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The Action of Soluble Antigen–Antibody Complexes in Perfused Guinea-Pig Lung

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Summary. When soluble antigen–antibody complexes, prepared in antigen excess, were injected into the perfused pulmonary artery of the unsensitized guinea-pig lung, they produced a bronchoconstrictor response which was associated with the appearance of histamine and a slow-reacting substance in the effluent. This activity was inhibited by the presence of normal rabbit serum or rabbit globulin; rabbit albumin and bovine γ-globulin produced no inhibition. Insoluble antigen–antibody complexes prepared at equivalence were at least 125 times less active than soluble complexes prepared in antigen excess. On comparing the activity of different solutions of soluble complex prepared in antigen excess ranging from $2\frac{1}{2}$ to 160 times that required for equivalence, all were found to be equal. The properties of soluble antigen–antibody complexes are consistent with the possibility that circulating antibody may participate in *in vivo* anaphylaxis, if reached by an amount of antigen greater than that required for equivalence.

INTRODUCTION

In studies dealing with the mechanism of anaphylaxis, it has been customary to make a distinction between antibody which is bound to cells and antibody not bound to cells. It is generally accepted that antibody which has first become fixed to cells has the capacity on subsequent combination with antigen to activate the system which results in anaphylaxis (Dale, 1912). On the other hand, there is a lack of agreement regarding the activity of antibody which combines with antigen before having become bound to cells.

Several studies have suggested that in systems which contain both free and cell-bound antibody, the presence of unbound antibody may be associated with a decreased susceptibility to anaphylaxis (Weil, 1912; Dale and Kellaway, 1921; Kabat, Coffin and Smith, 1947; Swineford and Samsell, 1962). The interpretation of this protective effect has been that free antibody on combination with antigen does not have the capacity to activate the process of anaphylaxis, and probably serves to decrease the access of antigen to cell-bound antibody. However, other work has failed to demonstrate a protective effect of unbound antibody (Morris, 1936; Kabat *et al.*, 1947). More recently it has been shown that antibody in the form of a soluble complex with antigen, may produce a variety of reactions resembling anaphylaxis (Germuth and McKinnon, 1957; Tokuda and Weiser, 1958; Trapani, Garvey and Campbell, 1958; Ishizaka, Ishizaka and Campbell, 1959; Treadwell, Wistar and Rasmussen, 1960; Weigle, Cochrane and Dixon, 1960) whereas insoluble complexes of antigen and antibody have been found inactive when tested in the same way. A possible explanation of these discordant observations is that unbound anti-

body may either activate or protect from anaphylaxis, depending on whether the ratio with antigens yields soluble or insoluble complexes.

Soluble antigen–antibody (Ag–Ab) complexes prepared *in vitro* under conditions of antigen excess have been shown to produce an anaphylactic syndrome when administered intravenously to unsensitized animals (Germuth and McKinnon, 1957; Tokuda and Weiser, 1958; Treadwell *et al.*, 1960; Weigle *et al.*, 1960) and an increase in capillary permeability when injected into the skin (Ishizaka *et al.*, 1959). They are also known to induce smooth muscle contraction *in vitro* (Trapani *et al.*, 1958).

The present experiments are a continuation of this line of investigation. They show that soluble complexes produce bronchoconstriction and histamine release in the isolated perfused lung. These effects are antagonized by normal serum, suggesting that Ag–Ab complexes, like antibody in anaphylactic sensitization, require attachment to cells before they can exert their effects. A preliminary report of this work has been published (Broder and Schild, 1964).

EXPERIMENTAL METHODS

Antigen

The antigen used in these studies was crystallized bovine plasma albumin (Armour Co., BPA). An azo dye C.I. Acid Blue 108 (Farbwerke Hoechst AG., Frankfurt), was diazotized and coupled to the BPA (dyed BPA) as described by Kruse and McMaster (1949); it was found by these authors that the dye did not otherwise bind to protein. Uncoupled dye was separated from the dyed BPA by passage through Sephadex G50 in columns 6 cm. in diameter $\times$ 30 cm. high, made up in 0·15 M sodium chloride (saline). The crude dyed BPA was placed on the column in 80 ml. volumes, containing approximately 10 mg. of BPA per ml. The dyed BPA passed through the column considerably more rapidly than the uncoupled dye, and the two could be readily distinguished by the ratio of their respective absorptivities at 225 and 645 mμ. The ratio for the dyed BPA was 20 : 25, while the dye alone was 1 : 2. In the course of four passages through the columns, the molar ratio of dye to BPA fell from 12 in the crude preparation to 4. The purity was considered acceptable at this point, since in an aliquot passed through the column a fifth time, only 2·5 per cent or less of the dye did not come through with the protein. The solution was stored at −20°.

All light absorption determinations were made in a Hilger and Watt H700 spectrophotometer, using cuvettes with a light path of 1·0 cm. and a capacity of 3 ml. The absorption peak for the dyed BPA was at 645 mμ, and the molar extinction coefficient, $2{\cdot}8 \times 10^{-4}$. The latter was based on an average of replicate absorption and micro-Kjeldahl determinations; two batches of dyed BPA made several months apart showed close agreement in this coefficient.

BPA and dyed BPA behaved similarly in horizontal starch gel electrophoresis at pH 8·6, using 0·08 M tris-citrate in the gels, and 0·3 M sodium borate in the electrode chambers (Poulik, 1959). Both showed a major band migrating to the positive electrode, and two lightly staining bands close behind. All of the dye in the dyed BPA moved with the protein-staining bands; the dye alone was markedly less mobile than the BPA and did not take up the protein stain (Amido black).

Antibody

Two groups of albino rabbits were immunized with 10 mg. of dyed BPA three times weekly for 4 weeks. During the first 2 weeks, alternate injections were given intravenously

and subcutaneously; the remainder of the course was given by the subcutaneous route. One week after the last injection, each rabbit was bled twice, 2–3 days apart (pools 1 and 3). Three weeks later, the rabbits which yielded pool 1, received a second course of three injections over 1 week and each was again bled twice (pool 2). All sera were separated from the clotted elements after 24 hours at 4°; they were subsequently kept at 4° for several days and then stored at −20°.

Quantitative precipitin tests and micro-Kjeldahl determinations were carried out using standard methods (Kabat and Mayer, 1961). For each tube in a precipitin series, 0·5 ml. of serum was mixed with an equal volume of antigen made up in saline. The tubes were kept at 37° for 30 minutes and at 4° for 48 hours. The precipitates were separated by centrifugation at 1500 ***g*** for 30 minutes at 4° and washed three times with 1·0 ml. volumes of cold saline. Each series was run in duplicate.

Soluble Ag–Ab Complexes

Soluble Ag–Ab complexes were prepared by two methods, using either BPA or dyed BPA in amounts calculated to be in antigen excess. (1) The required amount of excess antigen, made up in saline containing 0·033 M sodium phosphate, pH 7·4 (buffered saline), was mixed with serum which had been dialysed against buffered saline. After 30 minutes at 37° and 48 hours at 4°, any insoluble material was separated by centrifugation at 12,000 ***g*** for 30 minutes at 4°. (2) The equivalence amount of antigen was mixed with an equal volume of serum and treated as described for the precipitin tests, with one exception: the precipitate was very finely broken during the course of each wash in cold buffered saline, by repeated pipetting through a small orifice. The required amount of excess antigen in buffered saline was then added and the precipitate again finely broken. After 30 minutes at 37° with occasional agitation, the tube was left on a wrist-action shaker for 48 hours at 4°. These preparations were then centrifuged as described for the first method.

All preparations of soluble Ag–Ab complex were made at an antibody protein concentration of 2–8 mg./ml. In the course of making solutions with five times antigen excess or more, the amount of insoluble material lost in centrifugation was very small, and no correction for this was carried out. Solutions were stored in 1–2 ml. aliquots at −20° and only briefly thawed for each use. There was no evidence of instability over storage periods exceeding one year and following repeated freeze-thawing.

Since dyed BPA was not precipitated at 50 per cent saturation with ammonium sulphate (pH 7·0, 16–20°) the complexes could be separated from uncombined dyed BPA by repeated precipitation under these conditions until the supernatants were no longer coloured. The precipitates were finally re-dissolved and both they and the supernatants dialysed at 4° against buffered saline. A small amount of insoluble material was removed by centrifuging and the amount of dyed BPA present in both fractions estimated from the absorptivity at 645 mμ. The assumption was made that all of the antibody was precipitated in half-saturated ammonium sulphate and that all the dyed BPA so precipitated was specifically associated with antibody. The validity of these assumptions has been discussed by Farr (1958). The ratio of antigen in complex with antibody was calculated from the percentage of antigen which was insoluble in 50 per cent ammonium sulphate and the known antibody content of the solution.

For the purposes of calculation the molecular weights of rabbit antibody and of dyed BPA were taken as 160,000 and 69,000 respectively, and their nitrogen contents at 16 per cent. The contribution of dye to the BPA (3 per cent by weight) was neglected.

A pool of normal serum was collected from the rabbits before the onset of their course of immunization. This was fractionated at 50 per cent saturation in ammonium sulphate, as described for the soluble complexes. Both the whole serum and fractions were dialysed against buffered saline for use in the inhibition studies.

Lung Perfusion

The activity of soluble Ag–Ab complexes was studied in a perfused guinea-pig lung system (Arunlakshana and Schild, 1959), slightly modified for this work. The pulmonary artery was cannulated and perfused with Tyrode solution which drained through the excised left atrium. The perfusion fluid was supplied from a Mariotte reservoir and passed through a capillary resistance and warming coil; the height of the reservoir above the pulmonary artery was generally 90 cm., but this was varied to maintain a perfusion rate of 2·4–2·6 ml./minute. The trachea was perfused with air and this escaped through scarifications on the pleural surface made by light stroking with a needle; the air flow rate was 1·5 ml./minute. The method of recording the air inflow pressure was as described in the publication referred to above; pressure increases due to bronchoconstriction were recorded on a smoked drum.

The lung was suspended from the two cannulae in a large jacketed funnel the top of which was lightly covered with absorbent cotton soaked in Tyrode solution, and fitting closely about the cannulae. The jacketed funnel and coil were circulated with water at a temperature of 37°. With an indwelling thermocouple the temperature on the inner aspect of the funnel was found to be consistently in the range of 36°.

Injections were made with a syringe and needle into the pulmonary artery cannula. Aliquots of the test material were measured and frozen in advance. They were thawed within 30 minutes of use, and 5 minutes prior to injection were diluted with buffered saline and heated in a 37° bath. All injections were given in a volume of 0·5 ml. and gently infused over a 15-second interval. No test materials were used which had been in the diluted state for over 7–8 minutes.

Unsensitized albino guinea-pigs were used throughout. Preliminary work was done with animals of both sexes, weighing 300–500 g., and supplied by a number of different breeders; the majority of the studies were carried out with males of a single strain (Porton), weighing 300–400 g., and all obtained from the same source. The animals were killed by a blow on the head, and the trachea immediately compressed to prevent aspiration of blood and gastric contents. Any preparation which showed evidence of either was discarded. After tying off the trachea, the animal was exsanguinated by transecting the neck. The pulmonary artery was cannulated and the Tyrode perfusion started as soon as possible following this; no longer than 10 minutes elapsed from the starting of a preparation to this point. The trachea cannula was then tied in and the lungs were allowed to inflate for 30 minutes. Scarification was carried out as uniformly as possible, with four light strokes of a needle across the pleural surface of each lobe.

The inflow pressure of the Tyrode solution at the level of the pulmonary artery cannula was measured at intervals during the course of some experiments. Although there was variation between preparations, on the whole the pressure was below 4 cm. H_2O for the first 4 hours of perfusion, and then gradually rose to approximately 10 cm. over a period of several hours. This rise in pressure appeared to parallel the development of oedema. The effluent rate of the Tyrode solution was continuously monitored by a Thorp drop recorder; in addition, an absolute measure was made at intervals during each experiment

by using a graduated cylinder. The rate tended to fall slightly as the inflow pressure rose, and the feeding reservoir would be elevated several cm. in order to preserve a near constant flow of perfusate. For a variable period following a bronchoconstrictor response, the flow rate generally fell to approximately 2·2 ml./minute, but would then spontaneously rise to within the former range. The pH of the inflowing Tyrode solution was sampled at intervals and found to gradually rise from approximately 7·5 to 7·9 over a 9 hour perfusion period.

The air inflow pressure was continuously recorded, and found to maintain a fairly steady baseline, generally in a range between 12 and 16 mm.Hg. During bronchoconstrictor responses, this would temporarily increase to as high as 72 mm.Hg, the maximum pressure which the system was capable of generating.

The area of each bronchoconstrictor response was measured with a metric scale planimeter. This was converted to a linear term by taking the square root, and will be referred to as the response in 'centimetres'.

Statistical assessments were made with Student's *t*-test, and with the analysis of variance by a Latin square design.

Bioassays

Histamine assays were performed on the guinea-pig ileum at 37° using a semi-automatic procedure (Boura, Mongar and Schild, 1954). The unknowns were tested at two dose levels in randomized blocks, and compared at the beginning and end of each block with histamine standards of 2·5, 5, 10 and 20 mμg./ml. (base). Each cycle included a contact and rest period of approximately 20–40 seconds respectively. Slow reacting substance (SRS) was tested on the same preparation, but using Tyrode solution containing mepyramine maleate at a concentration of 1·0–0·1 μg./ml. (Brocklehurst, 1960). This level of antihistamine was sufficient to inhibit both the standard doses of histamine and the concentration of histamine in the samples assayed for SRS. For SRS assays the contact period of the test sample with the gut was 1½–3 minutes, and the rest period between tests 3½–8 minutes; in any given series, a fixed cycle was used for all assays and the samples assayed against each other. When testing solutions for both histamine and SRS activity, the procedure was to first assay the total activity as described for histamine. Since these assays were carried out with higher dilutions and at a faster cycling period than SRS assays, any interference by the latter was neglected and total activity expressed in terms of histamine base equivalents. Mepyramine was then added to the system and the residual activity measured as described for SRS.

Assays with bradykinin were carried out on the rat uterus in de Jalon's solution at 30°.

RESULTS

Precipitin Curves

The antibody protein content of the three serum pools, using dyed BPA as antigen, was 2·5, 4·6 and 2·6 mg./ml. respectively; using BPA as antigen, pool 3 was found to have 2·3 mg. antibody protein per ml. (Fig. 1). The similarity between the precipitin curves for pool 3 using BPA and dyed BPA as the antigen in separate studies, suggested that the antibody was principally directed towards the BPA rather than the dye. The molar ratio of antigen to antibody at the 'equivalence point' of the three sera, ranged from 0·33 to 0·37.

Antigen–Antibody Ratio

The ratio of antigen in soluble complex with antibody was determined in preparations of complex made from pool 2 in antigen excess ranging from 2½ to 160 times that needed for equivalence (Table 1). Preparations which were separately made from the same pool of serum and at a similar level of antigen excess, showed close agreement in their respective antigen–antibody ratios. The ratio in a given solution did not change after either 4 weeks at 4°, or several freeze-thawings and 4 weeks at −20°.

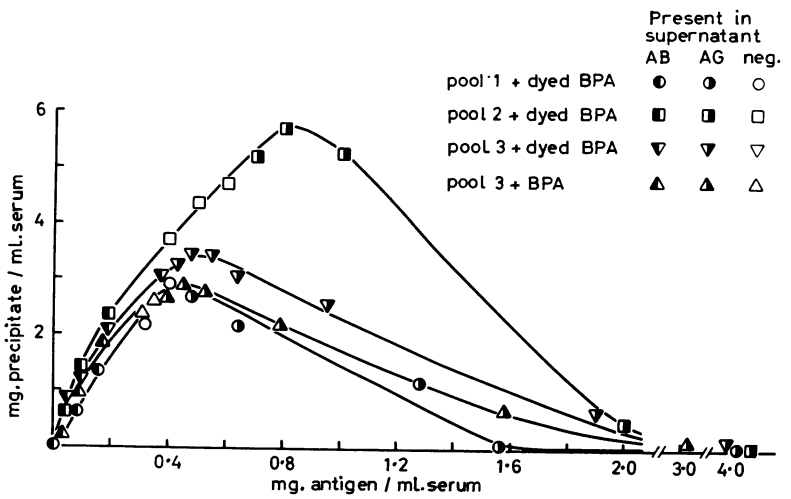

FIG. 1. Quantitative precipitin curves for rabbit serum pools 1, 2 and 3.

TABLE 1

THE MOLAR RATIO OF ANTIGEN TO ANTIBODY AT VARYING LEVELS OF ANTIGEN EXCESS

Antigen bound to antibody was measured spectrophotometrically after precipitation at 50 per cent saturation in ammonium sulphate. This quantity of antigen was expressed as a molar ratio of the known amount of associated antibody.

Ag excess	*Ab (mg./ml.)*	*Ag (mg./ml.)*	*Ag in precipitate* (%)	*Ag in supernatant* (%)	*Ag/Ab molar ratio*
2½*	8·1	3·2	96·2	3·8	0·9
5	7·5	5·8	75·0	25·0	1·4
10	5·4	7·8	57·2	42·8	1·9
40	7·5	46·0	23·8	76·2	3·4
160	5·2	128·0	7·0	93·0	4·1

* Correction was made in calculating the concentration of antigen and antibody in this solution, for the reactants which remained insoluble following re-equilibration of the equivalence precipitate in this level of antigen excess.

Lung Perfusions

When a solution of soluble antigen–antibody complexes was injected into the pulmonary perfusate, a bronchoconstrictor effect was observed (Fig. 2). The latency and magnitude of this effect depended on the dose used: a medium response would begin 30–60 seconds after injection and last about 45 minutes; a small response might be more delayed in onset, but all began within 4 minutes. These bronchoconstrictor effects were

similar in character to those following histamine injections, except that they developed a little more slowly.

Release of Active Substances

Pharmacologically active substances appeared in the venous effluent collected during each bronchoconstrictor response. Two kinds of activity were detected, histamine-like and slow-reacting.

(a) *Histamine-like activity.* When active effluent was assayed on the isolated guinea-pig ileum, a rapid histamine-like contraction was produced (Fig. 3) which could be antagonized by mepyramine. These effects were obtained with effluent dilutions of up to 100-fold,

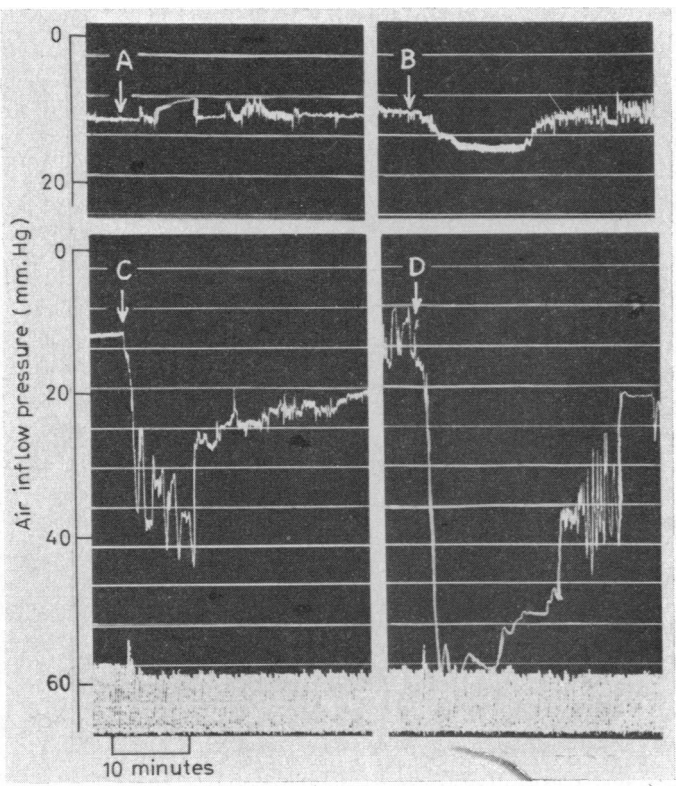

FIG. 2. Bronchoconstrictor effects of graded doses of soluble Ag–Ab complexes. Successive injections into a single lung contained at A, 0·4 μg. antibody protein; at B, 1·6 μg.; at C, 6·4 μg., at D, 25·6 μg.

depending somewhat on the magnitude of the associated bronchoconstrictor response. In different lungs, the mepyramine-inhibited activity ranged between approximately 70 and 99 per cent of the total.

(b) *Slow-reacting substance.* Effluent diluted for histamine assay produced no appreciable effect on the guinea-pig ileum in the presence of mepyramine at a concentration of 1·0–0·1 μg./ml. More concentrated samples still produced a response under these conditions, even though a histamine standard adjusted to the same increased concentration remained totally inhibited. This activity became more obvious when the assay cycle was lengthened

to permit contact with the ileum for 1½–3 minutes, rather than the 20-second interval used for the histamine-like activity (Fig. 3).

The mepyramine resistant activity could be distinguished from bradykinin, 5-hydroxytryptamine and acetylcholine. Effluent was compared in parallel quantitative assay against bradykinin on both the rat uterus and the mepyramine-treated guinea-pig ileum. A concentration of bradykinin which was equal to that of the effluent on the guinea-pig ileum was 80 times more active on the rat uterus. Similarly, using the mepyramine-treated guinea-pig ileum, equivalent responses to acetylcholine and 5-hydroxytryptamine

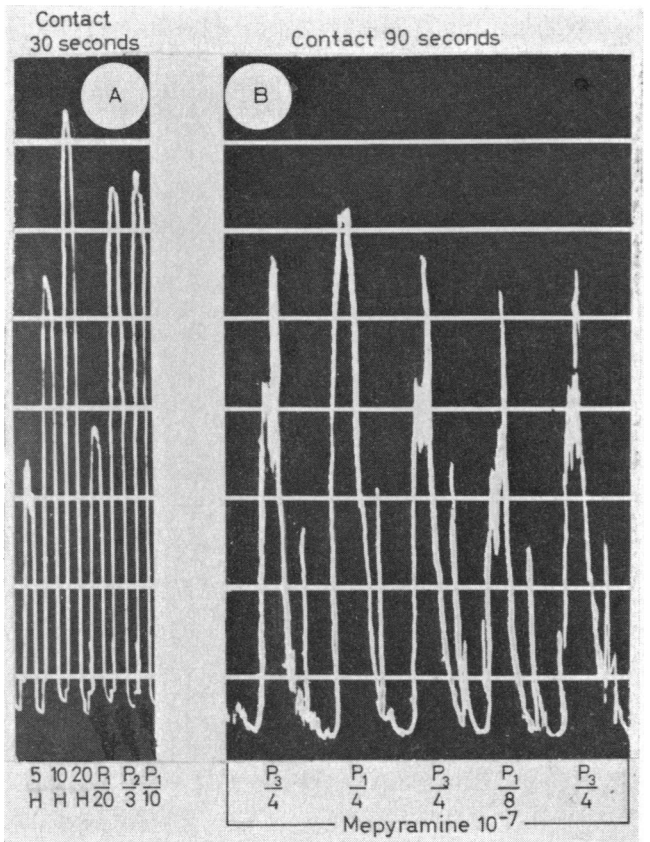

FIG. 3. The activity of effluent on the guinea-pig ileum. In A, effluent samples P_1 and P_2 are compared against histamine (H = μg./l.) standards. In B, P_1 is re-assayed against another sample as standard, in the presence of mepyramine maleate 0·1 μg./ml.

were completely inhibited by 1·0 μg./ml. of atropine sulphate, while the effluent activity remained unaltered (Fig. 4).

We concluded that the effluent obtained after injections of Ag–Ab complexes resembles that of anaphylactic shock, in containing a mixture of histamine and an unidentified slow-reacting substance (Brocklehurst, 1960).

Possible Direct Effects of Ag–Ab Complexes

As Trapani *et al.* (1958) have reported that Ag–Ab complexes produce a contractile

effect on guinea-pig ileum, it was necessary to determine if the effluent activity was due to the presence of Ag–Ab complex. Doses of Ag–Ab complex equal to those injected, and diluted in a volume corresponding to the first collected effluent fraction, were assayed on the guinea-pig ileum. These solutions were tested both before and after the addition of mepyramine to the bath, and found to be inactive under the conditions of our experiments. The preparations of complex examined had been made from the washed equivalence precipitate at either five or ten times antigen excess, and contained 1–100 μg. antibody protein per ml.

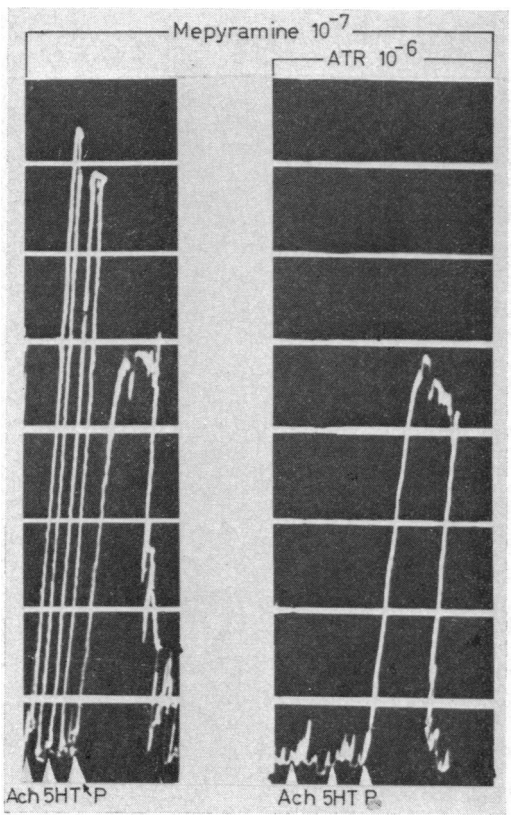

FIG. 4. Mepyramine-resistant effluent activity (P) is compared with acetylcholine (Ach) and 5-hydroxytryptamine (5HT) before and after the addition of atropine 1 μg./ml.

Relation between Effluent Activity and Bronchoconstriction

Effluent fractions collected at intervals during a bronchoconstriction response were assayed for histamine-like activity (Fig. 5). The peak activity appeared during the first 5 minutes following the injection of soluble complex, and subsequently fell off steeply. The associated bronchoconstrictor response ran in parallel with this, but with a more prolonged time-course.

No significant activity was released from the heart when it was opened and incubated alone for 5 minutes at 37° in 5 ml. of Tyrode solution containing 12·6 μg. antibody protein in the form of a soluble complex at five times antigen excess. The lung preparation from

which the heart had been taken was initially shown to release considerable activity on being perfused with the same amount of complex. This would seem to exclude the possibility that a significant amount of activity released during a bronchoconstrictor response might originate in the heart rather than the lungs.

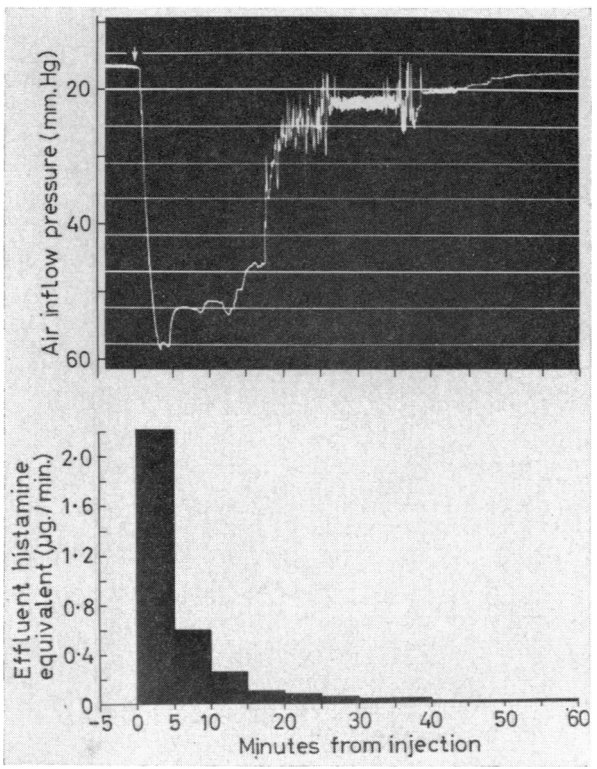

FIG. 5. Correlation between time-course of bronchoconstrictor response and appearance of histamine-like activity in the effluent. These responses followed an injection of soluble complexes containing 25·6 µg. of antibody protein, prepared from the washed equivalence precipitate at 5 times antigen excess.

Duration of Preliminary Perfusion

A prolonged preliminary perfusion with Ringer solution tended to increase the bronchoconstrictor response to a subsequent injection of complex. In a series of lungs, individual preparations were allotted at random to different preliminary perfusion times before being given a single injection of soluble complex. The preliminary perfusion periods were ½, 1½, 3, 5½ and 12 hours; five experiments were carried out for each period except the longest, which was tested only twice. Fig. 6 shows that despite considerable variation between individual lungs within a group, there was a trend of increasing responses up to 5½ hours of preliminary perfusion.

Rate of Perfusion

In another series of lungs, the duration of preliminary perfusion was kept constant at 3 hours, but the rate was varied: one group was perfused at the standard rate of 2·5 ml./minute throughout, and the other at a rate of 10 ml./minute for 2½ hours, followed by the

standard rate for ½ hour. Lungs from the two groups were paired. As shown in Table 2, the preparations perfused at the faster rate gave significantly larger responses ($0{\cdot}02<P<0{\cdot}05$ by *t*-test).

It can be concluded that the bronchoconstrictor effect of a dose of soluble Ag–Ab complex is related to both duration and intensity of the preceding perfusion with Ringer solution. Although we have not made a search for the factors which determine this relation, it seems likely that washing out of the guinea-pig extracellular proteins may be one (see Discussion). Others which may also be involved are the perfusion fluid pH and inflow pressure, both of which generally rise in the course of time.

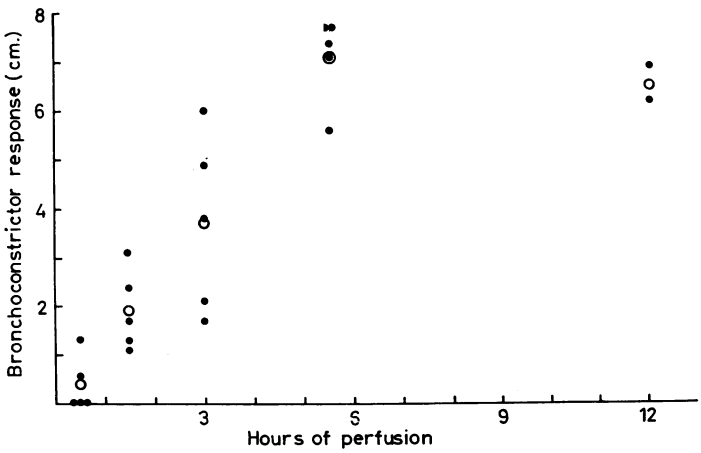

FIG. 6. Duration of preliminary perfusion. Individual lungs were perfused for ½, 1, 3, 5½ or 12 hours and then injected with a single dose of soluble complex containing 190 µg. of antibody protein, prepared from the washed equivalence precipitate at five times antigen excess. ○ = average.

TABLE 2

RATE OF PRELIMINARY PERFUSION

Perfusion (ml./min.)	*Bronchoconstriction (cm.)*							*Average*
2·5	1·8,	2·0,	2·1,	3·5,	4·2,	4·9,	2·5	3·0
10	4·7,	8·1,	1·6,	4·5,	4·7,	6·0,	7·0	5·2

Two groups of lungs were given a single injection of soluble Ag–Ab complex containing 12·6 µg. of antibody protein prepared from the washed equivalence precipitate at 5 times antigen excess. One group was perfused at the standard rate of 2·5 ml./minute throughout; the other was perfused at 10 ml./minute for the initial 2½ hours, and following this at the standard rate. The injection was given at 3 hours in both groups.

Serial Injections in the Same Preparation

Repeated injections of soluble Ag–Ab complex in a single perfused lung produced both repeated bronchoconstrictor effects and release of pharmacologically active material (Table 3). Each index first showed an increasing, followed by a decreasing sensitivity to successive equal doses of soluble complex. In the tabulated experiment, the histamine and SRS release ran parallel, reaching their peak at the time of the seventh injection. The highest bronchoconstrictor response occurred somewhat earlier. The relationship between release of pharmacological activity and magnitude of bronchoconstriction suggested that

if the former was the cause of the latter, the lungs tended to become less responsive to the released agents with successive stimulations.

Despite this type of variation, it was possible to compare the activity of soluble complex injections which contained different amounts of antibody. The dose–response relationship to four graded doses of a single soluble complex preparation was studied in experiments planned to a 4 × 4 Latin square design. Four injections were given to each of four lungs,

TABLE 3

SERIAL RESPONSES FROM A SINGLE LUNG

	Injection number										
	1	2	3	4	5	6	7	8	9	10	11
Bronchoconstriction (cm.)	4·9	7·4	5·7	5·5	5·4	5·8	5·4	3·6	2·4	1·7	1·4
Effluent histamine	1·3	1·0	1·6	2·6	3·8	4·8	6·3	5·1	2·8	2·6	1·9
Effluent SRS	0·9	1·0	1·8	1·8	2·3	2·2	3·5	2·7	2·3	2·3	2·2

Eleven successive injections were given at 1-hour intervals, beginning after a 3-hour preliminary perfusion period. Each injection contained 12·6 µg. antibody protein, in the form of a soluble complex prepared from the washed equivalence precipitate at 5 times antigen excess. The amounts of histamine and SRS appearing in the effluent are each expressed relatively to their respective concentrations in sample number 2. In absolute terms, sample 2 contained 1·16 µg. histamine base equivalents, when assayed against histamine standards.

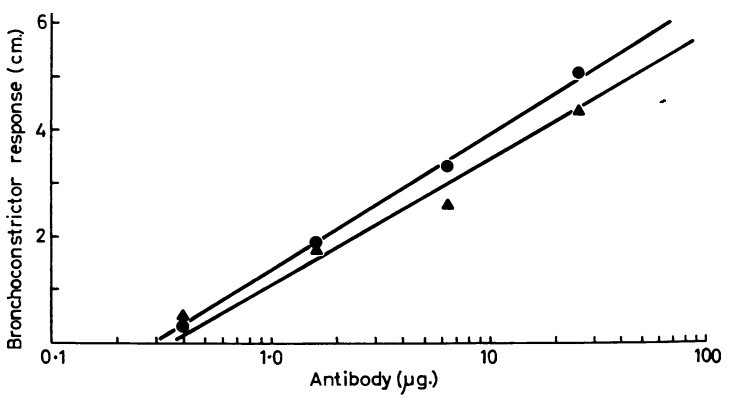

FIG. 7. Dose–response relationship using preparations of soluble complex made from the washed equivalence precipitate, at two different levels of antigen excess (●, 5 ×; ▲, 160 ×). The two sets of points were separately obtained from experiments planned to a 4 × 4 Latin square design, in which each of four lungs was injected once with each of the four doses of complex. Individual points on the graph represent the average of four responses, each given by a different lung.

each lung thus forming a self-contained assay block. When the bronchoconstriction data were plotted against log antibody dose (Fig. 7), a linear relationship was seen using in separate studies preparations of complex in both 5 and 160 times antigen excess. A similar relation could be shown between effluent histamine equivalents and antibody dose (Table 4). On the basis of either bronchoconstriction data, or the measured release of activity, it was found by the analysis of variance that dose pairs having an antibody content which differed by four-fold or higher produced significantly different responses (Table 4). Although doses with a two-fold difference in antibody content showed a trend toward

different activity, this could not be proven significant on the basis of data from only four lungs (Table 4).

In this type of experiment, only those lungs could be accepted which were responsive at the time of the first injection. The first injection was given in the earlier studies, after 4½ hours of preliminary perfusion at the standard rate of 2·5 ml./minute; it was later found that a 3-hour preliminary period would suffice, if the rate of perfusion was increased to 10 ml./minute for the first 160 minutes. The injections were always given at intervals of 1½ hours.

TABLE 4

SENSITIVITY OF EXPERIMENTS SET TO A 4 × 4 LATIN SQUARE DESIGN

	Dose of complex (μg. *Ab*)						*F value*†	
	0·4	1·6	3·2	6·4	12·6	25·6	*Pair 1 vs. pair 2*	*Regression*
Bronchoconstriction*								
Pair 1		1·9				5·1	30·8 (6·0, 13·8)	107 (6·0, 13·8)
Pair 2	0·3			3·3				
Histamine release*								
Pair 1				1·5		6·0	2·3 (6·0)	11·2 (6·0, 13·8)
Pair 2			1·1		3·3			

Bronchoconstriction and histamine release data are respectively shown for two different experiments. In each experiment, preparations of complex were used which had been prepared at 5 times antigen excess from the washed equivalence precipitate, and comparisons made between two dose pairs. Bronchoconstriction data from the experiment demonstrating histamine release showed the same relative differences between the pairs and for regression.

* Each recorded value is the average of four responses obtained in different lungs. Bronchoconstriction data are given in cm., and histamine release in μg. base.

† A single number in parentheses is the value of F yielding a P value of 5 per cent; two numbers represent the respective values for 5 and 1 per cent.

Varying Antigen Excess

Comparisons of activity were carried out between solutions of soluble complex made with dyed BPA in antigen excess ranging from 2½ to 160 times equivalence amounts. The preparations of complex used in these studies were those in which the ratio of antigen in complex with antibody had been measured (Table 1). The preparation made at 5 times antigen excess was taken as the standard and compared against the other preparations in paired experiments. Two dose levels of each were used, in a 4 × 4 Latin square design as described. The smaller dose of complex contained 1·6 μg. antibody protein and the larger 12·6. As seen in Table 5, no significant difference in bronchoconstrictor activity was detected between any of these preparations; neither was a trend towards a difference observed which might be proven significant in a larger series of lungs. In each assay set, the regression of response on dose was always statistically significant.

Additional similar comparisons were carried out as follows:

1. The solution of complex prepared at 160 times antigen excess which was used in the foregoing, was assayed against an aliquot of the same solution kept at 4° for an additional 7 days. No difference in bronchoconstrictor activity was found between the two.

2. Using BPA as antigen and complexes made from the washed equivalence precipitate at five times antigen excess, no difference in bronchoconstrictor activity was found on comparing one preparation made from serum which had been heated to 56°, against another made from serum which had not been heated. The remaining comparisons were all carried out with complexes prepared from serum which had not been heated to 56°.

3. Complexes made from the washed equivalence precipitate at 5 and 160 times with BPA, were equal in both their bronchoconstrictor effects and release of pharmacologically active materials.

4. At 5 times antigen excess with BPA, the bronchoconstrictor activity of a preparation made without precipitation at equivalence was equal to that of another made from the washed equivalence precipitate.

5. With either BPA or dyed BPA as antigen, and complexes made without precipitation at equivalence, no difference in bronchoconstrictor activity was found between preparations made at 5 and 160 times antigen excess.

TABLE 5

COMPARED BRONCHOCONSTRICTOR ACTIVITIES OF SOLUBLE Ag–Ab COMPLEX PREPARATIONS MADE AT ANTIGEN EXCESS RANGING FROM 2½ TO 160 TIMES EQUIVALENCE AMOUNTS

Ab (μg.)	5 × *vs.* 2½ ×		5 × *vs.* 10 ×		5 × *vs.* 40 ×		5 × *vs.* 160 ×	
12·5	5·5*	4·7	4·9	5·1	5·8	4·8	5·0	4·3
1·6	2·0	2·0	3·3	3·1	2·2	2·3	3·0	2·5
F value†								
Standard *vs.* Unknown	2·5 (6·0)		15·0 (234)		2·7 (234)		3·5 (6·0)	
Regression	29·2 (6·0–13·8)		8·5 (6·0–13·8)		15·0 (6·0–13·8)		37·4 (6·0–13·8)	

Each comparison was carried out in a 4 × 4 Latin square design. The preparation made at 5 times antigen excess was used as the standard in each compared set. All preparations were made from the washed equivalence precipitate.

* Each recorded value is the average of four responses obtained in different lungs.

† A single number in parentheses is the value of *F* yielding a *P* value of 5 per cent; two numbers represent the respective values for 5 and 1 per cent.

Controls

The doses of soluble complex used in the foregoing experiments contained from 0·4 to 190 μg. antibody protein and from 0·3 to 310 μg. antigen protein. Various control solutions were prepared to determine if the observed effects were due to soluble complex as such and not to its constituent parts. The control samples were assayed on the perfused lung against two flanking doses of standard Ag–Ab complex, the lower containing 1·6 μg. antibody with 1·2 μg. antigen, and the higher, 12·6 μg. antibody with 9·7 μg. antigen. The following preparations were all less active than the lower dose of standard, with regard both to bronchoconstrictor effect and release of pharmacologically active agents. Representative data are shown in Table 6.

(a) *Antigen alone.* BPA or dyed BPA was used in doses from 0·5 to 500 μg. antigen protein.

(b) *Antigen in serum.* Doses of 1·2–310 μg. antigen protein were prepared in dialysed normal rabbit serum. The preparation was carried out as with immune serum at 5 and 160 times antigen excess.

(c) *Antibody in serum.* Doses of 1·6–200 μg. antibody protein were prepared from serum dialysed against buffered saline. As these injections produced passive sensitization, comparisons were made only with preceding doses of Ag–Ab complex.

(d) *Normal serum.* The same dilutions of normal rabbit serum were used as for immune serum.

(e) *Ag–Ab precipitate.* Doses of 1·6–200 μg. antibody protein in the form of an equivalence precipitate were suspended in the original serum or in buffered saline. The highest dose produced a small effect.

(f) *Ag–Ab supernatant.* The supernatants of equivalence precipitates were prepared from volumes of immune sera yielding 1·6–200 μg. antibody.

TABLE 6

CONTROL SOLUTION COMPARED IN THE SAME LUNG AGAINST FLANKING DOSE OF SOLUBLE COMPLEX

Control used	*Results from single lungs*			
	Soluble complex standard (1·6 μg. *Ab* + 1·2 μg. *Ag*)		*Control*	
	Constriction (*cm.*)	*Histamine* (μg.)	*Constriction* (*cm.*)	*Histamine* (μg.)
Normal rabbit serum 1 : 800 + 1·2 μg. BPA	2·8	2·1	0	0
Normal rabbit serum 1 : 100 + 310 μg. dyed BPA	2·4	1·8	0	0
Immune rabbit serum 1 : 800 (1·6 μg. Ab)	3·7	8·1	0	0
Immune rabbit serum 1 : 6·5 (200 μg. Ab)	1·1	1·0	0	0
Equiv. precipitate in serum 1 : 800 (1·6 μg. Ab)	1·6	2·6	0	0
Equiv. precipitate in serum 1 : 13 (100 μg. Ab)	1·2	0·5	0	0
Equiv. precipitate in buffer (200 μg. Ab)	4·3	5·7	2·1	1·3

Antagonism between Ag–Ab Complexes and Serum

In view of the known inhibitory action of normal γ-globulins on passive sensitization *in vitro* (Halpern, Liacopoulos, Liacopoulos-Briot, Binaghi and Van Neer, 1959; Mongar and Schild, 1960), we investigated whether a similar antagonism exists between the bronchoconstrictor effects of rabbit Ag–Ab complexes and normal rabbit serum. The following preparations were tested: (1) normal rabbit serum; (2) the precipitate of normal serum which had been fractionated at 50 per cent saturation with ammonium sulphate (globulin fraction); (3) the corresponding supernatant (albumin fraction); (4) bovine γ-globulin (fraction 2, Armour).

The serum fractions were mixed with a standard dose of Ag–Ab complex, *in vitro* diluted and warmed to 37°, and at once injected into the pulmonary perfusate. A preparation of soluble complex was used which had been made from the washed equivalence precipitate at 5 times antigen excess. A concentration of 20 per cent and 5 per cent rabbit serum completely inhibited the bronchoconstrictor effect of soluble complex. Similar inhibition was obtained with the amount of globulin fraction contained in these concentrations of whole serum (2·13 and 0·43 mg. protein per 0·5 ml.), whereas comparable levels of the albumin fraction failed to inhibit (4·8 and 0·96 mg. protein per 0·5 ml.). A single experiment demonstrating these effects is shown in Fig. 8. Neither histamine-like activity nor SRS appeared in the effluent following an inhibited injection of complex. In an experiment where the standard was inhibited by 0·5 mg. protein of the rabbit globulin fraction, there was no inhibition by either 0·5 or 5 mg. of a bovine γ-globulin preparation. The bovine γ-globulin alone was inactive.

DISCUSSION

The ratio of antigen to antibody in solutions of soluble complex prepared at 40 and 160 times antigen excess was higher than would be expected for a molar ratio of 2 : 1. No correction was made in calculating these ratios, for any dyed BPA which may have been

non-specifically precipitated at 50 per cent saturation in ammonium sulphate, in association with other proteins which were insoluble at this concentration (Farr, 1958). If 3 per cent of the antigen were precipitated non-specifically, correction for this would yield a ratio of 2·9 at 40 times excess, and 2·3 at 160 times excess.

Soluble complexes were equally active when prepared either in the whole serum, without precipitation at equivalence, or from the washed equivalence precipitate, the soluble

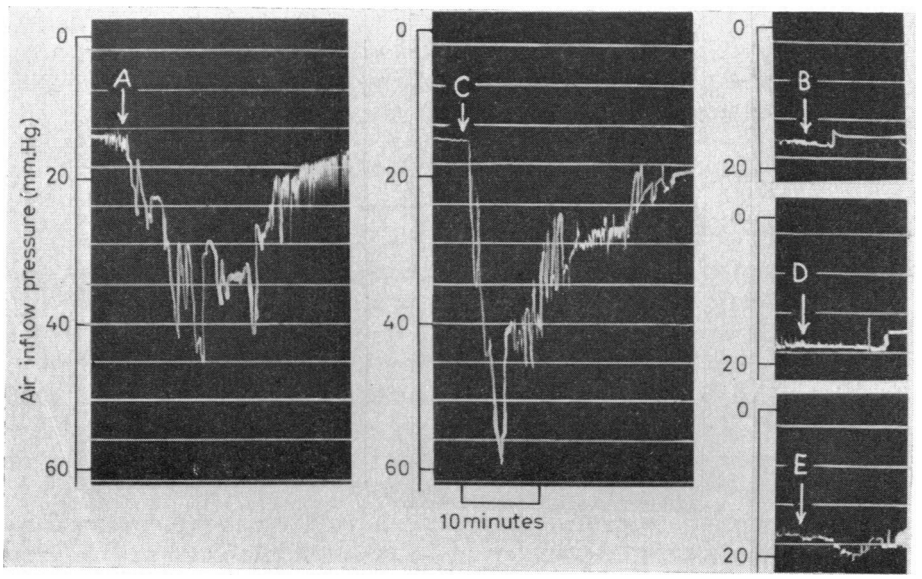

FIG. 8. Inhibition of bronchoconstrictor activity by normal rabbit serum and its globulin fraction. A series of injections were given to a single lung; at A, the standard of soluble complex was injected, containing 12·6 μg. antibody protein; at B, the standard was injected after being premixed in 5 per cent normal rabbit serum; at C, in 0·96 mg. protein of the albumin fraction, and at D, in 0·43 mg. protein of the globulin fraction; a final injection of complex was given at E which contained 1·6 μg. antibody protein.

serum factors being discarded. Also, interference with complement by heating at 56° prior to the formation of the complexes did not apparently modify the subsequent activity. These findings indicate that the activity in solutions of complex was neither due to the production of some soluble factor in serum, nor dependent on the presence of serum complement. It is also unlikely that the bronchoconstrictor activity was due to denaturation either from slight foaming while being prepared on a shaker, or from the high dilution used: preparations made without shaking were equally active; the activity of complexes was not dependent on their being diluted, and there was a direct relationship between concentration and activity.

There was no significant fall in the activity of soluble complexes prepared in antigen excess of as high as 160 times the amount needed for equivalence, contrary to most findings reported in other systems (Tokuda and Weiser, 1958; Trapani *et al.*, 1958; Ishizaka *et al.*, 1959; Treadwell *et al.*, 1960; Weigle *et al.*, 1960). It seemed possible that our failure to demonstrate this was due to a lack of re-equilibration between antigen and antibody at the time of use, but prolonging the equilibration period did not alter the activity. Furthermore, complexes in which re-equilibration from equivalence to antigen excess was not necessary,

had similar activity. A further possibility is that higher degrees of antigen excess were needed for diminished activity in this system. This would be difficult to reconcile with the mechanism proposed by Ishizaka and Campbell (1959b) as one would expect the molar antigen–antibody ratio of 2 to be for all practical purposes achieved at 160 times antigen excess. However, it is conceivable that if soluble complexes act by becoming bound to cells, the antigen not in complex with antibody may be quickly washed away in a perfusion experiment. If re-equilibration of complexes in the changed antigen environment took place very rapidly, the final effective ratio of antigen to antibody might become similar in preparations originally made at widely differing levels of antigen excess. Although the rate of re-equilibration in a test tube, from a given level of excess antigen to a higher level, would probably be too slow to fit this hypothesis (Talmage, 1960), the rate in tissue and from a given level of excess to a lower one is not known. Studies which may help to clarify this point are in progress.

Soluble Complexes and Anaphylaxis

As the bronchoconstrictor and releasing effects of soluble complexes bear a close resemblance to anaphylactic reactions, the question arises whether they involve a fundamentally similar mechanism.

Certain macromolecules are known to produce bronchoconstriction in intact guinea-pigs (Hanzlik and Karsner, 1920) and their isolated perfused lungs (Schild, 1936), but it seems unlikely that soluble complexes act simply as macromolecules. Insoluble Ag–Ab complexes prepared at equivalence were 125 times less active than soluble complexes in our system.

The inhibition of soluble complex activity by normal serum suggests on the contrary an immunological mechanism. Rabbit serum is capable of inhibiting passive anaphylactic sensitization of guinea-pig lung *in vitro* (Mongar and Schild, 1960), and the permeability increasing effect of soluble complexes in guinea-pig skin *in vivo* (Ishizaka and Campbell, 1959a). These inhibitory effects have been attributed to competition between antibody and non-specific globulin for cellular attachment sites to which either may bind. The inhibitory activity of rabbit serum in the present experiments can be similarly explained on the basis of receptor competition, especially as it resided in the globulin fraction. A ratio of non-specific globulin to antibody of 34 was sufficient for complete antagonism. Bovine γ-globulin is known not to interfere with passive sensitization of guinea-pig lung (Mongar and Schild, 1960), and failed to inhibit in the present studies.

The competition hypothesis implies that soluble complexes, like anaphylactic antibody, undergo cellular attachment, with the difference that the union of antigen with antibody precedes cellular fixation, whereas in anaphylaxis it follows fixation. It is not known whether the same processes are subsequently set in motion. The union of antigen and antibody in the anaphylactic reaction leads to the activation of a cellular enzyme system which is heat labile and calcium dependent (Mongar and Schild, 1957, 1958) but no comparable studies have as yet been carried out with soluble complexes. While it is thus too early for a full comparison of the two reactions, certain common features are apparent even at this stage.

Quantity of Antibody

The amount of antibody effective in the form of a soluble complex is probably of the same order of magnitude as in passive sensitization. The amount of antibody taken up *in*

vitro during maximal sensitization of guinea-pig lung has been estimated at less than 1 μg./g. (Brocklehurst, Humphrey and Perry, 1961) whilst the quantity of antibody in soluble complexes which produced threshold bronchoconstrictor effects in our experiments was 0·4–1·6 μg. per 3–6 g. lung. Ishizaka *et al.* (1959) have calculated that the amount of antibody in soluble complexes required for the production of skin reactions in the guinea-pig is similar to that required for anaphylactic sensitization when the incubation period is very short. It will be interesting to carry out quantitative studies on these lines in the perfused guinea-pig lung.

Rate of Attachment of Antibody

Although the time-course of the bronchoconstrictor effects of soluble complexes seems fast in relation to the accepted time-course of passive sensitization, it does not necessarily exclude cellular attachment. Recent studies of passive sensitization *in vitro* have suggested that it is a two-stage process, consisting of a fast reaction with low activation energy representing attachment to receptors initially free from γ-globulins, and a slower reaction with high activation energy representing attachment to receptors initially occupied by other γ-globulin (Mongar and Schild, (1960). The bronchoconstrictor effects of soluble complexes in the perfused lung may involve only receptors which are initially free. This could explain the greater activity of soluble complexes after an intensive preliminary perfusion which would tend to wash away any attached globulins from the lung.

Desensitization

Although repeated injections of soluble complexes resulted in a marked decline of bronchoconstrictor responses, there was a lesser reduction in the release of histamine and SRS. This may or may not represent the same phenomenon as anaphylactic desensitization. At least four apparent factors may be involved in desensitization: saturation of available antibody, exhaustion of the enzymatic system which antigen and antibody activate, exhaustion of the stores of pharmacological materials and their precursors, and a decreased responsiveness of the end organ(s) to the released mediator materials. The more usual type of anaphylactic desensitization is probably due mainly to the reduction in available antibody, as sensitivity may remain intact to an unrelated antigen–antibody system (Liacopoulos, Halpern and Frick, 1963), and also resensitization may be effected by providing more of the original antibody (Dale, 1912). As Ag–Ab complexes carry their own antibody, the question of this factor becoming exhausted does not arise. Our findings were most in keeping with a loss in sensitivity of the end organ response (Table 3). It is possible with a greater number of injections that we may have in addition been able to show a more marked decrease in histamine and SRS release, suggesting a definite exhaustion either of a presumably enzymatic system or the mediator materials.

In conclusion it would seem that the observed activity of soluble Ag–Ab complexes is best explained in terms of a reaction which involves cellular fixation. In this sense the effects provide an interesting confirmation of the 'cellular' theory of anaphylaxis. At the same time they also provide a link with 'humoral' theories because of the possibility that circulating antibody may participate in *in vivo* anaphylaxis, if it is reached by an amount of antigen greater than that required for equivalence.

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REFERENCES

ARUNLAKSHANA, O. and SCHILD, H. O. (1959). 'Some quantitative uses of drug antagonists.' *Brit. J. Pharmacol.*, **14**, 48.

BOURA, A., MONGAR, J. L. and SCHILD, H. O. (1954). 'Improved automatic apparatus for pharmacological assays on isolated preparations.' *Brit. J. Pharmacol.*, **9**, 24.

BROCKLEHURST, W. E. (1960). 'The release of histamine and formation of a slow-reacting substance (SRS-A) during anaphylactic shock.' *J. Physiol. (Lond.)*, **151**, 416.

BROCKLEHURST, W. E., HUMPHREY, J. H. and PERRY, W. L. M. (1961). 'The *in vitro* uptake of rabbit antibody by chopped guinea-pig lung and its relationship to anaphylactic sensitization.' *Immunology*, **4**, 67.

BRODER, I. and SCHILD, H. O. (1964). 'Bronchoconstrictor effects of soluble antigen–antibody complexes in isolated lung, and their inhibition by normal serum.' *Nature (Lond.)*, **201**, 1137.

DALE, H. H. (1912). 'The anaphylactic reaction of plain muscle in the guinea pig.' *J. Pharmacol. exp. Ther.*, **4**, 167.

DALE, H. H. and KELLAWAY, C. H. (1921). 'Anaphylaxis and immunity *in vitro*.' *J. Physiol. (Lond.)*, **54**, 143P.

FARR, R. S. (1958). 'A quantitative immuno-chemical measure of the primary interaction between I*BSA and antibody.' *J. infect. Dis.*, **103**, 239.

GERMUTH, F. G., JR. and MCKINNON, G. E. (1957). 'Studies on the biological properties of antigen-antibody complexes. 1. Anaphylactic shock induced by soluble antigen-antibody complex in unsensitized normal guinea pigs.' *Bull. Johns Hopk. Hosp.*, **101**, 13.

HALPERN, B. N., LIACOPOULOS, P., LIACOPOULOS-BRIOT, M., BINAGHI, R. and VAN NEER, F. (1959). 'Patterns of *in vitro* sensitization of isolated smooth muscle tissues with precipitating antibody.' *Immunology*, **2**, 351.

HANZLIK, P. J. and KARSNER, H. T. (1920). 'Anaphylactoid phenomena from the intravenous administration of various colloids, arsenicals and other agents.' *J. Pharmacol. exp. Ther.*, **14**, 379.

ISHIZAKA, K. and CAMPBELL, D. H. (1959a). 'Biological activity of soluble antigen–antibody complexes. 4. The inhibition of the skin reactivity of soluble complexes and the PCA reaction by heterologous complexes.' *J. Immunol.*, **83**, 116.

ISHIZAKA, K. and CAMPBELL, D. H. (1959b). 'Biological activity of soluble antigen–antibody complexes. 5. Change of optical rotation by the formation of skin reactive complexes.' *J. Immunol.*, **83**, 318.

ISHIZAKA, K., ISHIZAKA, T. and CAMPBELL, D. H. (1959). 'The biological activity of soluble antigen–antibody complexes. 2. Physical properties of soluble complexes having skin-irritating properties.' *J. exp. Med.*, **109**, 127.

KABAT, E. A., COFFIN, G. S. and SMITH, D. J. (1947). 'A quantitative study of passive anaphylaxis in the guinea pig.' *J. Immunol.*, **56**, 377.

KABAT, E. A. and MAYER, M. M. (1961). *Experimental Immunochemistry*, pp. 60, 72 and 476. Thomas, Springfield.

KRUSE, H. and MCMASTER, P. D. (1949). 'The distribution and storage of blue antigenic azoproteins in the tissues of mice.' *J. exp. Med.*, **90**, 425.

LIACOPOULOS, P., HALPERN, B. N. and FRICK, O. L. (1963). 'The effect of excess antigen on the anaphylactic reaction.' *J. Immunol.*, **90**, 165.

MONGAR, J. L. and SCHILD, H. O. (1957). 'Effect of temperature on the anaphylactic reaction.' *J. Physiol. (Lond.)*, **135**, 320.

MONGAR, J. L. and SCHILD, H. O. (1958). 'The effect of calcium and pH on the anaphylactic reaction.' *J. Physiol. (Lond.)*, **140**, 272.

MONGAR, J. L. and SCHILD, H. O. (1960). 'A study of the mechanism of passive sensitization.' *J. Physiol. (Lond.)*, **150**, 546.

MORRIS, M. C. (1936). 'The relation between anti-anaphylaxis and antibody balance. 1. The role of excess circulating antibody in hypersensitiveness.' *J. exp. Med.*, **64**, 641.

POULIK, M. D. (1959). 'Starch-gel immuno-electrophoresis. *J. Immunol.*, **82**, 502.

SCHILD, H. O. (1936). 'Histamine release and anaphylactic shock in isolated lungs of guinea pigs.' *Quart. J. exp. Physiol.*, **26**, 165.

SWINEFORD, O., JR. and SAMSELL, D. (1962). 'Purification of antibodies. 2. Precipitation, passive sensitization and passive desensitization with antibodies eluted from specific precipitates.' *J. Allergy*, **33**, 423.

TALMAGE, D. W. (1960). 'The kinetics of the reaction between antibody and bovine serum albumen using the Farr method.' *J. infect. Dis.*, **107**, 115.

TOKUDA, S. and WEISER, R. S. (1958). 'Anaphylaxis in the mouse produced with soluble complexes of antigen and antibody.' *Proc. Soc. exp. Biol. (N.Y.)*, **98**, 557.

TRAPANI, I. L., GARVEY, J. S. and CAMPBELL, D. H. (1958). 'Stimulating action of soluble antigen–antibody complexes on normal guinea pig smooth muscle.' *Science*, **127**, 700.

TREADWELL, R. E., WISTAR, R. and RASMUSSEN, A. F., JR. (1960). 'Passive anaphylaxis in mice with homologous antiserum. 1. Some quantitative aspects.' *J. Immunol.*, **84**, 539.

WEIGLE, W. O., COCHRANE, C. G. and DIXON, F. J. (1960). 'Anaphylactogenic properties of soluble antigen–antibody complexes in the guinea pig and rabbit.' *J. Immunol.*, **85**, 469.

WEIL, R. (1912). 'The nature of anaphylaxis, and the relations between anaphylaxis and immunity.' *J. med. Res.*, **27**, 497.