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ADSORPTION AND DIFFUSION OF γ-GLOBULIN DURING PASSIVE SENSITIZATION OF CHOPPED GUINEA-PIG LUNG

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WITH AN APPENDIX

DIFFUSION AND ADSORPTION IN TISSUES

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During passive sensitization it is usually considered that antibody molecules become 'fixed' to cell surfaces. It would be of considerable interest if measurements of the adsorption of γ-globulin on to cells could throw light on the process of 'fixation'.

The adsorption of ^{131}[I]-labelled antibody has been measured by several workers, e.g. Humphrey & Mota (1959), Nielson, Terres & Feigen (1959), Brocklehurst, Humphrey & Perry (1961) and Liacopoulos, Liacopoulos-Briot, Binaghi & Perramant (1961). In most of these experiments guinea-pig tissues were incubated with labelled γ-globulin (usually from the rabbit) and adsorption estimated by measuring the amount of radioactivity remaining in the tissue after washing it with a physiological saline for an arbitrary length of time. This method ignores both the removal of adsorbed antibody and the presence of non-adsorbed antibody in the extracellular space.

An attempt has been made to discover more about the adsorption process by (*a*) attempting to assess the effect of diffusion on the rate of adsorption and sensitization, (*b*) use of a method for the measurement of adsorption which avoids some of the uncertainties inherent in the method mentioned above, (*c*) making all measurements with sufficiently low protein concentrations to ensure that sensitization was submaximal, (*d*) using guinea-pig, as well as heterologous, γ-globulins and (*e*) assessing the effect of changing the ionic environment during the adsorption process. A preliminary account of the findings has been given by Brocklehurst & Colquhoun (1963).

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METHODS

Estimation of inulin and sucrose

Samples of tissue were washed into a ground-glass homogenizer tube (type TTH/20, Loughborough Glass Co. Ltd.) and homogenized for 2 min. The homogenate was washed quantitatively into a volumetric flask with distilled water, protein precipitated by adding 1·0 ml. 20 % metaphosphoric acid (British Drug Houses Ltd., and the volume adjusted to 10·0 ml. with distilled water. The suspension was centrifuged at 2000 *g* for 10 min and estimations were performed on the supernatant solutions with a Technicon Autoanalyser, using the method of R. B. Fisher and J. C. Gilbert (unpublished). Standard solutions were made from the solutions which had been incubated with the lung tissue, and contained the same concentration of metaphosphoric acid as the unknowns. Control solutions made from tissue which had not been incubated with inulin were also tested.

Isosmotic solutions

Wherever possible concentrations of isosmotic solutions were calculated from the molal freezing point depression data given in standard tables. Otherwise a 0·28 M solution of non-electrolyte was assumed to be isosmotic with plasma.

Antisera

Rabbit antisera against bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne) (BSA) were prepared by injecting intramuscularly 10 mg of BSA in 0·5–1·0 ml. of complete Freund's adjuvant made according to the first formula given by Colquhoun & Brocklehurst (1965). This was repeated twice at weekly intervals. After 6 weeks doses of 1, 2, 2 and 4 mg of BSA adsorbed on to aluminium hydroxide were given intravenously at 2-day intervals. The animals were bled 1 week after the last injection.

Guinea-pig anti-ovalbumin sera were prepared as described by Colquhoun & Brocklehurst (1965).

Purification of proteins

Rabbit γ-globulin was prepared in one step from whole serum by chromatography on columns made from diethylaminoethyl (DEAE) cellulose which was prepared from 100–200 mesh Solka Floc cellulose according to Peterson & Sober (1956), or bought from Kodak Ltd. (Kirkby, Liverpool). All columns and samples were equilibrated with 0·01 M, pH 8·2, sodium phosphate buffer. Up to 20 ml. of serum was applied to a column of 2·8 cm diameter and 40–45 cm height, and the first protein peak emerging was collected.

Guinea-pig γ_1- and γ_2-globulins were purified by preparative electrophoresis followed by DEAE cellulose chromatography as described by Colquhoun & Brocklehurst (1965).

Goat anti-rabbit serum and anti-rabbit γ-globulin sera were kindly given to us by Dr J. H. Humphrey (National Institute for Medical Research, London).

Radio-iodination of proteins

Purified γ-globulins were labelled with iodine-131 by the method of McFarlane (1958) (and Dr T. Freeman, personal communication). After iodination non-protein-bound radioactivity was removed by gel filtration on a small column of Sephadex G-50 (Pharmacia, Sweden). The proportion of radioactivity not precipitable by 12 % trichloracetic acid was frequently less than 1 % and nearly always less than 1·5 % at the start of the experiment.

In most preparations the average degree of iodination was less than 1·0 atom of iodine per molecule of γ-globulin and it was always less than 1·5. The specific activity of the protein was almost always between 25 and 50 μc/mg. Labelled protein was used within 24 hr from the time it was prepared.

In most experiments the iodination was performed by very rapid injection of the protein solution into the ^{131}[I]Cl solution (cf. McFarlane, 1964). It has been shown that the order

in which protein and iodine are mixed does not affect their metabolism (Dr T. Freeman, personal communication) or their adsorption (Colquhoun, 1964).

The dose of self-irradiation received by the labelled protein should never have been more than 160 rad and was usually much less (Colquhoun, 1964). If γ-globulin were similar in radiosensitivity to albumin, this dose would be enough to damage, at the most, 0·75 % of the protein.

Measurement of radioactivity

Radioactivity was measured in a well-type crystal scintillation counter. Corrections were made for background count rate, decay and dead time of the counter. Enough counts were registered to ensure that the coefficient of variation of the net count rate owing to the random disintegration process was always less (usually much less) than 5 %. All samples were contained in a volume of 1 ml. or less over which range the geometrical efficiency of the counter was almost independent of sample volume.

Measurement of adsorption and sensitization

Samples of perfused chopped guinea-pig lung tissue were prepared, and incubated with ^{131}[I]-γ-globulin, as described by Colquhoun & Brocklehurst (1965). At the end of the incubation a sample of the supernatant solution (0·025–0·25 ml.) was taken for radioactivity measurement; the tissue was drained, transferred to a clean tube and immediately weighed. Tyrode solution (0·96 ml.) was then added to the tissue sample, and its radioactivity measured. The tissue sample was replaced in the rocking incubator at 37° for 10 min and then 1·0 ml. of warmed double-strength antigen solution in Tyrode solution added to give a final concentration of 10 or 20 μg BSA per ml. The released histamine was collected and assayed in the way described by Colquhoun & Brocklehurst (1965).

Measurement of extracellular space

Samples of lung tissue were first washed, and then incubated for 30 min at 37° C, with between 4 and 5 ml. of 1 % solution of inulin (British Drug Houses Ltd., Poole) in Tyrode solution. At the end of the incubation a sample of the supernatant solution was taken for making standard solutions for the inulin determination. The tissue was drained in the same way as tissue which had been incubated with ^{131}[I]-γ-globulin, transferred to a clean tube, weighed, and assayed for inulin content as described above. Between five and ten replicate determinations of extracellular space were made in each experiment, in parallel with the determinations of antibody uptake.

Estimation of protein content, concentration of protein solutions and immunoelectrophoresis

The methods cited by Colquhoun & Brocklehurst (1965) were employed.

RESULTS

Purity of γ-globulins

When preparations were tested by immunoelectrophoresis only a single arc, corresponding with the slower part of the γ-globulin of whole serum, was visible. When stored for a few weeks at 4° γ-globulin solutions were altered, an additional arc appearing on immunoelectrophoresis. The solutions were routinely sterilized by passage through cellulose acetate filters (Oxoid Ltd., London), and stored in sealed ampoules. Such solutions kept well at 4° for at least 1½ years.

Diffusion of inulin and extracellular space determination in chopped guinea-pig lung

The size of lung-tissue fragments in the preparation. Although the method of preparation of the tissue might be expected to produce rectangular parallelepipeds of tissue, this shape was not obvious, and for diffusion calculations the tissue fragments were treated as cylinders. The radius of cylinders, measured by low-power microscopy, was found to be variable.

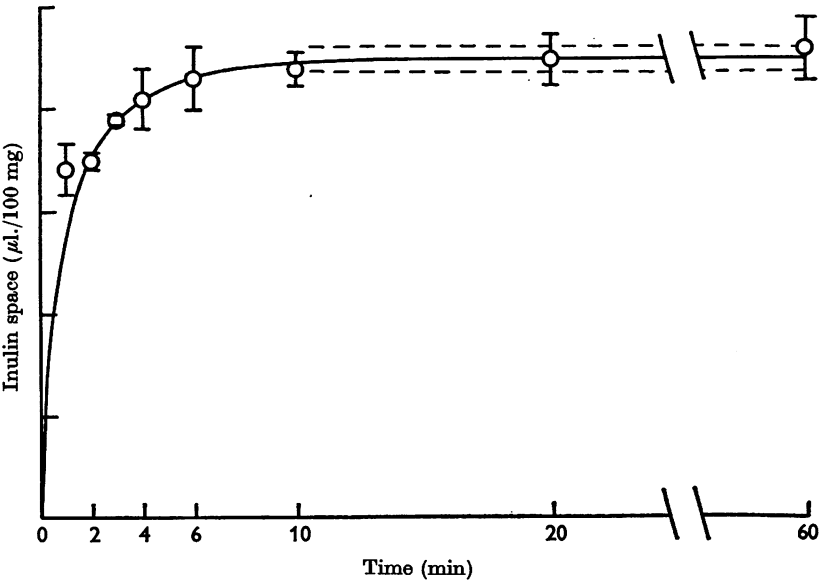

Fig. 1. The observed rate of inulin uptake with curve drawn by eye. The vertical bars show the 95 % confidence limits for the means and the interrupted lines show the 95 % confidence limits for the mean of all values from 10 min onwards.

The average radius was between 0·15 and 0·2 mm and the coefficient of variation of the radius was between 25 and 30 %. The mean radius did not change detectably after up to 7 hr incubation at 37° C and did not vary noticeably with the extent of inflation of the lungs before chopping. The length of most fragments was considerably (about 20 times) greater than the radius.

The mean wet weight of drained tissue samples was between 70 and 90 mg in most experiments (coefficient of variation per sample 9–13 %).

The rate of uptake of inulin and the extracellular space. The uptake of inulin as a function of time is shown in Fig. 1. Equilibration is virtually complete within 10 min and the inulin space remains constant for at least another hour. No consistent difference was seen between inulin spaces

determined at the beginning of an experiment and those determined at the end, about 3 hr later.

The equilibrium inulin space of drained tissue lay between 45 and 55 μl./100 mg in almost all experiments. The coefficient of variation of a single estimate of inulin space was usually less than 5%. If the drained tissue was blotted gently more fluid (about 16 μl./100 mg) was removed but this procedure was not routinely used since it impaired subsequent histamine release.

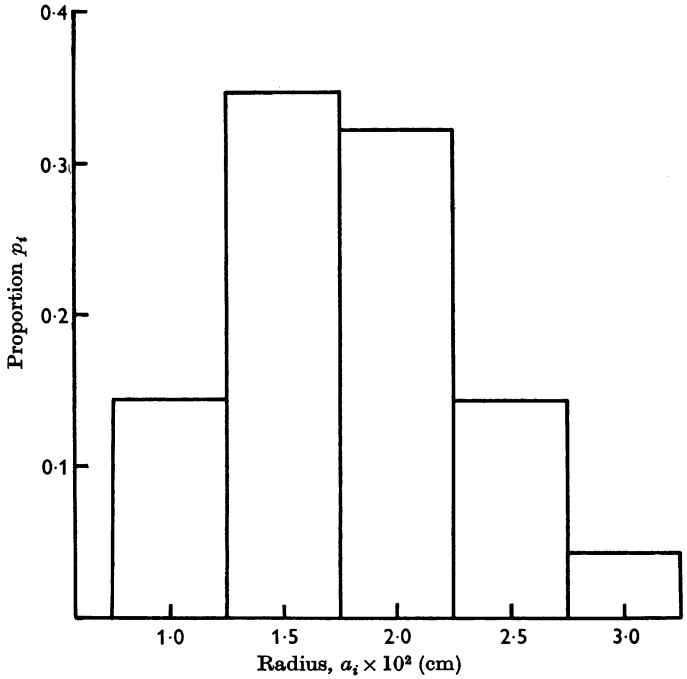

Fig. 2. Distribution of radius of lung-tissue particles. The distribution has been coarsely grouped for use in diffusion calculations.

Estimation of the effective diffusion coefficient of inulin in lung tissue. For the purposes of diffusion calculations the tissue samples were treated as a population of infinitely long cylinders of variable radius. In order to simplify calculations the observed distribution of radii was coarsely grouped as shown in Fig. 2.

The diffusion coefficient of inulin in free solution (D) in 0·15 M saline at 38° C was taken as $2{\cdot}29 \times 10^{-6}$ cm^2 sec^{-1}. This value was obtained on the batch of inulin actually used, by the method of Schantz & Lauffer (1962) and, when corrected to the same temperature and solvent, is similar to the values given by Bunim, Smith & Smith (1937) and by Conway & Fitzgerald (1942).

It was assumed that the diffusion of inulin through the extracellular space could be represented by the same equations as unobstructed diffusion in solution but with a lower 'effective' diffusion coefficient (D').

The observed values of inulin uptake (given in Fig. 1) expressed as a proportion of the final uptake, i.e. $M(t)/M(\infty)$, are compared in Fig. 3 with various theoretical curves. Curve 1 was calculated using eqn. (1) in the Appendix assuming that the tissue sample consisted of infinite cylinders,

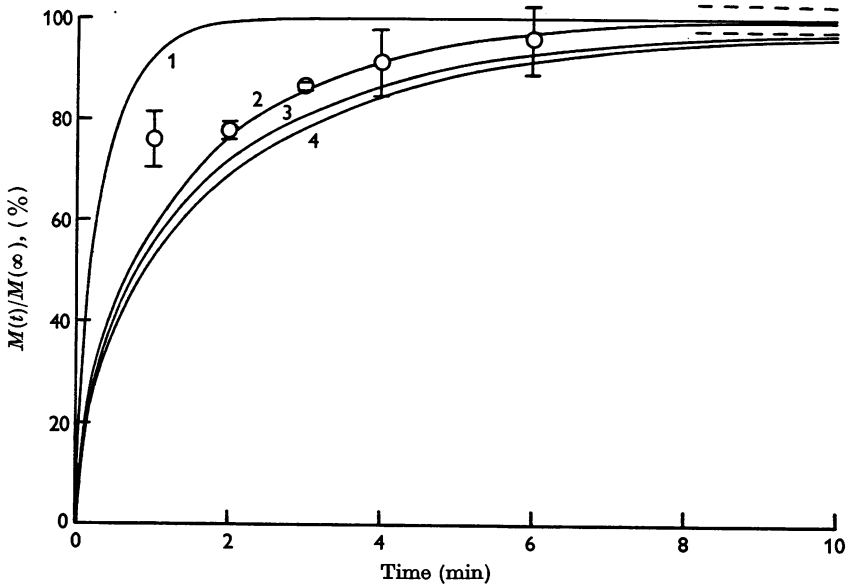

Fig. 3. The rate of inulin uptake during the first 10 min. The observed points are taken from Fig. 1. Curve 1: calculated diffusion curve for a population of cylinders all of the same (the observed mean) radius, using the diffusion coefficient of inulin in solution D. Curve 2: as curve 1, but using an effective diffusion coefficient $D' = D/4{\cdot}25$. Curve 3: calculated diffusion curve for a population of cylinders of constant length with radius distributed as in Fig. 2 and $D' = D/4{\cdot}25$. Curve 4: as curve 3 but assuming that the length of cylinders is proportional to their radius.

all with the mean observed radius ($1{\cdot}87 \times 10^{-2}$ cm in this case). The diffusion coefficient of inulin in the tissue was assumed to be the same as that in solution. As expected, the calculated rate is much faster than the observed rate. If the diffusion coefficient is reduced by a factor of 4·5, the calculated curve (Fig. 3, curve 2), again assuming constant radius, is seen to fit approximately the observed points. Thus the effective diffusion coefficient of inulin through the tissue might be taken as

$$D' = D/4{\cdot}25 = 0{\cdot}539 \times 10^{-6} \text{ cm}^2 \text{ sec}^{-1}.$$

In fact diffusion is being observed in a population of cylinders whose radii are far from constant, and it is of interest to see how far this affects

the results. For this purpose the distribution of radii shown in Fig. 2 was used. Equation (2) in the Appendix represents diffusion in this situation. Curve 3 in Fig. 3 was calculated from this equation, again using $D' = D/4{\cdot}25$. It can be seen that allowing for the variability of the radius slows the calculated approach to equilibrium considerably. Curve 3 assumes cylinders of constant length. In fact there was a tendency for cylinders of small radius to be short. If length were proportional to radius, curve 4 would be predicted. Clearly the assumption of constant length is not likely to affect the results much.

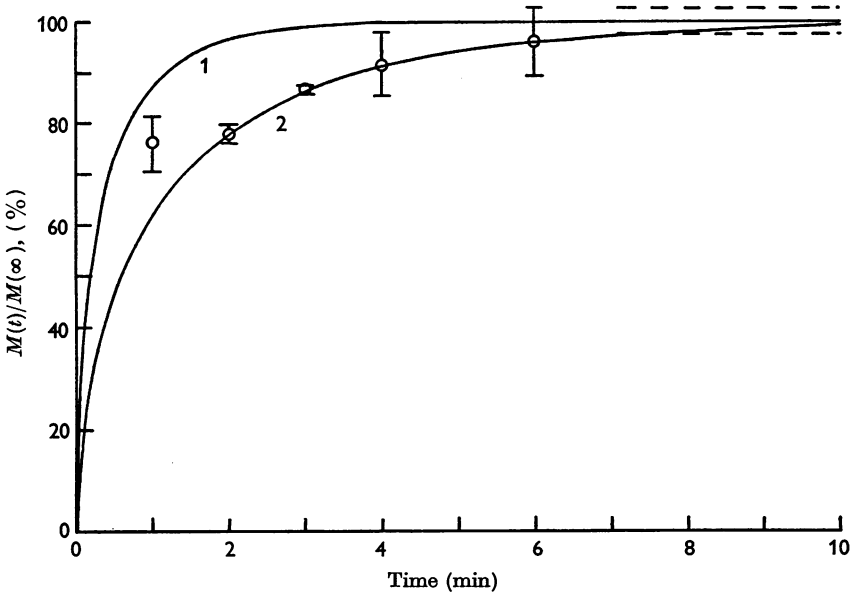

Fig. 4. The rate of inulin uptake. The observed points are as in Fig. 3. Curve 1; calculated diffusion curve for cylinders of constant length using the distribution of radius shown in Fig. 2 and the diffusion coefficient in solution, D. Curve 2; as curve 1 but using an effective diffusion coefficient $D' = D/3{\cdot}25$.

Figure 4 shows that, when the variability of radii is allowed for, the effective diffusion coefficient of inulin through the tissue is estimated to be $D' = D/3{\cdot}25 = 0{\cdot}705 \times 10^{-6}$ cm^2 sec^{-1}.

In this experiment, as in others, the 1 min point fell rather above the predicted curves. Since systematic errors will be greatest at short time intervals this point should not be given too much weight. However, a somewhat closer fit can be obtained if it is assumed that 16 μl./100 mg of extracellular fluid (which, as mentioned earlier, can be removed by gentle blotting) lies outside the cylinders of lung tissue, and therefore equilibrates instantaneously. Using this model, the effective diffusion coefficient, D', is estimated to be $\frac{1}{4}$ or $\frac{1}{5}$ of the value in solution.

The accuracy of the above, and of all subsequent results, depends on the assumption that inulin is neither adsorbed on nor penetrates cells.

Diffusion and adsorption of γ-globulin during passive sensitization

The rate of diffusion and adsorption. Adsorption has been estimated both by washing the tissue for an arbitrary time and assuming that the remaining γ-globulin is adsorbed, and by subtracting from the total γ-globulin present in the drained tissue the amount of γ-globulin calculated to be present in the extracellular space (hereafter referred to as the inulin method).

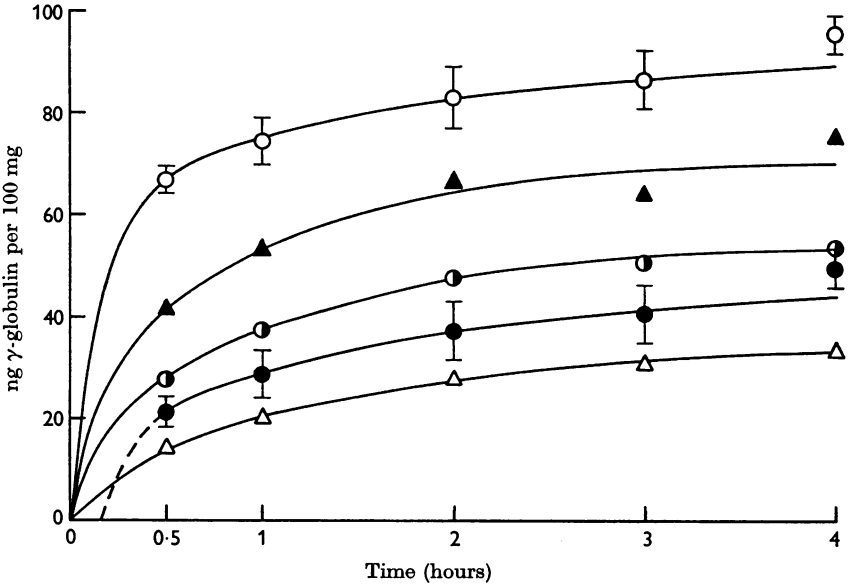

Fig. 5. The adsorption of rabbit γ-globulin (at a concentration of 1·0 μg/ml.) measured by different methods. The vertical bars show the 95 % confidence limits of the means. ○, The total γ-globulin content of the tissue; ▲, the amount of γ-globulin remaining after one wash with Tyrode solution; ◑, the amount of γ-globulin remaining after three washes; ●, inulin method (assuming equilibration of the extracellular space); △, the amount of γ-globulin remaining after three washes followed by 15 min incubation in about 4 ml. of Tyrode solution.

The results of adsorption measurements at different times are shown in Fig. 5. It can be seen that the inulin method gave somewhat lower values than were obtained by washing the tissue three times with Tyrode solution (a procedure occupying roughly 3 min), but higher values than were obtained if the tissue was gently agitated for 15 min in about 4 ml. of Tyrode solution after washing. These results also suggest that the assumption that the extracellular space contains the same concentration of

γ-globulin as the external solution is unlikely to be true at times much shorter than 30 min.

The diffusion coefficient of rabbit γ-globulin has been determined by Cammack (1962). When corrected to the conditions of the present experiments it is $D = 7{\cdot}20 \times 10^{-7}$ cm^2 sec^{-1}. If it is assumed that the diffusion coefficient of γ-globulin through the tissue is reduced by the same factor as that of inulin (most theories of diffusion through obstructing media indicate that this is likely to be true), so that $D' = D/3{\cdot}25 = 2{\cdot}22 \times 10^{-7}$ cm^2 sec^{-1}, the rate at which the extracellular space equilibrates with the external γ-globulin solution can be predicted. There is, however, the additional complication of concurrent adsorption. This will tend to slow down diffusion to an extent dependent on the rates of adsorption and desorption. As it is not possible to estimate these two rate constants, one of the following simpler models must be adopted.

(*a*) If it is assumed that adsorption is a slow process compared with diffusion, the effect of the former can be ignored so the effective diffusion coefficient of γ-globulin will be $D' = 2{\cdot}22 \times 10^{-7}$ cm^2 sec^{-1}. In this case the equilibration of the extracellular space would be virtually complete before any adsorption had taken place.

(*b*) If adsorption were a rapid process compared with diffusion, every point on the surface being in equilibrium with the concentration of γ-globulin in solution at that point, and if the adsorption isotherm were linear (as it is in the present case), then the effective diffusion coefficient would be reduced by a factor of $K+1$, where K is the adsorption equilibrium constant (Crank, 1956). It is shown in the Appendix that $K \simeq 1{\cdot}0$, so in this case the effective diffusion coefficient of γ-globulin would be $D' = 1{\cdot}11 \times 10^{-7}$ cm^2 sec^{-1}.

The γ-globulin content of the extracellular space, $M(t)$, can now be calculated as a function of time using eqns. (1) and (2) in the Appendix. It is predicted that 93% of the equilibrium γ-globulin content, $M(\infty)$, would be reached in about 15 min if adsorption were slow (model *a*) or in about 30 min if adsorption were fast (model *b*). The application of these calculations to experimental results is illustrated in Fig. 6. Curves 2 and 3 are the calculated γ-globulin contents of the extracellular space, $M(t)$, using models *a* and *b* respectively. By subtracting these from the total γ-globulin content of the tissue (curve 1), the estimated adsorption (curve 4) is found. Figure 7 shows a similar experiment but in this case $M(t)$ has been calculated assuming, as explained earlier, that 16·0 μl./100 mg of the extracellular fluid equilibrates instantaneously with γ-globulin, and that $D' = D/4{\cdot}25$. It appears that the predicted diffusion rate is approximately correct, but the results are not sufficiently precise to distinguish between the theoretical models.

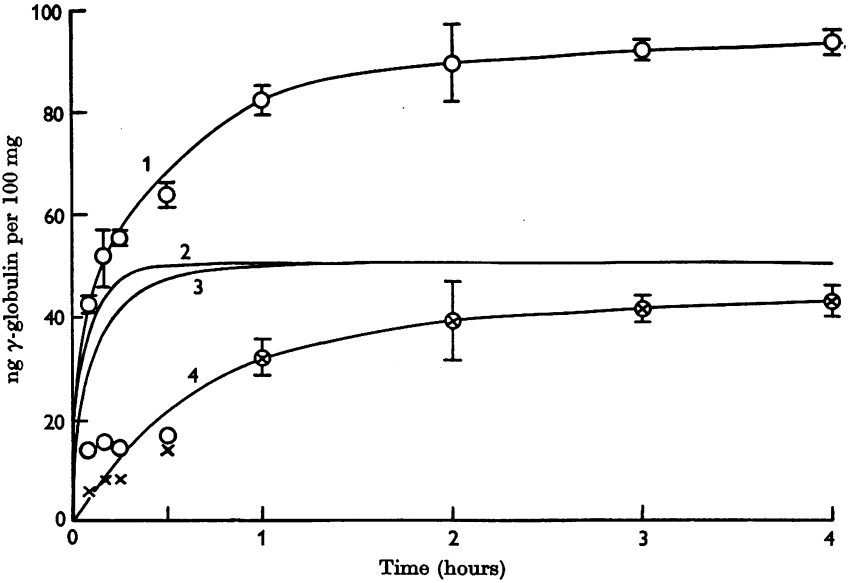

Fig. 6. The adsorption of rabbit γ-globulin at a concentration of 1·0 μg/ml. Curve 1; observed total γ-globulin content of the tissue with standard deviations of means. Curves 2 and 3; γ-globulin content of extracellular space calculated using models *a* and *b* (see text). Curve 4; net adsorption of γ-globulin using models *a* (×) and *b* (○).

The rate of sensitization. The time course of sensitization over 4 hr is shown in Fig. 8. The adsorption of γ-globulin in this experiment has been shown in Fig. 7. The sensitization is seen to be increasing quite rapidly at least up to 4 hr. Similar results were seen in five other experiments. There is no sign of an initial fast phase of sensitization. After 4 hr the amount of γ-globulin adsorbed was increasing only slowly.

These results indicate that neither the rate of sensitization nor the rate of adsorption is entirely diffusion controlled, but that diffusion is sufficiently slow to exert a considerable influence on the rates in the first 15 or 30 min.

The validity of the assumption of equilibrium. Between 1 and 4 hr there was usually a slow continuous uptake of γ-globulin, as can be seen in Fig. 7. While γ-globulin is still being taken up there must be a concentration gradient between the external solution and the extracellular space, so the assumption that the concentration in the extracellular space is the same as that in solution will not be strictly true. It is therefore interesting to see whether an estimate can be made of the error from this source.

Although the mechanism of the slow uptake is not known, an approximation to the answer required can be obtained by assuming that

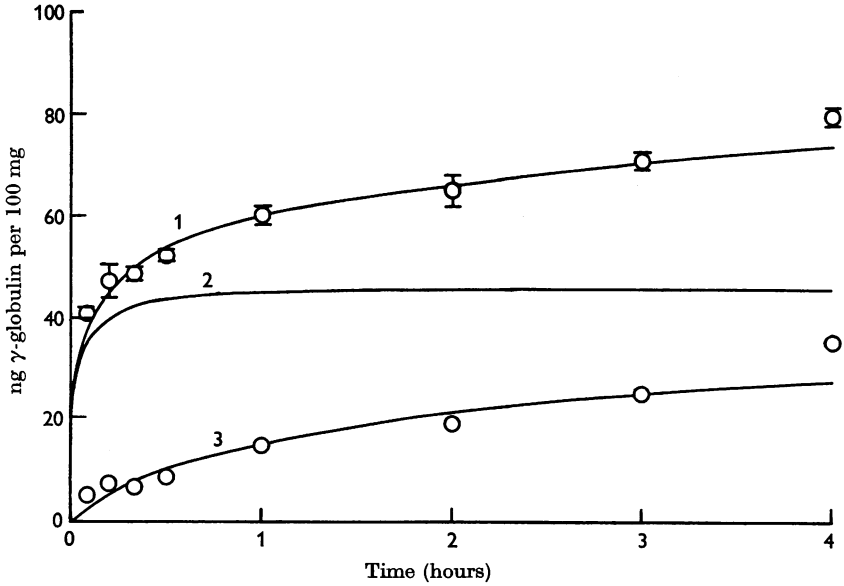

Fig. 7. The adsorption of rabbit γ-globulin at a concentration of 1·0 μg/ml. Curve 1; observed total γ-globulin content of the tissue with standard deviations of means. Curve 2; calculated γ-globulin content of the extracellular space using model *b* and assuming that 16 μl. interparticle fluid per 100 mg equilibrates instantaneously. Curve 3; net adsorption, i.e. curve 1 minus curve 2.

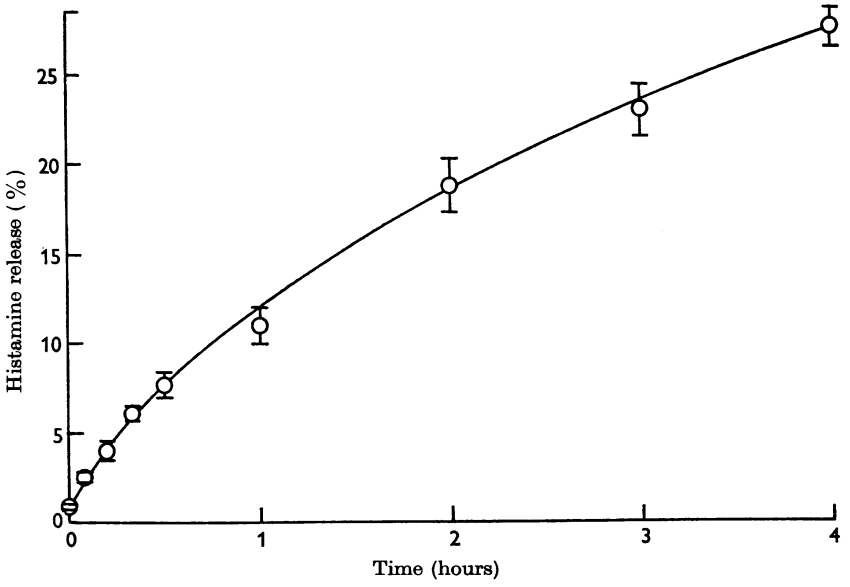

Fig. 8. The rate of sensitization with rabbit γ-globulin at a concentration of 2·25 μg/ml. (0·72 μg precipitating antibody per ml.). The vertical bars show the standard deviations of the means.

γ-globulin is being removed from the extracellular space by a first-order reaction (it turns out that assumption of a zero-order reaction would not alter the conclusions), and that after about 1 hr a steady state had been attained. Using the effective diffusion coefficient, the amount of γ-globulin which would be present in the extracellular space in the steady state can then be calculated. The method of calculation is outlined in the Appendix.

The fastest rate of steady uptake seen was about 16 ng γ-globulin/100 mg of tissue per hour. From the equations in the Appendix it has been calculated that the quantity of γ-globulin in the extracellular space in the steady state would be about 1·8% below the amount that would be present if true equilibrium were attained. The concentration in the centre of the cylinder would be about 3·5% below the external concentration in these circumstances. The error resulting from the fact that true equilibrium may not be reached is thus quite small.

Adsorption and sensitization near equilibrium

The adsorption isotherm for rabbit γ-globulins. Measurements were made only over the low range of antibody concentrations in which sensitization was submaximal. The adsorption isotherm in this range is shown in Fig. 9. In this, as in all other experiments, no significant deviations from linearity were detectable.

The affinity of the protein for the tissue is simply the slope of the isotherm, i.e. the adsorption for unit concentration. If the adsorption is measured in ng γ-globulin/100 mg wet weight of tissue, the slope, K', will have the dimensions μl./100 mg. The relation between this slope and the adsorption equilibrium constant, K, is discussed in the Appendix. The determination of K' requires knowledge of the radioactivity of the tissue and of the supernatant solution, and—when the inulin method is used—of the inulin space. It does not, however, require knowledge of the actual protein concentration in the supernatant solution.

After 4 hr incubation with antibody, K' was found to be about 40 μl./100 mg (i.e. about 40 ng γ-globulin/100 mg was adsorbed when the protein concentration was 1·0 μg/ml.). After 1 hr incubation K' was usually between 20 and 30 μl./100 mg.

No correlation was detectable between the affinity of γ-globulin for lung tissue and its extent of labelling (atoms iodine/mole protein), specific activity (μc/mg) or proportion of non-protein-bound radioactivity (Colquhoun, 1964).

Sensitization by rabbit γ-globulin. The relation between sensitization and concentration is shown in Fig. 10. After 1 hr incubation 50% of maximum sensitization is achieved with less than 0·5 μg γ-globulin/ml.

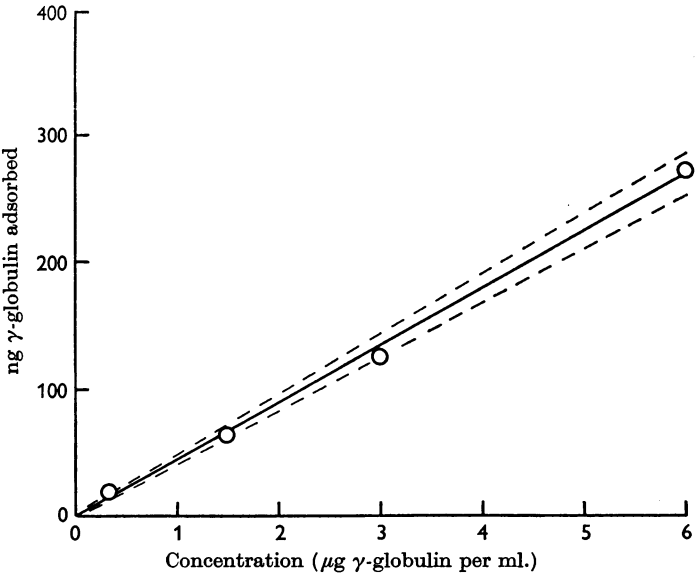

Fig. 9. Adsorption isotherm for rabbit γ-globulin determined by the inulin method. The interrupted lines are the 95 % confidence limits for the slope of the isotherm (constrained to pass through the origin).

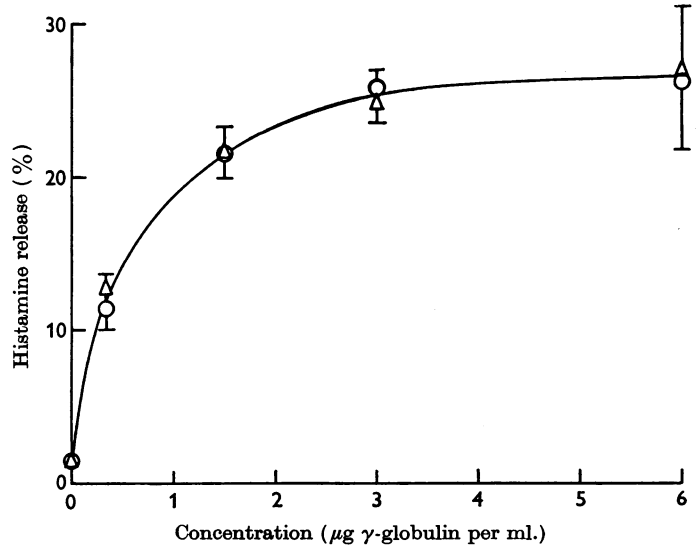

Fig. 10. Sensitization as a function of concentration. Same experiment as shown in Fig. 9. ○, Drained tissue (no washes); △, three washes with Tyrode solution. The vertical bars show the 95 % confidence limits for the combined means.

This concentration of protein corresponded to less than 0·15 μg precipitating antibody/ml. It is clear from Fig. 8 that this amount of protein would have produced even more histamine release if left in contact with the tissue longer.

Adsorption of guinea-pig γ_1- and γ_2-globulins. It was uniformly found in six experiments that no difference could be detected between the amounts of labelled γ_1- and γ_2-globulins adsorbed by lung tissue at, or near, equilibrium.

Table 1 shows the results of adsorption measurements using equal low concentrations of γ_1- and γ_2-globulin, such that good sensitization of the lung was produced by the γ_1-globulin antibody, but no detectable sensitization by the γ_2-globulin antibody (Colquhoun, 1964). Table 2 shows the results obtained using a much larger concentration of γ_2-globulin

TABLE 1. Adsorption of guinea-pig γ_1- and γ_2-globulins, both in low concentration. Lung tissue was incubated with labelled protein for 2 hr; adsorption was measured by the inulin method

Protein	Concentration (μg/ml.)	Adsorption (K' with 95% confidence limits) (μl./100 mg)
γ_1-globulin	0·75	24·9 ± 6·5
	3·0	25·8 ± 3·9
γ_2-globulin	0·75	25·8 ± 3·2
	3·0	22·8 ± 1·9

TABLE 2. Adsorption of guinea-pig γ_1- and γ_2-globulins, in concentrations producing comparable sensitization. Lung tissue was incubated with labelled protein for 4 hr; adsorption was measured by the inulin method

Protein	Concentration (μg/ml.)	Adsorption (K' with 95% confidence limits) (μl./100 mg)	Histamine release (%)
γ_1-globulin	0·433	71·8 ± 14·1	21·6
	0·134	96·1 ± 8·3	9·8
γ_2-globulin	250	70·4 ± 9·6	10·3
	77·7	70·6 ± 29·4	5·3

(labelled at 13·9 nc/mg, 1·0 atoms iodine/mole protein) than of γ_1-globulin (labelled at 57·1 μc/mg, 0·7 atoms iodine/mole protein). The concentrations were such as to produce similar degrees of sensitization. The fact that there is no sign of different adsorption in either of these experiments (analysis of variance gave variance ratios of less than one for the difference between proteins in both experiments) implies that the adsorption isotherm of γ_2-globulin must be linear up to a concentration of at least 250 μg/ml.

Lack of effect of changing calcium concentration during sensitization. Reducing the calcium concentration of the Tyrode solution used during sensitization had no effect on the inulin space of lung tissue, on the adsorption of rabbit γ-globulin, or on the ensuing sensitization as shown

by the results in Table 3. The tissue was washed with low-calcium Tyrode before and after incubation with labelled γ-globulin. The calcium concentration was returned to normal (1·8 mM) 15 min before addition of antigen.

TABLE 3. The effect of Ca^{2+} concentration on inulin space, adsorption and sensitization of lung *in vitro*

Calcium concentration (mM)	Inulin space (μl./100 mg)	Adsorption constant, K' (μl./100 mg)	Histamine release (ng/sample)
0·0	52·2	22·9	152
0·008	50·1	21·9	160
0·05	49·7	26·9	175
0·3	52·2	25·9	145
1·8	51·9	27·4	163

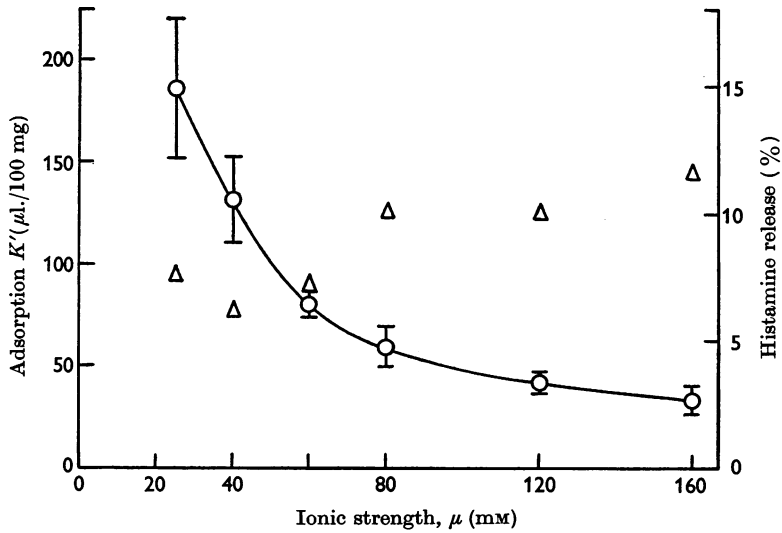

Fig. 11. The effect of reduction of ionic strength on the adsorption of, and sensitization by, rabbit γ-globulin. Varying proportions of the sodium chloride in Tyrode solution were replaced by isosmotic concentrations of sucrose. ○, Adsorption (measured by the inulin method) with 95% confidence limits; △, histamine release (measured at normal ionic strength).

Effect of ionic strength on adsorption and sensitization. It was consistently found that reduction of ionic strength caused a large increase in the amount of γ-globulin adsorbed before and at equilibrium. Figure 11 shows the results of an experiment in which varying proportions of the sodium chloride in Tyrode solution were replaced by isosmotic amounts of sucrose. Adsorption increases rapidly at low ionic strength, roughly in the way expected on physicochemical grounds. However, no corresponding increase in histamine release was seen on addition of antigen (after restoration of the normal ionic strength).

Even greater adsorption was seen when all electrolytes were omitted. For example, in isosmotic glycine the adsorption constant, K', was 586 μl./100 mg $\pm$ 6·7% (95% confidence limits), and in isosmotic buffered sucrose $K' = 623$ μl./100 mg $\pm$ 7·2% compared with $K' = 40{\cdot}7$ μl./100 mg $\pm$ 12·6% in normal Tyrode in the same experiment. Similar results were obtained with glucose, mannitol, sorbitol and raffinose solutions, but in no case (with the possible exception of glycine) was any significant increase in sensitization found to accompany the increased adsorption of antibody.

Some evidence was found that tissue was damaged when it was incubated in solutions containing no electrolyte. Such tissue showed increased weight and inulin space and the whole tissue water appeared to be penetrated by sucrose. If actively sensitized tissue was used, histamine release from it was reduced. These effects were, however, not noticeable when tissue was incubated in solutions in which part of the sodium chloride had been replaced by non-electrolyte, but even so no increase in sensitization was observed.

The loss of rabbit γ-globulin from lung tissue. Diffusion out of the extracellular space is represented by the same curves as diffusion into it if the ordinate, $M(t)/M(\infty)$, is interpreted as the proportion of γ-globulin lost from the cylinder at time t. It is therefore predictable that the standard washing procedures, which do not occupy longer than 3 or 4 min, cannot be expected completely to remove γ-globulin from the extracellular space. For example, if adsorption were slow compared with diffusion, it is easily calculated that 67% of the total (free and adsorbed) γ-globulin content of the tissue would remain after washing for 3½ min. If, on the other hand, adsorption equilibrium were rapid compared with diffusion, 52% of the total γ-globulin would be predicted to remain after washing. As expected, the experimental results fall between these two extreme models. For example, in Fig. 5 between 55 and 60% of the total γ-globulin is seen to remain after three washes at the 2, 3 and 4 hr points.

In order to test whether γ-globulin becomes more strongly adsorbed with time, lung tissue was incubated for various lengths of time with labelled γ-globulin; then the adsorption was measured by the inulin method and the tissue washed three times and agitated gently for 15 min in 5 ml. of Tyrode solution. The amount of γ-globulin remaining in the tissue, as a proportion of the net amount adsorbed at the end of the incubation, as shown in Table 4, was about 70% in all samples and was not seen to increase with time. No difference was observed in the amounts of histamine released from tissue washed in different ways, as shown in Fig. 12 (see also Fig. 10).

When the amount of γ-globulin remaining in tissue which had been

incubated with antibody for 2 hr was measured after various periods of washing with Tyrode solution, about 50% of the adsorbed material was removed within 1 hr, but thereafter loss of γ-globulin was very slow as shown in Fig. 13.

TABLE 4. Amount of γ-globulin remaining in lung tissue after three washes and 15 min incubation in Tyrode solution following different periods of incubation with labelled γ-globulin. Results are expressed as a percentage of the amount of γ-globulin adsorbed (estimated by the inulin method) at the end of the period of incubation with antibody

Incubation time (hr)	Expt. 36 (%)	Expt. 41 (%)
$\frac{1}{2}$	70·1	67·3
1	67·4	69·8
2	70·2	74·2
3	68·9	75·0
4	75·6	66·4

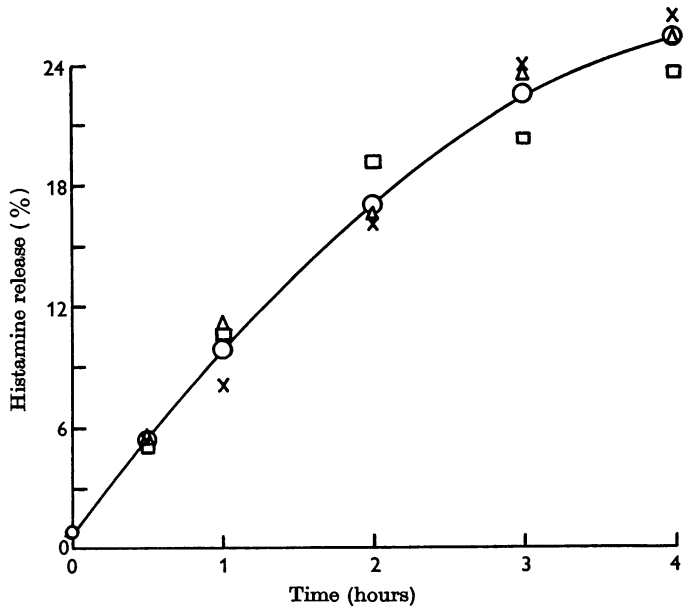

Fig. 12. The rate of sensitization, histamine release being measured after treatment of the tissue by different methods. △, One wash with Tyrode soluion; ×, three washes with Tyrode solution; □, three washes followed by 15 min incubation with Tyrode solution; ○, mean of all results.

DISCUSSION

The results presented show that even in small pieces of chopped lung tissue diffusion is not fast enough to be ignored. The time courses of events occurring within less than 30 min are likely to depend substantially on diffusion. It is apparent that the short washing periods used by some

workers cannot be expected to empty the extracellular space of non-adsorbed γ-globulin, but that error from this source is partially cancelled by loss of adsorbed material so estimates of adsorption obtained in this way do not vary grossly from those obtained by the inulin method. The results and calculations presented also show that a slow, steady uptake of γ-globulin (usually seen even after long periods of incubation) does not result in serious error in the assumption that the concentration of diffusing material in the extracellular space is the same as that in the external solution.

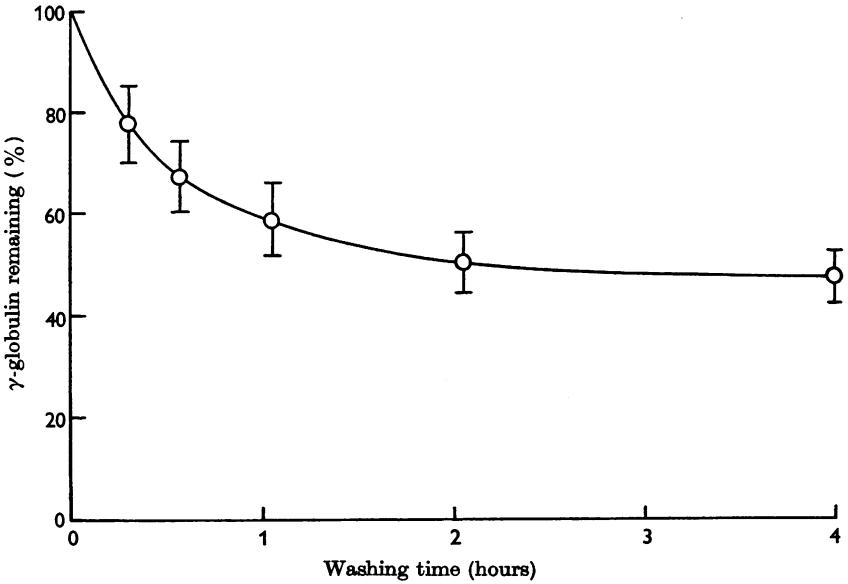

Fig. 13. The desorption of γ-globulin. The amount of γ-globulin remaining in the tissue, expressed as a percentage of the net adsorption (measured by the inulin method) at the end of 2 hr incubation with antibody, is plotted against the duration of incubation of the tissue in a large volume of Tyrode solution. The vertical bars show the 95 % confidence limits of the means.

The possibility of the existence of a fast initial phase in the sensitization process has been suggested by Mongar & Schild (1960, 1962). No consistent evidence for this has been observed when measurements were made after a little as 5 min incubation, even though the tissue had been perfused free of blood and thoroughly washed with Tyrode solution, which might be expected to favour the appearance of a fast phase. Since the calculated rate of diffusion into the tissue is very fast initially it is possible that diffusion-controlled phenomena might give rise to the appearance of a fast initial phase. It has also been consistently observed that sensitization was still increasing after 4 hr, whereas Austen & Humphrey (1963) mention

that, using low concentrations of antibody, sensitization was complete within 40 min even at 0° C.

In the present work no consistent evidence has been obtained against a linear adsorption isotherm. This might be expected in the low range of γ-globulin concentrations usually employed. However, in one experiment in which a concentration of 250 μg/ml. of guinea-pig γ_2-globulin was used no sign of non-linearity was detectable. Although adsorption isotherms of various forms have been reported (Nielsen *et al.* 1959; Brocklehurst *et al.* 1961; Feigen, Nielsen & Terres, 1962) inspection of the results in the literature shows that none of them is inconsistent with a linear adsorption isotherm up to a γ-globulin concentration of at least 250 μg/ml.

The amount of antibody required to produce sensitization was less than most of the values previously reported. For example, guinea-pig γ_1-globulin produced good submaximal sensitization at a concentration of 0·1 μg total protein/ml., i.e. about 6×10^{-10} M. This corresponds to about 40 ng of adsorbed protein per gram of wet tissue. Since only part of this protein is antibody, and since about half of it is only loosely bound and can be removed without loss of sensitization it appears that less than 10 ng (i.e. 6×10^{-14} mole) antibody/g tissue is required to produce sensitization.

The finding that neither adsorption nor sensitization are affected by altering the calcium concentration of the medium is consistent with the work of Mongar & Schild (1960), who found that sensitization was not inhibited by lack of calcium. It suggests that calcium is not involved in the binding of γ-globulin on to cells.

The increased adsorption at low ionic strengths is what would be expected on physicochemical grounds. The fact that lowering the ionic strength reduces the solubility of γ-globulin when it is on the alkaline side of its isoelectric point (Edsall, 1947) shows that the activity coefficient of the protein rises with decreasing ionic strength, as expected on theoretical grounds, and adsorption would be expected to increase as a consequence of this. Lowering the ionic strength should also increase the potential of the surface of the cells with respect to the solution. Binaghi, Liacopoulos, Halpern, Liacopoulos-Briot & Bloch (1961) found that the rate of sensitization of guinea-pig ileum by rabbit antibody was greatly increased at low ionic strengths, which may be explained by the increase in adsorption of antibody which presumably occurred. The reason why no important increase in the sensitization of guinea-pig lung was found to accompany the increased adsorption at low ionic strengths is not understood.

It would be of interest in connexion with theories of passive sensitization

if the binding of γ-globulin could be shown to become more firm with time (see, for example, Mongar & Schild, 1962), but no such effect has been observed over a 4 hr period. The proportion of the adsorbed γ-globulin (measured by the inulin method) removed by a standard washing procedure was constant. It may be noted that if the 'adsorption' were measured by a method which did not completely remove material from the extracellular space a spurious fall in the proportion of material 'adsorbed' which was removed by the standard washing procedure would be observed while the amount actually adsorbed was still rising.

The results of skin sensitization inhibition experiments obtained by Ovary, Benacerraf & Bloch (1963) imply that guinea-pig γ_1-globulin is 'fixed' on cutaneous tissues, whereas γ_2-globulin is not. There is circumstantial evidence for the belief that this means, in physical terms, that γ_1-globulin has a higher affinity than γ_2-globulin for the receptor sites supposed to be involved in passive sensitization of the tissue. However, this has never been shown directly and other explanations may be possible. In the present experiments no difference could be detected between the affinities of γ_1- and γ_2-globulins for lung tissue. The most plausible explanation for this is that most of the adsorbed antibody is not adsorbed on the supposed specific receptor sites for sensitization. This proposition is, of course, not incompatible with the observed correlation (a sigmoid curve) between the amount of antibody adsorbed and the extent of sensitization. Such a correlation is *a priori* inevitable when adsorption increases with concentration and sensitization is not an all-or-nothing process.

SUMMARY

1. Adsorption of γ-globulin over a range of concentrations producing submaximal sensitization has been studied by a new method.

2. The diffusion of inulin and γ-globulin into chopped lung-tissue particles has been studied from a theoretical and experimental point of view. The rate of uptake of γ-globulin was found to be at least partly controlled by diffusion.

3. No evidence was found for a fast initial phase of sensitization or adsorption.

4. Calculations show that a slow continuous uptake of γ-globulin would not seriously invalidate the assumption that the concentration of γ-globulin in the extracellular space is the same as that in the external solution.

5. The adsorption isotherm was linear over the range of concentrations studied.

6. Guinea-pig γ_1- and γ_2-globulins were adsorbed to the same extent. Sensitization was produced by as little as 6×10^{-14} mole (10^{-8} g) of γ_1-

globulin antibody per gram of tissue but very much larger amounts of γ_2-globulin antibody were required.

7. Neither adsorption nor sensitization were altered by reducing the calcium concentration during passive sensitization.

8. Reduction of the ionic strength of the medium caused a large increase in the amount of γ-globulin adsorbed but no important increase in sensitization was observed.

We should like to thank Kenneth Renton and Kathleen Godfrey for their skilled assistance and Dr J. H. Humphrey for many useful discussions. This work was supported by the Scottish Hospitals Endowment Research Trust.

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APPENDIX

DIFFUSION AND ADSORPTION IN TISSUES

BY D. COLQUHOUN

Diffusion in a population of cylinders

For the purposes of diffusion calculations lung-tissue particles were treated as long cylinders surrounded by a solution of constant concentration at $t > 0$. Under these conditions the solution of the diffusion equation

$$\frac{\partial c}{\partial t} = D\nabla^2 c$$

may be put in the form

$$f_i(t) = \frac{m_i(t)}{m_i(\infty)} = \left[1 - 4\sum_{j=1}^{\infty} \frac{1}{\alpha_j^2} \exp\left(-\alpha_j^2 Dt/a_i^2\right)\right], \qquad (1)$$

where $m_i(t)$ is the amount of material, of diffusion coefficient D, which has diffused into a cylinder of radius a_i at time t; α_j is the jth root of $J_0(x) = 0$ where J_0 is the Bessel function of the first kind of zero order. Values of Bessel functions and their roots were taken from the tables of Jahnke & Emde (1945). The function $f(t)$ is plotted in fig. 5·7 of Crank (1956). It may be noted in this connexion that Crank's equations 5·21 and 5·22 both contain misprints. Since $m_i(\infty) = \pi a_i^2 h_i c(\infty)$, where h_i is the length of the cylinder and $c(\infty)$ is the equilibrium concentration of diffusing material (mass per unit volume of tissue), eqn. (1) can be rearranged giving

$$m_i(t) = \pi a_i^2 h_i c(\infty) f_i(t).$$

In a population of cylinders containing a proportion p_i of cylinders of radius a_i (as shown, for example, in Fig. 2), the average amount of material which has diffused into a cylinder at time t will be

$$M(t) = \Sigma p_i m_i(t) = \pi c(\infty) \Sigma f_i(t) p_i h_i a_i^2.$$

Hence the proportion of the final uptake in the population attained at time t will be

$$\frac{M(t)}{M(\infty)} = \frac{\Sigma f_i(t) p_i h_i a_i^2}{\Sigma p_i h_i a_i^2}.$$

If all the cylinders are the same length, this reduces to

$$\frac{M(t)}{M(\infty)} = \frac{\Sigma f_i(t) p_i a_i^2}{\Sigma p_i a_i^2} \tag{2}$$

and, if the lengths of the cylinders are proportional to their radii, it reduces to

$$\frac{M(t)}{M(\infty)} = \frac{\Sigma f_i(t) p_i a_i^3}{\Sigma p_i a_i^3}.$$

The adsorption equilibrium constant

The affinity of protein for lung tissue has been expressed as the slope, K', of the linear adsorption isotherm. When adsorption is measured in ng/100 mg and protein concentration in μg/ml., this has the dimensions μl./100 mg.

However, the use of the solution of the diffusion equation for a cylinder (eqn. 1) implies that the cylindrical tissue particle has been treated as a homogeneous cylinder in which diffusion takes place with a lower diffusion coefficient (D') than that found in solution (D). The effect of adsorption, if adsorption equilibrium is rapidly attained, is further to reduce the effective diffusion coefficient to $D'/K + 1$ (see, for example, Crank, 1956), where K is the adsorption equilibrium constant. K must be dimensionless; i.e. the concentrations of both free and adsorbed material must be expressed in the same units, e.g. mass per unit volume of cylinder. It follows that

$$K = \frac{K'}{E},$$

where E is the extracellular space in μl./100 mg. From the values of K' and E given in the text it can be seen that the value of K was usually equal to, or a bit less than, 1·0.

The assumption of equilibrium: diffusion in the presence of a first-order reaction

It has been assumed that after about 60 min the concentration of γ-globulin in the extracellular space is equal to that in the external solution, but in fact slow continuous uptake was often observed at times between 1 hr and 4 hr. The assumption is therefore not strictly true.

To calculate the error involved, the period of slow continuous uptake may be represented to a first approximation by the steady state of a process of diffusion accompanied by first-order removal of material. The diffusion equation to be solved is

$$\frac{\partial c}{\partial t} = D\nabla^2 c - kc = 0,$$

where k (sec^{-1}) is the rate constant of the first-order reaction. It is known that the solution of this equation for the concentration $c'(r)$ of diffusing material at a distance r from the centre of the cylinder is

$$\frac{c'(r)}{c'_o} = \frac{I_0[r\sqrt{(k/D)}]}{I_0[a\sqrt{(k/D)}]},$$

where c'_o is the external concentration. By integrating this it can be shown that the solution required is

$$\frac{M_s}{M_e} = \frac{2}{a\sqrt{(k/D)}} \frac{I_1[a\sqrt{(k/D)}]}{I_0[a\sqrt{(k/D)}]}, \tag{3}$$

where M_s is the amount of material (with diffusion coefficient D) in the extracellular space in the steady state in the presence of a first-order reaction, and M_e is the amount which would be present at equilibrium if there were no such reaction. I_0 and I_1 are modified Bessel functions of the first kind of zero and first order, defined by $I_n(x) = i^{-n}J_n(ix)$ where $i = \sqrt{-1}$. It can be shown that, in the limiting case when $k = 0$ (or $D \to \infty$), eqn. (3) reduces to $M_s = M_e$ as it should.

It is necessary to obtain a value of the rate constant, k, in order to evaluate eqn. (3). This cannot be obtained directly from the observed rate of uptake since the concentration (to which the rate is proportional) varies throughout the tissue. However, in the steady state the rate of uptake per unit length of cylinder, $F(\infty)$ ($\mu g\ sec^{-1}\ cm^{-1}$), is given by

$$F(\infty) = 2\pi a c_o \sqrt{(kD)} \frac{I_1[a\sqrt{(k/D)}]}{I_0[a\sqrt{(k/D)}]}$$

(Danckwerts, 1951), where a is the radius of the cylinder and c_o the concentration per unit volume of cylinder which would be attained if equilibrium were reached. This may be expressed in more convenient units as follows. The uptake per unit *mass* of cylinder, $F'(\infty)$, is given by

$$F(\infty)(\pi a^2 \rho)^{-1},$$

where ρ is the density of the cylinder. Also if c'_o is the equilibrium concentration in the extracellular space (i.e. the concentration in the external solution), $c'_o V_e = c_o V$ where V_e is the volume of extracellular space per unit length and V is the total volume per unit length. Denoting the extracellular space per unit mass, $V_e/V\rho$, by E gives

$$F'(\infty) = \frac{2c'_o E\sqrt{(kD)}}{a} \frac{I_1[a\sqrt{(k/D)}]}{I_0[a\sqrt{(k/D)}]}, \tag{4}$$

which has the dimensions $ng\,(100\ mg)^{-1}\ sec^{-1}$. Equation (4) was solved graphically for k using the appropriate values of c'_o (1·0 μg/ml.), a ($1{\cdot}87 \times 10^{-2}$ cm), E (48 μl./100 mg) and D. For the fastest rate of slow

continuous uptake observed, about 16 ng (100 mg)$^{-1}$ (hr)$^{-1}$, the result was $k = 9{\cdot}8 \times 10^{-5}$ sec^{-1}. Substitution of this value in eqn. (3) gives $M_s/M_e = 0{\cdot}982$; i.e. an error of 1·8% would be made if it were assumed that equilibrium had been reached.

Since M_s/M_e is close to 1, it would be expected that a similar result would be obtained if k were calculated assuming that the concentration throughout the extracellular space was uniform and equal to that in the external solution. In this case equation (4) reduces to $F'(\infty) = kc'_o E$ (by allowing $D \to \infty$ or $a \to 0$) which gives $k = 9{\cdot}5 \times 10^{-5}$ sec^{-1}, not much different from the value obtained from the unreduced equation.

I am very grateful to Dr B. L. Ginsborg for many helpful discussions and suggestions.

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