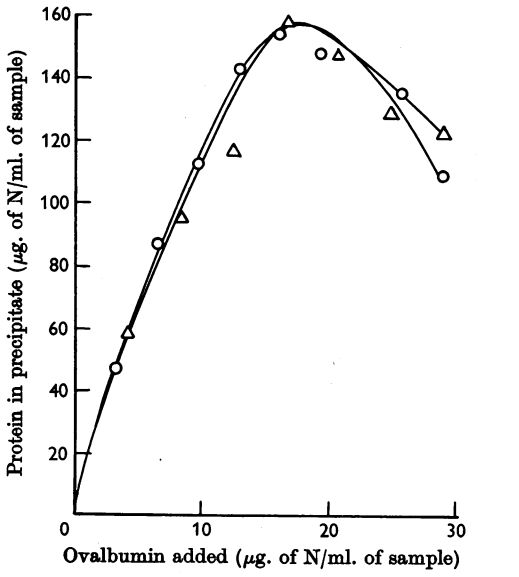

Fig. 4. Precipitin curves of ovalbumin with: ○, rabbit antiovalbumin I; △, carbon dioxide-dissociated antibody from a complex of antiovalbumin I.

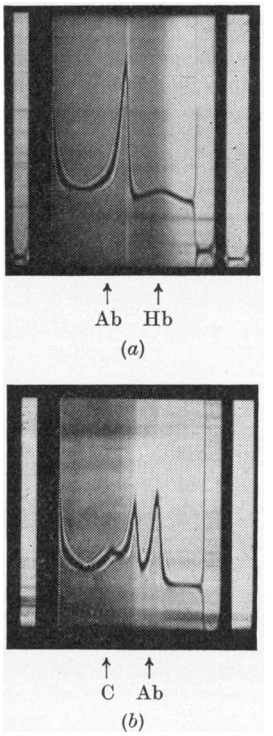

Fig. 5. Sedimentation diagrams of aqueous carbon dioxide solutions (39 mM; pH 5) showing: (*a*) complete dissociation of haemoglobin–rabbit-antihaemoglobin III complex (0·90%); (*b*) partial dissociation of haemoglobin–rabbit-antihaemoglobin IV complex (1·0%). Sedimentation diagrams were photographed after (*a*) 82 min. and (*b*) 52 min. at 59780 rev./min. Hb, haemoglobin; Ab, antibody; C, complex.

which had been dissolved in saturated aqueous carbon dioxide. Here the dissociation was only partial. The slower boundary ($S^*_{20,w}$, approx. 5·2s) now corresponded to antibody and the faster boundaries to residual complexes containing 40–60% of the antibody and all of the antigen as shown by the association of light-absorption with these boundaries. A precipitate from antihaemoglobin I behaved similarly.

Isolation of rabbit antihaemoglobin by ion-exchange chromatography. Calculations from sedimentation data on the two types of haemoglobin precipitates dissolved in saturated aqueous carbon dioxide showed that antihaemoglobin could not be separated efficiently either from the free haemoglobin or residual complex by preparative ultracentrifuging. Chromatographic methods were therefore investigated for this separation. Some separation of antibody (15–25%) was achieved with calcium phosphate (Tiselius, Hjertén & Levin, 1956) or cross-linked polydextran (Sephadex 50; Pharmacia, Uppsala). However, the best material proved to be carboxymethylcellulose (Peterson & Sober, 1956).

Our original use of phosphate buffer (pH 7·1; I 0·044) to elute the material from carboxymethylcellulose (Tozer *et al.* 1958) produced a strongly acid (pH 2–3) eluate, owing to displacement of H^+ ions from the ion-exchanger which had been equilibrated at pH 4 with saturated aqueous carbon dioxide; this phosphate eluent was discarded in favour of sodium hydrogen carbonate solution which produced an eluate of more neutral pH.

A column (3·6 cm. diam. × 4 cm. long) of carboxymethylcellulose (5 g.) was equilibrated with saturated aqueous carbon dioxide (20 l. for 48 hr.). The preparation of the column and subsequent chromatography were carried out at 2–3° in an atmosphere of carbon dioxide. A specific precipitate (130 mg., from antihaemoglobin III) in saturated aqueous carbon dioxide (15·5 ml.) was added to the column, followed by the rinsings (15 ml. of saturated aqueous carbon dioxide) of the flask. The supply of carbon dioxide to the head of the column was disconnected and the column eluted with 50 mM-sodium hydrogen carbonate solution (flow rate 60 ml./hr. at 8 cm. Hg pressure). Fractions (25 ml.) were analysed for nitrogen content. (Absorption measurements at 280 mμ were invalidated by the removal of non-nitrogenous dialysable material, which also absorbed light at 280 mμ, from the carboxymethylcellulose under these conditions.) The fractions containing the eluted protein were combined and concentrated by pressure dialysis against phosphate-buffered saline. A small amount of residual haemoglobin precipitated as a complex during the dialysis and was removed by centrifuging. The yield was 40–50% of the original antibody in the precipitate.

Increase in the column loading from 20–40 to 90–140 mg. of complex/g. of carboxymethylcellulose increased the relative amount of antibody eluted, but unfortunately there was also increased contamination of the antibody with haemoglobin; although the latter could be precipitated with antibody on addition of sodium chloride, so much antibody was removed in the resulting haemoglobin complex that the overall yield (40–50%) was not improved.

Properties of rabbit antihaemoglobin

Precipitability. Five samples of rabbit antihaemoglobin, isolated as described above, contained 47–63% of antibody precipitable under standard conditions of precipitin analysis. The amount of antigen present in the precipitate formed at the point of maximum antibody precipitation varied from 19 to 62% of the added haemoglobin in different preparations. The low precipitability of antigen suggested that much of the non-precipitated antihaemoglobin was present as soluble complex and the results of solubility experiments carried out on a precipitated complex in phosphate-buffered saline (at 3°) supported this.

Solubility in water. Exhaustive dialysis of an aqueous suspension of dissociated antihaemoglobin (3·9 mg./ml.) against water indicated a euglobulin content of about 77% (cf. dissociated antiovalbumin).

Physicochemical properties of rabbit antiovalbumin and rabbit antihaemoglobin

The physicochemical properties of single batches of antiovalbumin and antihaemoglobin were examined as follows.

Homogeneity. In the ultracentrifuge both preparations resolved into major ($S_{20,w}$, approx. 7s) and minor ($S_{20,w}$, approx. 9s; 18% in antiovalbumin and 8% in antihaemoglobin) components [see Fig. 6 (*a*), (*b*)]. The 9s component has been observed in several normal and immune rabbit γ-globulins stored at −20° and this may be due to dimerization of the 7s molecule (Albert & Johnson, 1961). Also, γ-globulin undergoes reversible dimerization at low ionic strength (Cann, 1953; Cann & Phelps, 1955).

The refractive increment derived from the sedimentation diagram (including both components before resolution) and a direct measurement in a differential refractometer were expressed as $(\Delta n)_{uc}/(\Delta n)_{dr}$. When all the macromolecular solute is represented by the velocity boundary (or boundaries), $(\Delta n)_{uc}/(\Delta n)_{dr}$ should be unity; this ratio measures the weight fraction of solute actually observed in the ultracentrifuge and the difference from unity indicates the amount of highly polydisperse, aggregated or degraded material (e.g.

denatured products) which may escape direct observation. The values for $(\Delta n)_{uc}/(\Delta n)_{dr}$ were 0·97 for both antiovalbumin and antihaemoglobin; hence at least 97% of these materials could be accounted for in the ultracentrifuge, which suggested that antibody was not extensively degraded by dissociating it from the complex.

Antiovalbumin produced a single diffuse boundary on electrophoresis in sodium veronal (pH 8·6; I 0·10), with average mobility $-1{\cdot}44 \times 10^{-5}$ cm.^{2} sec.$^{-1}$v^{-1}, in general agreement with mobility values for 'normal' rabbit γ-globulins. Antihaemoglobin was examined in the isoelectric region only [pH 6·98; sodium cacodylate (I 0·02) plus sodium chloride (I 0·08)], and under these conditions there was no evidence of additional components. The 9s component observed in the ultracentrifuge in both samples, and thought to be a dimer of the 7s component, could not be identified in electrophoresis and presumably migrated as γ-globulin.

Approximate molecular weights. Sedimentation and diffusion data were combined in the Svedberg equation, $M = RTS/D(1-\bar{v}\rho)$, in which the partial specific volume, $\bar{v}$, was assumed to be 0·745 for γ-globulin. Molecular weights of 155 400 ($S_{20,w}$, 7·00s, and $D_{20,w}$, $4{\cdot}29 \times 10^{-7}$ cm.^{2}/sec.) and 150 400 ($S_{20,w}$, 6·68s, and $D_{20,w}$, $4{\cdot}15 \times 10^{-7}$ cm.^{2}/sec.) were calculated for antiovalbumin and antihaemoglobin respectively. These values correspond to the lower limit of a wide range of values for normal and immune rabbit γ-globulins (150 000–188 000) quoted by Porter (1960), but they are only approximate for the following reasons. First, $S_{20,w}$ and $D_{20,w}$ were determined for protein concentrations of 0·24–0·34% instead of extrapolating them to zero concentration. Secondly, both of the isolated antibodies contained the 9s component in addition to the major 7s component, and $D_{20,w}$ for the latter could not be determined accurately from a simple analysis of the Gouy interference pattern (cf. Baldwin, Gosting, Williams & Alberty, 1955); the true $D_{20,w}$ values for the major component are almost certainly higher than those reported here, and consequently any revision of the above molecular weights would produce even lower figures (Cammack, 1962).

Average isoelectric points. The electrophoretic mobility as a function of pH under constant ionic strength conditions (I 0·10) for dissociated antiovalbumin is plotted in Fig. 7; also included is a single mobility determination on dissociated antihaemoglobin at pH 6·98. Both preparations are isoelectric in the region of pH 6·68–6·96, i.e. higher than the pH$_t$ (5·85) generally accepted for normal and immune [anti-(*Pneumococcus* polysaccharide) and antiovalbumin] rabbit γ-globulin [Tiselius & Kabat (1939); also cf. Porter (1960)]. When normal ethanol-fractionated γ-globulin (Nichol & Deutsch, 1948) was examined (see Fig. 7),

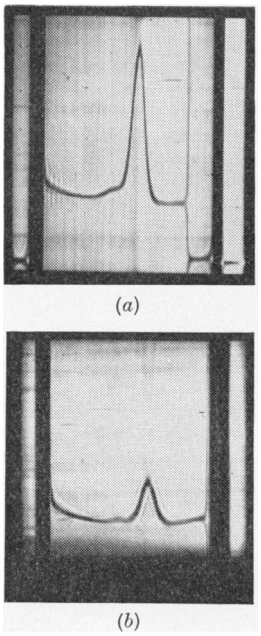

Fig. 6. Sedimentation diagrams obtained for: (*a*) rabbit antiovalbumin I, and (*b*) rabbit antihaemoglobin III, both dissociated from the respective serological complexes by aqueous carbon dioxide (35 mM). Sedimentation (right to left) was at 59 780 rev./min. in phosphate-buffered saline [pH 7·0; sodium phosphates (I 0·045) plus sodium chloride (I 0·125)] for: (*a*) 42 min.; (*b*) 50 min. The initial protein concentrations were: (*a*) 1·07%; (*b*) 0·31%. The phase-plate angle was 45°.

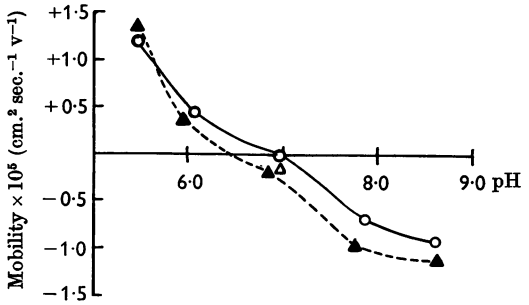

Fig. 7. Electrophoretic mobilities of normal and immune rabbit γ-globulins as a function of pH. Solvents: cacodylate buffer [sodium cacodylate (I 0·02) plus sodium chloride (I 0·08)] for final pH in the range 5·45–6·98; veronal buffer [veronal (I 0·02) plus sodium chloride (I 0·08)] for final pH 7·88 and 8·60. The protein concentration was approx. 0·35%. ○, Carbon dioxide-dissociated antiovalbumin; △, carbon dioxide-dissociated antihaemoglobin; ▲, normal γ-globulin isolated by ethanol fractionation.

a similar high isoelectric point was indicated. This apparent discrepancy cannot be explained by the different buffer conditions used, because under the conditions of Tiselius & Kabat (1939) [pH 5·85; sodium acetate (I 0·02) plus sodium chloride (I 0·15)] the same normal γ-globulin was not isoelectric and had a mobility of $+0{\cdot}40_4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$v^{-1}, only slightly lower than that shown in Fig. 7. Variable results of this nature might be expected from the known heterogeneity of γ-globulin and antibodies (Porter, 1960), and the narrow range of average isoelectric points obtained in this work is therefore somewhat surprising.

Antibody release from many serological complexes with salt-free saturated aqueous carbon dioxide

The results of numerous experiments with many different serological precipitates, summarized in Table 4, show that the use of saturated aqueous carbon dioxide is a general method for dissociating antigen–antibody precipitates. The pH of all these dissociating systems (0·5–1·5% protein) was 4·8–5·1, except for the diphtheria toxin–antitoxin system which had a pH of 4·4. The extent of the dissociation depends on the antigen and the particular antiserum used, e.g. rabbit anti-(sperm-whale myoglobin) III appeared to contain a more avid antibody than either I or II, and this was supported by the fact that buffers of more extreme pH (< 2·5, > 11·5) were needed to dissolve precipitates from antibody III. In some cases, aqueous carbon dioxide extracted almost pure antibody, leaving an insoluble residual complex.

Isolation of various antibodies

Rabbit anti-(bovine serum albumin) (23% yield, 81% precipitable) was isolated by a method comparable with that described for rabbit antiovalbumin. Rabbit antiserum to antigen 3 of *P. pestis* (12% yield, 80% precipitable), rabbit anti-

Table 4. *Solution and dissociation of various antigen–antibody precipitates in salt-free saturated aqueous carbon dioxide*

Precipitates were formed in the equivalence zone unless otherwise stated. Antibody release was estimated by sedimentation analysis except for sparingly soluble precipitates where the values quoted were the amounts (%) of antibody isolated after aqueous carbon dioxide extraction and purification.

Nature of precipitate		Dissociation in aqueous CO_2		
Antigen	Antiserum	Solubility (%)	Antibody released (approx. %)	$S^*_{20,w}$ of residual complex (s)
Sperm-whale myoglobin	Rabbit I	>1·5	30–40	11·7†
	Rabbit II	>1·5	30–40	—
	Rabbit III	0·6	—	—
Human serum albumin	Rabbit	>1	15–25	19·6
Bovine serum albumin	Rabbit			
(*a*) antibody:antigen, 6:1		>1	15	33
(*b*) antibody:antigen, 11:1		>1	20–30	~100
Ribonuclease	Rabbit	>1	30	7·9 and 11·5
Lysozyme	Rabbit	>1	20	14
Polysaccharide of *S. shiga*	Rabbit			
(*a*) Degraded		>1	>50	9·7
(*b*) Undegraded		1	25	17·6 and 31
Horse serum albumin	Rabbit	0·7	15–20	Insoluble‡
Antigen 3 of *P. pestis*	Rabbit	<0·1	15–20 (extracted)	Insoluble
Pneumococcus polysaccharide SIII	Rabbit	0·1	30 (extracted)	Insoluble
Diphtheria toxin	Horse I	>0·7	15–20	Very polydisperse
	Horse II	>0·5	15–20	Very polydisperse
Pneumococcus polysaccharide SI	Horse			
(*a*) Antibody:antigen, 20:1		0·3	50	Insoluble
(*b*) Antibody:antigen, 16:1		0·3	33	Insoluble
(*c*) Antibody:antigen, 8:1		0·2	17	Insoluble

† Light-absorption due to myoglobin associated with the complex peak cf. dissociation of antihaemoglobin IV [Fig. 5 (*b*)].

‡ Sedimented before the ultracentrifuge had reached full speed.

(*Pneumococcus* polysaccharide SIII) (30% yield, 71% precipitable) and horse anti-(*Pneumococcus* polysaccharide SI) (48% yield, 56% precipitable) were isolated by extraction of the corresponding specific precipitates with aqueous carbon dioxide followed by a purification similar to that described (cf. Fig. 3) for the supernatants in the rabbit-antiovalbumin preparation.

Dissociation of complexes by various acids at low ionic strength and pH 5

The marked effect of salt-free aqueous carbon dioxide on antigen–antibody reactions prompted a brief examination of the effects of other acids at about pH 5 under salt-free conditions. We were unable to find in the literature any record of such experiments. Although Turnbull (1959) quotes several references to dissociation of antigen–antibody complexes by aqueous carboxylic acids, only one (Friend, 1953) in fact relates to antigen–antibody systems; in this case a concentrated salt solution [8% (w/v) sodium salicylate] was used and not the free acid. Previous work has been done almost exclusively with hydrochloric acid or acetic acid at pH 2–4 and at moderate ionic strength.

The results showed that ovalbumin–rabbit-antiovalbumin precipitates dissolved at about pH 5 in aqueous solutions of low ionic strength of many acids (including a number of amino acids, succinic acid, benzoic acid, propionic acid, acetic acid, telluric acid, sulphurous acid and hydrochloric acid). The concentration of acid necessary to give a solution of complex (0·5–1·5% protein) at about pH 5 varied according to the pK of the acid: from 0·7 mM for hydrochloric acid and sulphurous acid to 200 mM for telluric acid. Many of the acid solutions were examined in the ultracentrifuge but only propionic acid and sulphurous acid were found to compare with aqueous carbon dioxide in effectiveness of dissociation. The effect of small concentrations of salt in reducing the solubility of complexes was observed in all the acid systems examined and the decrease in dissociation was more marked in aqueous sulphurous acid than in aqueous carbon dioxide.

Alkaline reagents which were successful in dissolving ovalbumin complexes at low ionic strengths included sodium hydroxide, veronal buffer, aqueous hydrazine and aqueous ethanolamine. Sedimentation analysis showed that dissociation into free globulin and residual complexes had taken place in a solution at pH 7·0 prepared by adding sodium hydroxide (mN; 1·7 ml.) to a salt-free suspension of complex (27 mg./ml.), but that no significant degree of dissociation was observed in the other alkaline reagents even at higher pH (8·2–8·9). The effect of slight increases in ionic strength on alkaline solutions of complex was similar to that observed in acid solution.

DISCUSSION

There is sufficient evidence in this paper to indicate that many serological complexes undergo some degree of dissociation in salt-free saturated aqueous carbon dioxide at pH 5. As might be expected, the extent of the dissociation depends on the antigen and the immunization procedure used for the preparation of the antibody. It can vary from complete dissociation of antigen from antibody, as for certain haemoglobin–rabbit-antibody complexes, to the removal of only a small amount of antibody from an insoluble residue, as found with some of the serological complexes of pneumococcal polysaccharides.

A salt-free medium plays an important role in the dissociation, and the action of aqueous carbon dioxide is only one special example of dissociation in salt-free systems at a relatively neutral pH which can be produced by a number of acids and even alkalies. None of the systems tested proved to be more effective than aqueous carbon dioxide, which has certain practical advantages to offer, namely in stabilizing the pH and in being readily removed. The unusual 'buffering' mechanism of aqueous carbon dioxide may have some bearing on its action. The presence of a hydrogen carbonate salt is unnecessary since a considerable potential adjustment in $[H_2CO_3]$ is provided by the reaction: CO_2 (dissolved) $+ H_2O \rightleftharpoons H_2CO_3$, which is largely displaced (K approx. 10^{-3}) in favour of dissolved but unhydrated carbon dioxide. Any readjustment of the equilibrium: $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$, demanded by the protonation of the antigen–antibody system, can thus occur without significant change in $[H_2CO_3]$. The other acids are not effective buffers at the concentration necessary to protonate the antigen–antibody system to a final pH of 5, since most of the acid is ionized. If a salt of the acid is added to provide buffer capacity then the increased ionic strength of the system results in decreased dissociation or even precipitation of the complex.

Dissociation of serological complexes in aqueous carbon dioxide would appear to be milder than the majority of methods employed previously. Final proof obviously rests on the physical integrity of the isolated antibody (or antigen). This was judged to be satisfactory from physicochemical and immunological tests.

Antibodies and γ-globulins are heterogeneous as shown by immunological and physical criteria (Porter, 1960); the immunological heterogeneity of our dissociated antibodies was evident from the following observations: (*a*) the first and second aqueous carbon dioxide extracts of some antiovalbumin complexes showed differences in precipitability with ovalbumin; (*b*) the dissociated anti-

ovalbumin proved to be almost completely soluble in water (pseudoglobulin) but dissociated anti-haemoglobin was largely insoluble (euglobulin); (c) over a period of 3 years there were changes in the pattern of precipitability of ovalbumin antisera prepared by the same method. However, it is unlikely that resolution of antibody activity within the γ-globulin mobility spectrum could be achieved by electrophoresis of the original antisera, since electrophoretic 'internal heterogeneity' [as judged by boundary-spreading analysis in the isoelectric region (Alberty, 1948)] was very similar for the normal γ-globulin and these dissociated antibodies.

Clearly, antibodies isolated by methods based on the dissociation of a complex with aqueous carbon dioxide would be suitable for further work on the fractionation of antigens by immunochemical means. The monospecific antigen–antibody precipitates may be separated by the aid of a gel-diffusion method (Smith *et al.* 1962) and dissociated in aqueous carbon dioxide. The isolated antibody can then be covalently linked to an insoluble matrix (Isliker, 1957) to produce a monospecific immunological absorbent from which antigen might be dissociated more easily than from the normal serological complex.

Finally, the dissociation of serological complexes in aqueous carbon dioxide and other systems of low ionic strength should be discussed in relation to currently held views on the nature of the union between antigen and antibody. The reversibility of the antigen–antibody reaction is generally accepted (Doty & Epstein, 1954; Singer & Campbell, 1955; Steiner, 1955), and the results described here demonstrate it quite clearly, e.g. the solubility and dissociation of complexes in aqueous carbon dioxide are reversibly decreased by either increase in ionic strength or decrease in carbon dioxide content of the system.

Electrostatic attraction between dissociated carboxyl groups and protonated basic groups on the proteins has been proposed for the fundamental linkage between antigen and antibody (Singer & Campbell, 1955; Marrack, 1958). Increased screening by counter-ions on increasing the ionic strength would explain the dissociation of complexes by concentrated salt solutions (Steiner, 1955); but under our conditions of low ionic strength attractive electrical forces are intensified and yet dissociation occurs, suggesting that even stronger repulsive forces, presumably due to similarly charged adjacent groups, come into play. Apart from this intensification of intermolecular forces between antigen and antibody at low ionic strengths, one must also consider similar intramolecular forces which may distort either antigen or antibody to the point where their molecular surfaces are no longer complementary. As with many protein–protein interactions [see Waugh (1954) and McMenamy & Oncley (1958)], it is unlikely that the union of antigen and antibody will depend solely on the electrostatic forces discussed so far, but will probably involve a complicated pattern of interaction including hydrogen bonding and van der Waals forces; this might explain the wide variation in the strength of the linkage which has been underlined by the work described here.

SUMMARY

1. The use of salt-free saturated aqueous carbon dioxide at pH 5 has proved to be a general method for dissociating antigen–antibody complexes. The extent of the dissociation depends on the nature of the antigen and the course of immunization used to produce the antibody. It varies between complete dissociation of antigen from antibody (a haemoglobin complex) to the liberation of a small amount of antibody from a residual insoluble complex.

2. The salt-free environment has proved to be essential for the dissociation, and the application of aqueous carbon dioxide in such a system was a useful example of a general effect in salt-free systems produced at relatively neutral pH by a number of other acids and alkalis.

3. A number of antibody preparations were obtained in good yield from specific precipitates after dissociation with aqueous carbon dioxide. The properties of these preparations underline the general heterogeneity of antibody as regards precipitation, solubility etc.

4. The results are discussed in relation to the molecular forces involved in breaking the union between antigen and antibody; it is suggested that, as in other protein–protein interactions, the total antigen–antibody union is due to a complicated pattern of different interactions not all of which may have to be present for some combination to take place. This would explain the enormous variation in the strength of the antigen–antibody linkage and the heterogeneity of antibody which has been confirmed by the work described here.

We should like to thank Professor J. R. Marrack, Dr M. Barr, Dr D. A. L. Davies, Dr J. H. Humphrey, Dr R. A. Kekwick, Dr M. C. Lancaster, Dr K. A. Munday, Dr J. H. Pearce and Dr C. G. Pope for gifts (see text) of various antigens and antisera. We are also indebted to Mr F. S. Scanes, Mr R. C. Gallop, Mr K. H. Grinstead and Mr V. M. C. Longyear for excellent technical assistance.

REFERENCES

Albert, A. & Johnson, P. (1961). *Biochem. J.* **81**, 658.
Alberty, R. A. (1948). *J. Amer. chem. Soc.* **70**, 1675.
Alberty, R. A. (1949). *J. phys. Chem.* **53**, 114.
Allison, A. C. & Humphrey, J. H. (1960). *Immunology*, **3**, 95.

Baldwin, R. L., Gosting, L. J., Williams, J. W. & Alberty, R. A. (1955). *Disc. Faraday Soc.* **20**, 13.
Boyd, W. C. & Malkiel, S. (1944). *J. infect. Dis.* **75**, 262.
Brice, B. A. & Halwer, M. (1951). *J. opt. Soc. Amer.* **41**, 1033.
Cammack, K. A. (1962). *Nature, Lond.*, **194**, 745.
Campbell, D. H. & Lanni, F. (1951). In *Amino Acids and Proteins*, p. 673. Ed. by Greenberg, D. M. Springfield: C. C. Thomas.
Cann, J. R. (1953). *J. Amer. chem. Soc.* **75**, 4213, 4218.
Cann, J. R. & Phelps, R. A. (1955). *J. Amer. chem. Soc.* **77**, 4266.
Cinader, B. (1957). *Annu. Rev. Microbiol.* **11**, 371.
Coulson, C. A., Cox, J. T., Ogston, A. G. & Philpot, J. St L. (1948). *Proc. Roy. Soc.* A, **192**, 382.
Creeth, J. M. (1952). *Biochem. J.* **51**, 10.
Doty, P. & Epstein, S. I. (1954). *Nature, Lond.*, **174**, 89.
Friend, C. (1953). *J. Immunol.* **70**, 141.
Gilbert, G. A. & Jenkins, R. C. Ll. (1956). *Nature, Lond.*, **177**, 853.
Gosting, L. J. (1956). *Advanc. Protein Chem.* **11**, 476.
Gosting, L. J., Hanson, E. M., Kegeles, G. & Morris, M. S. (1949). *Rev. sci. Instrum.* **20**, 209.
Gosting, L. J. & Morris, M. S. (1949). *J. Amer. chem. Soc.* **71**, 1998.
Grant, R. A. (1959). *Brit. J. exp. Path.* **40**, 551.
Harned, H. S. & Owen, B. B. (1950). *The Physical Chemistry of Electrolytic Solutions*, 2nd ed., p. 138. New York: Reinhold Publishing Corp.
Heidelberger, M. & Kabat, E. A. (1938). *J. exp. Med.* **67**, 181.
Hummeler, K. & Ketler, A. (1958). *Virology*, **6**, 297.
Isliker, H. C. (1957). *Advanc. Protein Chem.* **12**, 387.
Jones, G. & Bollinger, G. M. (1931). *J. Amer. chem. Soc.* **53**, 411.
Kabat, E. A. (1939). *J. exp. Med.* **69**, 103.
Katz, J. J. (1954). *Nature, Lond.*, **174**, 509.
Kegeles, G. & Gosting, L. J. (1947). *J. Amer. chem. Soc.* **69**, 2516.
Kleinschmidt, W. J. & Boyer, P. D. (1952). *J. Immunol.* **69**, 247.
McMenamy, R. H. & Oncley, J. L. (1958) *J. biol. Chem.* **233**, 1436.
Marrack, J. R. (1958). *Immunology* **1**, 251.
Marrack, J. R. Hoch, H. & Johns, R. G. S. (1951). *Brit. J. exp. Path.* **32**, 212.
Mitz, M. A. (1957). *Biochim. biophys. Acta*, **25**, 426.
Nichol, J. C. & Deutsch, H. F. (1948). *J. Amer. chem. Soc.* **70**, 80.
Oudin, J. & Grabar, P. (1944). *Ann. Inst. Pasteur*, **70**, 7.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Pope, C. G. (1957). *Brit. J. exp. Path.* **38**, 207.
Porter, R. R. (1960). In *The Plasma Proteins*, vol. 1, p. 267. Ed. by Putnam, F. W. New York: Academic Press Inc.
Rees, E. D. & Singer, S. J. (1956). *Arch. Biochem. Biophys.* **63**, 144.
Schachman, H. K. (1959). *Ultracentrifugation in Biochemistry*, p. 116. New York: Academic Press Inc.
Shedlovsky, T. (1930). *J. Amer. chem. Soc.* **52**, 1793.
Singer S. J. & Campbell, D. H. (1955). *J. Amer. chem. Soc.* **77**, 4851.
Smith, H. Tozer, B. T. Gallop, R. C. & Scanes, F. S. (1962). *Biochem. J.* **84**, 74.
Steiner, R. F. (1955). *Arch. Biochem. Biophys.* **55**, 235.
Svedberg, T. & Pederson, K. O. (1940). *The Ultracentrifuge*, p. 23. Oxford: Clarendon Press.
Tiselius, A., Hjertén, S. & Levin, Ö. (1956). *Arch. Biochem. Biophys.* **65**, 132.
Tiselius, A. & Kabat, E. A. (1939). *J. exp. Med.* **69**, 119.
Tozer, B. T. Cammack, K. A. & Smith, H. (1958). *Nature, Lond.*, **182**, 668.
Turnbull, J. H. (1959). *Experientia*, **15**, 304.
Wagner, M. L. & Scheraga, H. A. (1956). *J. phys. Chem.* **60**, 1066.
Waugh, D. F. (1954). *Advanc. Protein Chem.* **9**, 361.
Waugh. D. F. & Yphantis, D. A. (1952). *Rev. sci. Instrum.* **23**, 609.

Biochem. J. (1962) **84**, 93

Sterol Biosynthesis in Neoplastic Cells: Utilization of [^{14}C]Acetate and of [2-^{14}C]Mevalonate

By IRENE YOUHOTSKY GORE and G. POPJÁK
Medical Research Council Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12

(*Received* 1 *January* 1962)

It was observed some years ago in this laboratory that mouse Ehrlich ascites-carcinoma cells failed to incorporate [^{14}C]acetate into sterols or fatty acids and that they also failed to oxidize it to carbon dioxide. When tumour-bearing animals were fed with ^{14}C-labelled egg-yolk lipids ([^{14}C]cholesterol, [^{14}C]phospholipids and [^{14}C]triglycerides) the tumour cells 'scavenged' the lipid from the host; the specific activities of the lipids isolated from the tumour cells and from the liver of the host were identical (G. Popják & J. Berthet, unpublished work). These observations were recently confirmed by us when tumour-bearing mice were fed with ^{14}C-labelled lipids of *Chlorella vulgaris* (I. Y. Gore & G. Popják, unpublished work).

These findings were in agreement with those reported by others. Busch & Baltrush (1954) concluded from experiments in which [^{14}C]acetate was