THE CHARACTERIZATION AND ADSORPTION OF SENSITIZING ANTIBODIES

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SUMMARY

1. A method of measuring adsorption without washing the tissue has been devised and studied.
2. Diffusion through chopped lung tissue has been studied from a theoretical and experimental point of view. Predicted curves for the γ-globulin content of the extracellular space have been calculated and applied to experimental results. The rate of uptake was found to be at least partly diffusion controlled. Adsorption equilibrium and rate constants have been rigorously defined. No evidence was found for a fast initial phase of sensitization or adsorption.
3. Calculations have been performed concerning the consequences of slow steady γ-globulin uptake after long periods of incubation.
4. The amounts of rabbit γ-globulin adsorbed onto lung tissue were found to be of the same order of magnitude, or somewhat lower, than those previously reported.
5. No evidence against a linear adsorption isotherm was found in any experiments.
6. Neither adsorption nor sensitization were altered by reducing the calcium concentration during passive sensitization.

7. Reduction of the ionic strength of the medium caused a large increase in the amount of γ-globulin adsorbed but did not increase sensitization.
8. Two guinea pig antibodies have been separated by preparative electrophoresis and ion exchange chromatography.
9. The antigenic relationship and purity of γ_1- and γ_2-globulins have been studied. No contamination was detectable in γ_2-globulin, but γ_1-globulin contained fast γ_2-globulin and sometimes β-globulins as well.
10. Ovalbumin (crystallized 5 times) has been shown to contain at least four proteins.
11. It has been shown that γ_1-globulin antibodies sensitize lung tissues in very low concentrations, but no evidence was found that the very low sensitizing power of γ_2-globulin antibodies was not due to impurity.
12. Quantitative passive cutaneous anaphylaxis experiments performed in parallel with the tests on lung tissue showed that whenever the γ_2-globulin fraction contained antibody it appeared more potent relative to γ_1-globulin than when tested on lung tissue although the γ_1-globulin was always considerably more potent in both tests.

13. The skin sensitization produced by the γ_2-globulin fraction disappeared faster than that produced by the γ_1-globulin fraction.

14. It was not possible to detect enough γ_1-globulin contaminant in the γ_2-globulin fraction to account for the skin sensitizing ability of the latter.

15. It was concluded that γ_2-globulin antibody must have some skin sensitizing ability of its own, but that it is considerably less potent than the γ_1-globulin antibody.

16. No difference was detectable between the extents of adsorption of γ_1- and γ_2-globulins onto lung tissue.

17. An equation has been derived describing the loss of thiosulphate by radiative oxidation in iodine-131 solutions.

18. An analysis has been presented of interpolation and other errors in a rapid method for assaying large numbers of histamine solutions using visual linear interpolation between two standards.

ABBREVIATIONS

BSA	bovine serum albumin
c.p.s.	counts per second
DEAE	diethylaminoethyl
e.c.s.	extracellular space
GP	guinea pig
IGPS	immune guinea pig serum
IS	inulin space
MIE	microimmunoelectrophoresis
NGPS	normal guinea pig serum
PCA	passive cutaneous anaphylaxis
R	rabbit

SYMBOLS

a	radius of cylinder (cm)
c	concentration per unit volume of cylinder (ng μl^{-1})
c'	concentration per unit volume of fluid (ng μl^{-1})
D	diffusion coefficient in solution (cm^2 sec^{-1})
D'	effective diffusion coefficient in cylinder (cm^2 sec^{-1})
E	extracellular space (μl/100 mg)
$f_i(t)$	proportion of equilibrium uptake reached at time t in cylinders of radius a_i
$F(t)$	rate of uptake through surface of cylinder per unit volume of cylinder at time t in presence of 1st order reaction (ng μl^{-1} sec^{-1})

$F'(t)$	the same but per 100 mg of cylinder ng $(100\ \text{mg})^{-1}\ \text{sec}^{-1}$
h	length of cylinder (cm)
i	$\sqrt{-1}$
$I_n(x)$	modified Bessel function of first kind of order n $\left[= i^{-n} J_n(ix)\right]$
$J_n(x)$	Bessel function of first kind of order n
k	first order rate constant (sec^{-1})
k'	first order rate constant ($\mu\text{l}\ 100\ \text{mg}^{-1}\ \text{sec}^{-1}$)
K	adsorption equilibrium constant (dimensionless)
K'	adsorption equilibrium constant. Uptake per 100 mg of tissue for bulk concentration of 1 μg/ml ($\mu\text{l}\ 100\ \text{mg}^{-1}$)
M	mass of cylinder per unit length ($100\ \text{mg}\ \text{cm}^{-1}$)
$M(t)$	amount of material present in extracellular space ($\text{ng}\ 100\ \text{mg}^{-1}$) at time t
m_a	mass adsorbed per unit length of cylinder ($\text{ng}\ \text{cm}^{-1}$)
m_f	mass free in solution per unit length of cylinder ($\text{ng}\ \text{cm}^{-1}$)
n	counts per second per ml of supernatant
n'	counts per second per 100 mg tissue
P_i	proportion of cylinders in population of radius a_i
r	radial distance from centre of cylinder
s	amount of material adsorbed per unit volume of cylinder ($\text{ng}\ \mu\text{l}^{-1}$)
t	time
T	temperature ($^{\circ}$K)

V	volume of cylinder per unit length ($\mu l\ cm^{-1}$)
V_e	volume of e.c.s. inside cylinder per unit length ($\mu l\ cm^{-1}$)

REVIEW

Part I

Previous work on adsorption of antibody onto tissues

In the 1919 Croonian Lecture on "The Biological Significance of Anaphylaxis" Dale said "You will not expect or desire that I shall attempt a detailed review of the enormous literature which has grown up, with almost unique luxuriance, round the study of anaphylaxis". As a complete review in the space available would now be far less possible, in the following section an attempt will be made to look critically at only those published works concerned directly with the relation between the adsorption of antibody and the sensitization of tissues.

The literature on adsorption must, if anything, be even larger than that on anaphylaxis. Adsorption isotherms of almost every possible shape have been observed and many theories put forward to account for them, though most theoretical isotherms are, even to-day, at best semi-empirical. Adsorption will be considered from the dynamic point of view (see de Boer, 1953) in the discussion, but a brief discussion of the two most used theoretical isotherms is relevant at this point.

The Langmuir isotherm (Langmuir 1916, 1918) was derived to describe the adsorption of gases by "smooth" glass, mica and platinum surfaces. It assumes that adsorption takes place on discrete non-interacting homogeneous sites on the surface, no further adsorption taking place once all the sites have been filled. As Langmuir points out adsorption takes place whenever there is a time lag between the condensation of a molecule on the adsorption site, and its evaporation. He insists that adsorption cannot be said to be a "physical" process as distinct from a "chemical" process. In fact these terms are still commonly used, usually without it being made clear what they are intended to mean. It seems that most commonly the term "chemical" is applied to a process involving bonds with a considerable degree of covalent character, but there is, of course, a continuous variation of bond types and energies and if such a distinction is made it would seem to be necessary to lay down some arbitrary value for the bond energy (or preferably for the equilibrium constant) above which the process is considered to be "chemical".

The assumption of homogeneity of the binding sites is unlikely to be fulfilled with living tissues. In fact even in a much simpler system Bull (1957) found that the adsorption of egg albumin onto pyrex glass powder (measured by the decrease in optical density of the supernatant) indicated that the glass surface was heterogeneous. In the case of γ-globulin adsorption there is an additional complication in the heterogeneity of the adsorbate as well as of the adsorbent, though there is circumstantial evidence that the major part of the heterogeneity of γ-globulin may not involve the part of the molecule responsible for "fixation" in the immunological sense.

The other frequently used adsorption isotherm which will be discussed is that commonly known as the Freundlich adsorption isotherm (though it was used by earlier workers)

$$A = Kp^{1/n}$$

where A is a measure of the amount adsorbed, p is the pressure (or concentration), and K and n are arbitrary parameters. 1/n is the slope of the usual plot of log A against log p. This equation is a simple power function and is entirely empirical. It will approximately fit any number of functions over a restricted range, and in fact usually only fits adsorption observations over a limited range. Since it is entirely empirical it has been suggested (Glasstone, 1955) that its chief use is as an interpolation formula and inferences concerning the mechanism of adsorption cannot be made from the observation that the above equation will fit results over a limited range. The "Freundlich" isotherm has the property that no saturation limit is reached. Adsorption goes on increasing as long as the concentration increases. It has often been pointed out (e.g. Hinshelwood, 1940) that heterogeneity of the adsorbing surface could give rise to an isotherm which looked like the Freundlich isotherm though adsorption on individual classes of adsorbing sites was in fact Langmuirian (or, at least, saturable). Langmuir (1918) said "As a matter of fact this equation agrees very poorly with experiment when the range of pressures is large. An examination of the data presented by Freundlich shows that for low pressures or high temperatures $^1/n$ approaches unity while at high pressures or low temperatures $^1/n$ often becomes as small as 0.1". Langmuir (1918) himself gave an equation for adsorption on a surface with a continuous distribution of site affinities but did not solve it. This equation (or at least one equivalent to it) was solved by Sips (1948) who showed that, if it is assumed that there is a continuous distribution of site affinities, adsorption on each (infinitesimal) class of sites being Langmuirian, it is possible to derive the form of the affinity distribution from the observed isotherm. It was shown that an exponential distribution of affinities among Langmuirian sites would give rise to a Freundlich isotherm. It has, however, never been shown that such a distribution actually exists.

At low concentrations (or pressures) all isotherms become linear and this will be so even if the surface is heterogeneous (see, for example, Barrer and Rees, 1961).

Since the work of Dale (1912), Weil (1913), Hartley (1939) and Benacerraf and Kabat (1949) it has been increasingly accepted that at least a major part of the anaphylactic syndrome is dependent on the attachment of antibodies to cells. It has been shown that γ-globulin and other proteins can be adsorbed onto various inanimate surfaces as well as onto cells.

For example Eley and Hedge (1956) have studied the adsorption of proteins (BSA and insulin) onto monolayers of lipid and phospholipids, the latter being positively charged. They thought that probably ionic interactions were important for binding. However this seems a priori less likely for γ-globulin as most cells have a net negative charge and many molecules of γ-globulin itself, which has a distribution of isoelectric points (Alberty 1948a and b, 1949), will have a small negative charge at physiological pH. The molecule is probably quite large enough for the net charge to be unimportant compared with the local conditions at the site (if there is a definite site) of adsorption. Abramson, Moyer and Gorin (1942) discuss many experiments on adsorbed proteins. They point out that particles of various materials such as quartz, collodion, carbon, mineral oil and glass all take on the electrophoretic properties of the protein (or of the partially surface denatured protein) even though the uncoated particles may have differed considerably in their electrophoretic mobility. Electrophoretic mobility has been used to follow the adsorption of positively charged polyelectrolytes onto human red cells by Nevo, de Vries and Katchalsky (1955) and Katchalsky, Danon, Nevo and de Vries (1959).

The affinity of various compounds for red cells was found to be quite high. For example a bulk concentration of 1 μg/ml of poly-DL-lysine (average degree of polymerization = 36) resulted in the adsorption of 0.75 x 10^{-7} μg/cell, and when the surface potential of the cells was reduced from its normal value of -15.6 mV to between -6.4 and -11.4 mV agglutination resulted. This figure for adsorption may be compared with the adsorption of human γ-globulin onto tanned sheep cells reported by Singer, Orestes and Altmann (1962). They found that at a concentration of roughly 300 μgN/ml approximately 2 x 10^{-9} μgN HGG was adsorbed per tanned cell. Also Nevo et al. (1955) found that erythrocytes were saturated with polylysine at a bulk concentration of 70 to 80 μg/ml (i.e. about 1.4 x 10^{-5} M) whereas Singer et al. (1962) found that the adsorption isotherm for human γ-globulin did not reach a plateau even when the protein concentration was 25.4 mg γ-globulin per ml. (i.e. about 16 x 10^{-5} M).

Although no exact comparison is possible between the affinities of these (and other) substances for cell surfaces it is clear that, as might be expected, the affinity of the positively charged polymer for red cells is considerably greater than that of γ-globulin (especially as in the above example the mass concentration units are, if anything, more relevant than the molar units).

It is of interest that the only plasma protein with which γ-globulin has been shown to interact is β-lipoprotein though the interaction is not very powerful or specific (Oncley, Ellenbogen, Gitlin and Gurd, 1952).

There have been many studies on the combination of haemolytic antibody with erythrocytes. This, of course, is an antigen-antibody reaction rather than passive sensitization but it is conceivable that similar cell-γ-globulin interactions are involved in both processes. Arrhenius (1907) found that antibody was taken up by red cells according to a power function of the Freundlich isotherm type and interpreted this as evidence for partition of the antibody between serum and cell phases with complex formation in one phase. It was pointed out by Cromwell (1922) that the results could be interpreted equally well as adsorption, and that in many cases the Freundlich isotherm did not fit the observations well. The combination of iso-haemagglutinins with erythrocytes has been studied by Wurmser and Filitti-Wurmser (1957). It was found that the combination was reversible and obeyed the law of mass action. The thermodynamic parameters of the reaction depended on the iso-haemagglutinin and also on the genotype of the individual from which it came.

Fluorescent labelling. In principle the presence of γ-globulin on cell surfaces should be detectable using fluorescent antibody methods. Although there are many studies of γ-globulins in plasma cells and in pathological lesions there appears to be no work on the presence of γ-globulins on the surfaces of normal cells. In fact if such staining occurred it would be regarded in most studies as background or "non-specific" staining and attempts made to get rid of it. The one interesting result using this method was reported by White, Jenkins and Wilkinson (1963) who showed thatthe mast cells of the mouse tongue were made fluorescent when treated with guinea pig γ_1-globulin (but not γ_2-globulin) followed by fluorescein labelled rabbit anti-guinea pig γ-globulin serum. White (personal communication) has said that this result is only to be considered as a preliminary finding and that the interpretation of the result is complicated by the technical necessity to use mouse rather than guinea pig mast cells since, as far as is known, guinea pig antibodies will not sensitize mouse tissues.

Antigen adsorption. Antibodies adsorbed onto cells in such a way that they can still combine with antigen should be detectable using labelled antigen. Experiments of this sort have been carried out by Boyden and Sorkin (1960, 1961). They revealed a substance in rabbit, horse and guinea pig antisera which was capable of adsorbing onto spleen cells so that, after the cells were washed, the antigen (labelled with ^{131}I) was found to be adsorbed specifically to the cells. This substance was called cytophilic antibody. Since removal of all detectable cytophilic antibody from rabbit anti-HSA serum results in only a small drop in the precipitating antibody content of the serum (Boyden and Sorkin, 1961) it was concluded that cytophilic antibody is responsible for only a small proportion (0.1 to 1.0%) of the antigen combining capacity of rabbit anti-protein sera. Cytophilic antibody has been shown to be taken up by various normal rabbit tissues (but not by erythrocytes), by spleen cells of various species (Sorkin, 1963) and by rat mast cells and liver cells (Keller and Sorkin, 1963).

Cytophilic antibody is not destroyed by heating at 56°C for 30 min. It can be precipitated by $\frac{1}{3}$ saturated ammonium sulphate and has the electrophoretic mobility of a γ-globulin. In the ultracentrifuge it is found to belong to the class of 7S globulins. It behaves like a γ-globulin on DEAE cellulose columns (Sorkin, 1963).

The uptake on rabbit spleen cells is not prevented by various enzyme inhibitors, but is prevented when the cells have been treated with trypsin, chymotrypsin or papain. Wheat germ lipase, 5% phenol, 8M urea, chloroform, ether, methanol and Tween 80 (polyoxyethylene sorbitan mono-oleate) all cause a partial reduction in the capacity of the cells to take up cytophilic antibody. On these grounds Sorkin (1963) suggests that the cellular receptor for cytophilic antibody may be a lipoprotein, but the evidence is, of course, very indirect.

Heating spleen cells at 56°C or above destroys their ability subsequently to adsorb cytophilic antibody and if cells already treated with antiserum are so heated active cytophilic antibody is eluted (Boyden and Sorkin, 1961). The substance eluted in this way has a ratio of cytophilic to precipitating activity at least 100 times greater than in the original serum.

The function (if any) of cytophilic antibody has yet to be discovered. This question is discussed by Boyden (1963) and by Sorkin (1963). It has been shown to be different from the antibodies which cause anaphylactic sensitization and it does not sensitize skin. Although it is adsorbed by rat mast cells it does not sensitize them. There is no evidence for its participation in delayed hypersensitivity, autoimmune, or graft rejection phenomena though the possibility is not ruled out.

Other speculations are discussed by Boyden (1963).

Rose and Brown (1962) found that the cytophilic antibody content of various sera correlated roughly with the tanned cell haemagglutination titre, but not with the content of precipitating or of anaphylactic antibody. They also found that the uptake of cytophilic antibody onto cells was greatly increased in media of low ionic strength.

Studies using labelled antibodies. The measurement of adsorption using labelled antibodies has been criticized by Boyden (1964) on the grounds that adsorption of the label may not imply adsorption of specific antibody (most preparations contain non-specific γ-globulin and often other proteins too), and that even if antibody is adsorbed it may not be in a physiologically active state and able to combine with antigen. Some of these objections are overcome by using labelled antigen but using labelled antigen no quantitative estimate of the amount of antibody adsorbed is possible at the moment and indeed the adsorbed molecules which combine with antigen may be denatured or partially denatured as there is no means of knowing quantitatively whether the molecules responsible for specifically adsorbing labelled antigen are showing their normal antigen binding capacity, or whether these molecules are physiologically active in the sense that their combination with antigen will result in an anaphylactic reaction (the experiments with "cytophilic antibody" reviewed in the previous section suggest that they are not active in this sense).

The uptake of ^{131}I labelled antibody was studied by Humphrey and Mota (1959). They found that the rates of uptake of rat, rabbit and horse γ-globulin onto guinea pig mesentery in vitro were substantially the same, whether the tissue was at 37° or 10°C and whether metabolic inhibitors were present or not, even though only rabbit γ-globulin was capable of sensitizing the tissue. They also found that goat and rabbit antibodies adsorbed onto guinea pig mesentery in vivo washed off at similar rates in vitro, and that mesentery which contained adsorbed rat and rabbit antibodies could both specifically take up labelled antigen to similar extents. There was thus no evidence that the failure of rat, horse and goat antibodies to sensitize guinea pig tissues is due to their not being adsorbed. The fact that the mesentery is a very thin tissue makes it unlikely that slow diffusion through the tissue might be a limiting factor in loss or uptake of γ-globulin. It is however perhaps worth noting that the antibody concentrations used in these experiments (1.5 and 3 mg/ml) were considerably higher than is needed for sensitization of the tissues when rabbit or guinea pig antibodies are used.

Nielsen, Terres and Feigen (1959) studied the adsorption onto guinea pig ileum of a partially purified ($\frac{1}{3}$ saturated ammonium sulphate) γ-globulin fraction of rabbit anti-ovalbumin labelled with ^{131}I. In guinea pig ileum diffusion would be expected to take place much more slowly than in a thin tissue such as mesentery. Nevertheless the rate of loss of γ-globulin during repeated washing seemed smaller than could be reasonably accounted for by diffusion although this point was not investigated. The relation between the concentration of antibody in solution and the amount remaining in the ileum after 3 washes with Tyrode following incubation for 1 hour with labelled antibody was stated to follow the Langmuir isotherm with saturation being reached at a concentration of 0.1 mg γ-globulin per ml. However it is quite possible to draw a curve through the points produced by linearizing transformation of the results, and replotting of the results on an arithmetic scale shows that no smooth curve can be drawn through all the points and that the evidence for the attainment of saturation (and in fact for non-linearity of any sort) depends on a single point. As no indication of experimental error was given, it is not possible to be sure of the shape of the isotherm.

Another study using the same rabbit antibody -guinea pig ileum system was made by Feigen, Nielsen and Terres (1962). It is stated in a footnote that "The terms 'adsorption' and 'equilibrium' as used in this report must be qualified in order to avoid the imputation that these experiments were rigorous in the physical chemical sense". The velocity of adsorption (measured as the amount remaining in the tissue after 4 one minute washes with Tyrode) was measured at 20°, 27° and 37°C. No difference between the rates was detectable though the experimental variability (when plotted on an arithmetic scale) appeared to be rather large. Adsorption was stated to be a first order rate process but the deviations from linearity of the appropriate linearizing transformation are considerable and the evidence for this assertion is correspondingly small. It is doubtful whether the observed rate curve could be distinguished from any other curve with continuously decreasing slope and two arbitrary parameters. No allowance was made for the possibility of diffusion-limited adsorption rate and the point was not discussed. As most of the "adsorption" was complete within 60 min. it is quite probable that diffusion influenced the rate considerably (c.f. calculation of diffusion rate in chopped lung in the results section). No effect of temperature on the rate and extent of elution of γ-globulin was demonstrable. The adsorption isotherm was also found not to depend on the temperature of uptake. It was however a different shape from that previously reported for the same system by these authors. It was said to be slightly sigmoidal, and when plotted on a double logarithmic scale probably did not deviate significantly from linearity up to a concentration of 0.273 mg γ-globulin per ml. The double logarithmic transformation will linearize the Freundlich adsorption isotherm, but since in this case the slope of the plot was, if anything, greater than unity the original figures must have represented an approximately linear adsorption isotherm over the range tested, rather than a Langmuir isotherm as reported earlier.

It was also found by Feigen et al. (1962) that when the temperature at which adsorption was performed was altered the amount of histamine release on subsequent challenge with antigen was changed even though the challenge was always performed at 37°C. (Preincubation of actively sensitized tissues at different temperatures, in contrast, had little effect on the amount of histamine subsequently released at 37°C). When the amount of antibody adsorbed (at 20°, 27° and 37°C) was plotted against histamine release (at 37°C) the curves were somewhat sigmoidal and were found to be linearized by the logistic transformation:

$$\log X = \log k + 1/n \log \left(\frac{y}{1-y}\right),$$

where X is adsorption (mg/g dry weight), y the histamine release (as proportion of the estimated maximum histamine release) and k and n are estimates of arbitrary parameters. It can be seen that k is the amount of antibody bound at equilibrium when the histamine release is 50% of its maximum value. It was shown that the three points obtained by plotting -log k against 1/T were more or less linear and from this graph it was stated that the relation "satisfied the Arrhenius function in which the slope is a measure of the average energy of activation" and that "the average activation energy for the 50%

reaction between the antibodies and their combining sites was found to be 18.1 kcal". The latter statement would appear to be meaningless since it is obviously not possible to have an activation energy for a "50% reaction" and in any case the derivation of the energy is clearly theoretically incorrect since the Arrhenius equation can be applied only to the variation of rate constants with temperature. There is an interesting history of the misapplication of this equation to all sorts of rates other than rate constants. For example it is not uncommon for the reciprocal of the time to reach some arbitrary end point to be used as a measure of rate but this is of course not a rate constant and cannot be expected to give meaningful values for activation energies even if the Arrhenius plot is linear. It appears that deviations from linearity are often rationalized as two phase processes in which the "activation energy" changes when a certain temperature is exceeded. Crozier (1924 and many other papers) has attempted to calculate in this way "activation energies" from the variation with temperature of the rate of creeping of ants and diplopods, the rate of chirping of tree crickets and the frequency of flashing of fireflies. However the results of Feigen et al. (1962) appear to be even further from the theoretical basis of the Arrhenius equation since the values of k they use are not measures of rate of any sort. Since they derive from equilibrium studies their variation with temperature should, if anything, reflect the enthalpy of reaction ($\triangle$ H) of antibody with specific sensitizing sites. A plausible working hypothesis to explain the results obtained would be that $\triangle$ H was different for the combination of antibody with those sites which lead to histamine release on addition of antigen and for "non-specific" sites which do not lead to histamine release.

Halpern, Liacopoulos, Liacopoulos-Briot, Binaghi and Van Neer (1959) found a similar effect of temperature. When the adsorption of rabbit antibody onto guinea pig ileum was carried out at various temperatures followed by challenge with antigen at 38°C it was found that a longer time (or higher antibody concentration) was required to reach a constant level of sensitization (Schultz-Dale response) when the adsorption was carried out at low temperatures than at high temperatures. It was also found the concentration of antibody (c) was inversely proportional to the square of the incubation time (t) when the response (submaximal) was kept constant, over a very large (16384-fold) range of antibody concentrations. Inspection of Table 1 in Halpern et al. (1959) shows that the proportionality constant is 1, i.e. c (μgN/ml) is actually equal to $1/t^2$ (with t in hours) and that for 8 of the 9 pairs of figures given the equality is exact. No indication of sampling error is given. The temperature coefficient (Q_{10}) of the 'sensitization process' was also calculated. As might be expected this was not a constant and perhaps results on temperature dependence can only be usefully interpreted when they allow calculation of the activation energy or enthalpy of reaction. Bayliss (1959) comments 'The attempts that have been made to segregate physiological processes into those primarily of chemical origin, and those primarily of physical origin, on the basis of the temperature coefficient alone, are mostly quite unreliable'.

Liacopoulos, Liacopoulos-Briot, Binaghi and Perramant (1961) found after passive sensitization (in vivo or in vitro) guinea pig ileum (with partially purified (18% sodium sulphate) rabbit anti-ovalbumin γ-globulin labelled with ^{131}I) that washing for 4 hours at 38°C produced little loss of sensitization though 60 or 70% of the original content of radioactivity of the gut was lost. Further washing for 24 or 48 hours at 5°C caused some further loss of antibody and some loss of sensitization. Although it is stated that ileum treated in this way rapidly recovers its histamine sensitivity and anaphylactic response, it seems probable that a precise quantitative study would be needed to ensure that the loss of sensitization, which was not large even at 48 hours, was not due to changes in the tissue brought about by long storage in vitro. It was also found that labelled bovine serum albumin was taken up by, and eluted from, ileum in an exactly similar way to rabbit γ-globulin. No allowance was made for the possible effects of diffusion through the tissue.

Brocklehurst, Humphrey and Perry (1961) also used ^{131}I labelled antibody to measure adsorption during passive sensitization in vitro. Perfused chopped guinea pig lung tissue was used. The adsorption isotherm was determined by exposing lung tissue to labelled antibody for 30 min. at 0-4°C and then washing. This is probably not long enough for equilibrium to be reached. The isotherm was linear when plotted on a double logarithmic scale so the results fitted the Freundlich equation. The slope of the isotherm was about 0.84 - not far from unity which would be the value if the original results followed a linear isotherm. In fact replotting the results on an arithmetic scale shows that the first five points (up to an antibody concentration of 0.1 mg/ml) are in fact linear, the evidence for non-linearity resting on the sixth point (0.5 mg/ml), though unless the sampling error of this point was rather large it seems likely that the isotherm had deviated significantly from linearity at this concentration. It was found that the rates and extents of adsorption were different at 37°C and 0°C. This appears to disagree with the results of Feigen et al. (1962) but this might be attributed to the fact that different tissues were used, or the disguising of a rather small effect in Feigen's experiments by sampling error (the lowest temperature used was 20°C). The results of Brocklehurst et al. (1961) agree with the finding of Feigen et al. (1962) that the amount of histamine released at 37°C depends on the temperature at which antibody was adsorbed even when the amount adsorbed was constant. Sensitization was maximal long before adsorption was complete but the antibody concentrations used (e.g. 400 µg/ml) were much higher than was needed for sensitization, as the authors pointed out. The results also show that the amount of histamine released depends on the ratio of antigen added to antibody adsorbed which complicates any quantitative interpretation of experiments in which a constant amount of antigen is added to tissue containing varying amounts of antibody. Attempts to find a γ-globulin fraction responsible for sensitization showed no significant differences in the adsorption of 6 DEAE chromatographic fractions of rabbit anti-ovalbumin and, with possibly one exception, no difference in their sensitizing abilities.

The work of Ovary and Karush (1961) has shown that although neither the part of the rabbit γ-globulin molecule containing the antibody combining sites (Porter's papain fragments I and II) nor the crystallizable part (Porter's papain fragment III) could sensitize guinea pig skin for PCA reactions, it was found that fragment III, but not I and II, could sensitize the guinea pig skin for reversed PCA using horse anti-rabbit γ-globulin as antibody. The failure of fragments I and II to produce reversed PCA was not attributable to lack of antigenic determinants since this fraction produced a precipitation line with the horse anti-rabbit γ-globulin serum in gel diffusion experiment. Evidence was cited indicating that fragments I and II, although univalent could still combine with antibody so as to produce skin reactions, since these fragments from rabbit anti-human γ-globulin sera can elicit reversed PCA reactions in guinea pig skin sensitized with human γ-globulin. It was therefore concluded that fragments I and II could not "fix" onto guinea pig skin, whereas fragment III could. The relation between "fixation" and adsorption is considered further in the discussion.

The experiments summarized in this section seem to provide no evidence for any specificity in the adsorption of γ-globulin as measured with labelled antibody during passive sensitization and there seems to be little evidence concerning even the adsorption isotherm. The available results suggest, if anything, that the isotherm is linear at least up to a γ-globulin concentration of 0.2 or 0.3 mg/ml. It seemed possible that new information might be obtained if adsorption measurements were made (a) over the low range of antibody concentrations in which sensitization is submaximal, (b) with some attempt to allow for the influence of diffusion through the tissue on the rate of adsorption and elution (c) using a method which would measure adsorption without washing off adsorbed antibody or leaving non-adsorbed antibody in the tissues and (d) using homologous (i.e. guinea pig) rather than heterologous γ-globulins.

REVIEW

Part II

Guinea pig γ-globulins

In 1962 Ovary and Benacerraf reported briefly that guinea pig antisera prepared against haptenic antigens contained two populations of precipitating antibody molecules and that only the electrophoretically fast component, when eluted from agar gel, could sensitize guinea pig skin. When the fast and slow components were separated by preparative electrophoresis on starch blocks it was found that both had sedimentation coefficients of about 7S and both precipitated with antigen. It was also reported that both proteins could fix complement, and that the two components gave a reaction of identity on Ouchterlony plate testing, but these findings were not confirmed in the later reports.

At the same time Yagi and Maier (1962) also published a preliminary report of the finding of two anti-insulin antibodies in guinea pig antisera, the two being separable by DEAE cellulose chromatography and visible by autoradiography of immunoelectrophoretic patterns developed with ^{131}I insulin.

Further details of the two guinea pig anti-insulin antibodies were given by Yagi, Maier and Pressman (1962 a and b). It was found that the two peaks of antibody activity eluted from DEAE cellulose, when rerun on DEAE cellulose columns produced single peaks. It is, however, shown later that this is not an adequate criterion for purity. The binding constants of the two antibodies for ^{131}I insulin were found to be very similar as were their sedimentation rates in sucrose density gradients, both being probably in the light (about 7S) class. Starch block electrophoresis showed that the 2nd peak from DEAE cellulose columns was of faster electrophoretic mobility than the first. Immunoelectrophoresis developed with anti-guinea pig serum plus ^{131}I insulin, followed by staining or autoradiography, showed that faster component corresponded with a faint spur of β_2 mobility in normal serum. Absorbtion experiments showed that the two antibodies had antigenic determinants in common and that peak II antibody had some determinants not present on peak I, but no evidence was obtained for the presence of antigenic determinants unique to peak I.

White, Jenkins and Wilkinson (1963) found that when guinea pigs were immunized in one foot pad with 2 mg ovalbumin in Freund's adjuvant containing human type M. tuberculosis, two distinct populations of anti-ovalbumin molecules were found when the immunoelectrophoretogram was developed with ovalbumin. But when the mycobacteria were omitted only the electrophoretically faster sort of precipitating antibody appeared. It was found that peptidoglycolipids from 4 strains of human type M. tuberculosis were effective in producing slow as well as fast precipitating antibody but bovine and avian types were inactive in this respect although all these mycobacteria increased antibody levels. Density gradient centrifugation showed that both antibodies appeared in the same fraction as labelled 7S human γ-globulin (which was used as an internal marker), and were therefore both about 7S. Serum was fractionated on DEAE cellulose columns and two separate antibody peaks appeared, the first to appear containing the electrophoretically slow γ-globulin. Antibody concentrations in the fractions were estimated in weight units from the ratio of the haemagglutination titres of the fraction and of whole serum, and the known precipitating antibody content of the whole serum. There would appear to be some difficulty in the quantitative interpretation of these results owing to uncertainty concerning the nature of antibodies measured by the haemagglutination method (see, for example, Sehon, 1962 and Rose and Brown, 1962). It was found that the antibody in the second peak to be eluted from the DEAE cellulose columns was at least 25 times as active in sensitizing guinea pig skin as that in the first peak. As mentioned earlier, evidence was found that only the skin sensitizing antibody was adsorbed onto the mast cells in frozen, methanol-fixed sections of mouse tongue.

In a preliminary communication Goodman, Robbins and Exum (1963) reported that when guinea-pig antisera against thyroid extract were examined by the methods of Yagi et al. (1962a) two populations of antibodies were present. It was also found that a third component corresponding to β_{2M}-globulin was present but the criteria for its identification were not given.

Benacerraf, Ovary, Bloch and Franklin (1963) immunized guinea pigs with haptenic antigens in complete Freund's adjuvant containing M. butyricum. Immunoelectrophoretic analysis showed two populations of antibodies as seen by White et al. (1963). When no adjuvant was used only the electrophoretically fast antibody was seen. Antibody was purified by dissociation of precipitated antibody-antigen complexes, precipitation of antigen and removal by dialysis of as much as possible of the hapten used for dissociation. The product was mostly precipitating antibody combined with some residual hapten. The total precipitating antibody was separated into populations of fast and slow mobility by preparative starch block electrophoresis, though there was some overlap between the fractions. The total precipitating antibody showed two antigenically distinct components using a rabbit anti-guinea pig serum, but sometimes extra lines were seen in the fast and slow antibody regions. These could not be interpreted. The patterns observed in these experiments are commented on in the discussion. Ultracentrifugation showed that both antibodies belonged to the 7S class.

Ovary, Benacerraf and Bloch (1963) confirmed that only the fast precipitating antibody eluted from agar electrophoresis experiments could sensitize guinea pig skin for PCA. This was found also to be the case with fast and slow precipitating antibodies purified according to Benacerraf et al. (1963), and intravenous passive sensitization experiments showed that "As for PCA, the ability to mediate systemic anaphylaxis is an exclusive property of the fast migrating antibody fraction". These experiments were really qualitative, since the two antibodies were given in equal doses. Inhibition experiments showed that 0.6 μg of fast antibody would produce passive cutaneous anaphylaxis, and that no PCA response was produced by 69 μg of slow antibody. When fast and slow antibodies were mixed in these doses no PCA sensitization resulted. However if the slow antibody was directed against an antigen different from that which the fast antibody was prepared with, no inhibition of PCA resulted although fast antibody from one system would non-specifically inhibit PCA sensitization by fast antibody antibody from another. From these observations it was inferred that only the fast antibody could "fix" (in the immunological sense) to guinea pig tissues, and that inhibition of sensitization by slow antibody took place only by competition for antigen. It also shows that, in the experiments illustrated, the potency ratio between fast and slow antibodies was greater than 100, i.e. rather larger than was observed by White et al. (1963).

Bloch, Kourilsky, Ovary and Benacerraf (1963) found that when specifically precipitated guinea pig anti-hapten antibodies were separated on starch block as above, that complement fixation and sensitization of antigen coated tanned erythrocytes for passive immune haemolysis (in the presence of complement) were properties of only the γ_2 (slow) antibody, whereas both γ_1 and γ_2 antibodies would produce passive haemagglutination. Similar distribution of skin sensitizing and complement fixing activities was found in anti-ovalbumin antibodies prepared from ammonium sulphate precipitated globulins (which would therefore contain non-specific γ-globulins and possibly non-precipitating antibodies in addition to precipitating antibodies). It was also shown that the γ_2 anti-ovalbumin antibodies were more active than the γ_1 antibodies (per unit weight of precipitating antibody) in producing reversed passive Arthus reactions in guinea pig skin. This is interesting in connection with the possibility that complement may play a role in the Arthus reaction (see, for example Siqueira and Nelson, 1961; Boyden, 1962). However, although the number of animals used in the published results was rather small, it appears that the difference between γ_1 and γ_2-globulins in this respect may be quantitative rather than qualitative. No estimate of their potency ratio was made. In the rat both γ_1- and γ_2-globulin antibodies were found to be able to provoke PCA.

Kourilsky, Bloch, Benacerraf and Ovary (1963) found that the passive lysis of antigen coated erythrocytes by γ_2-globulin antibody in the presence of complement was inhibited by prior or simultaneous incubation with γ_1-globulin antibody against the same antigen, when the γ_1-globulin was about 50 times as concentrated as the γ_2-globulin. Normal γ_1-globulin and γ_1-globulin antibody directed against a different antigen were ineffective, so the mechanism of inhibition was inferred to be one of competition for antigen.

If the structure and functions of guinea pig γ-globulins are like those of rabbit and human γ-globulins (see, for example, Fleischman, Porter and Press, 1963; Porter, 1963; Porter and Press 1962; Taranta, Franklin and Ovary, 1962; Ishizaka, Ishizaka and Sugahara 1962) the results reviewed above would suggest that guinea pig γ_1- and γ_2-globulins have similar papain digest fragments I and II (Porter, 1959) but differ in the structure of fragment III. This interpretation was supported by the study of Thorbecke, Benacerraf and Ovary (1963) who found that γ_2-globulin had antigenic determinants not possessed by γ_1-globulin and that when γ_1- and γ_2-globulins were tested by the Ouchterlony method, using a rabbit anti guinea pig γ-globulin serum which had been precipitated with the S fragments (equivalent to Porter's fragments I and II) of papain-digested guinea pig γ_2-globulin, they no longer cross reacted with each other, or with S fragment, implying that the antigenic determinants responsible for the antigenic difference between the two globulins are present in fragment III (or F). This inference is consistent with the findings that the antigenic differences between 7S γ-globulin and γ_{1A}-globulin in man (Franklin and Stanworth, 1961) and mouse (Fahey, 1963) are in fragment F (or III). Thorbecke et al. (1963) also observed a third line of intermediate γ-mobility when certain guinea pig antibodies were tested immunoelectrophoretically with rabbit anti guinea pig γ-globulin serum. This phenomenon could not be explained.

The structure of guinea pig γ-globulin was investigated by Edelman, Benacerraf, Ovary and Poulik (1961) and Edelman, Benacerraf and Ovary (1963). Antibody γ-globulin (prepared by specific precipitation followed by dissociation with hapten) was reduced with mercaptoethanol in 8M urea and then alkylated with iodoacetamide. The products of this process are denatured, insoluble and without biological activity. When this material was examined by starch gel-urea-formate electrophoresis a slow band common to all types of γ-globulin was seen. This was called the H chain. The smaller molecular weight reduction product, the L chain, appeared heterogeneous on starch gel electrophoresis and the pattern of bands produced by the L chains was found to be characteristic of the haptenic antigen used (but not much influenced by the nature of the carrier protein bearing the hapten). Cross reacting antigens produced similar L chain patterns. Since the L chains are probably entirely within papain fragments I and II (Olins and Edelman, 1962; Fleischman, Pain and Porter 1962; Cohen, 1963) which contain the antibody combining sites, it was suggested that the L chain contained, wholly or partly, the antibody combining site (Edelman et al. 1961), or that specificity is a result of L chain -H chain interaction (Edelman et al. 1963).

Fleischman, Pain and Porter (1962) and Fleischman, Porter and Press (1963) showed that reduction of γ-globulin in aqueous solution followed by fractionation by gel filtration in acetic or propionic acid solution produced polypeptide chains, of M.W. about 105,000 and 40,000 (both probably dimers), which they called the A and B chains. These are probably the same as the H and L chains of Edelman et al. (1961), but are not denatured and retain biological activity. However the only antibody activity (detected by production of time lag in the precipitation of homologous antigen and antibody, by polarization of fluorescence, and by specific inhibition of precipitation in a flocculating antigen-antibody system) found by Fleischman et al. (1963) was present in the A (or H) chains of horse and rabbit γ-globulins. No trace of antibody activity was detectable in the B (or L) chains. Also the banding of the L chain seen by Edelman, Benacerraf, Ovary and Poulik (1961) on starch gel-urea electrophoresis with chains from antibody but not with chains from "non-antibody" guinea pig γ-globulin, was not observed by Fleischman et al. (1963) with chain B (except at high pH when chain B from all sorts of horse and rabbit γ-globulin produced a banded pattern). Although their evidence suggested that chain B was not concerned in the antibody combining site, no reason for the complexity of chain B has been found. Dependence of the mobility of whole γ_1- and γ_2-globulin antibodies on specificity has also been observed, the mobility of the whole proteins being correlated with that of their S fragments (Nussenzweig and Benacerraf, 1964).

It seems that the immunoglobulins of the guinea pig are in many ways analagous with those of other mammalian species in which two different 7S γ-globulins are found. The first example of the production of two sorts of antibody γ-globulins to be discovered was that of horse antitoxins. During immunization γ_2-globulin antitoxin appeared first but later increasing amounts of antitoxin of γ_1 mobility (T component) were formed (van der Scheer, Wyckoff and Clarke, 1941; Kekwick and Record, 1941; Relyveld, 1959). It is now well established that in man (Heremans, 1960) and in the mouse (Fahey, 1961) there are two groups of 7S γ-globulins differing in electrophoretic mobility and antigenic structure, the γ_2-globulins and the γ_{1A}- (or β_{2A}-) globulins. In both of these species the antigenic difference between the two proteins has been found to be in the F fragment (or fragment III) (Franklin and Stanworth, 1961; Fahey, 1963), and hence presumably in the A (or H) chain. It is fragment III of rabbit γ-globulin which appears to be involved in "fixation" (in the immunological sense) to skin (Ovary and Karush, 1961); and the F fragment of human γ-globulin and fragment III of rabbit γ-globulin which is probably responsible for complement fixation (i.e. contains Ehrlich's complementophilic haptophore group) (Taranta, Franklin and Ovary (1962).

The analogy between guinea pig and human γ-globulins is carried further by the recent discovery that, in man, reaginic antibodies appear to be γ_{1A}-globulins (see Vaerman, Epstein, Fudenberg and Ishizaka, 1964, and the earlier work cited therein).

MATERIALS AND METHODS

ANTIGENS

Crystalline bovine serum albumin (BSA) (Armour Pharmaceutical Co. Ltd. Eastbourne) and Ovalbumin (OA) (5 x crystallized Mann Research Laboratories Inc., New York) were used throughout.

<u>Aluminium hydroxide adsorbed antigens.</u>
10 ml of 5% protein solution was mixed with 4.6 ml 1 M sodium bicarbonate and 10% potassium aluminium sulphate added dropwise with constant stirring until pH 6.8 (Pye direct reading metre). After leaving overnight in the refrigerator the precipitate was centrifuged down and washed with distilled water and resuspended in 10 ml of 0.154 M saline. The protein concentration of the suspension was measured by adding acid until the precipitate dissolved and measuring the optical density at 280 mμ.

PREPARATION OF ANTISERA

(a) Rabbit Anti-BSA sera

Rabbits were injected intramuscularly with 10 mg BSA in 0.5 or 1.0 ml of Freund's adjuvant of the following composition (hereafter referred to as Weybridge type Freund's adjuvant):

Protein solution in 0.154 M saline	4 vol.
Light liquid paraffin B.P. + mycobacteria	4 vol.
Grill K-16 (sorbitan mono oleate Croda Ltd., Goole, Yorks)	1 vol.

The light liquid paraffin contained 4 mg/ml of heat killed mycobacteria (M. tuberculosis human type. Strains C, DT and PN mixed Central Veterinary Laboratory, Weybridge, Surrey). After 1 week the same dose was given I.M. in a different site and after another week subcutaneously. After a 6 week interval booster doses of 1, 2, 2 and 4 mg of aluminium hydroxide adsorbed BSA were given intravenously at 2 day intervals. The animals were bled one week after the last injection.

(b) <u>Rabbit anti- (guinea pig serum) sera</u>

R 87-90 and R 94-97. Two or four doses of 1 ml normal guinea pig serum (NGPS) in Freund adjuvant of the composition given above, were given I.M. and S.C. at weekly intervals. After 1 month 5 doses of aluminium hydroxide adsorbed NGPS were given intravenously at intervals of a few days, and the rabbits bled 1 week after the last dose.

<u>R 104 and R 105</u> were made by injecting immune guinea pig serum (IGPS) in Difco complete Freund adjuvant (Baird and Tatlock Ltd., 124 Great Ancoat Street, Manchester), which has the following composition:

Protein solution in 0.154 M saline	10 ml
Arlacel A (mannide monooleate)	1.5 ml
Bayol F (paraffin oil)	8.5 ml
Mycobacterium butyricum (dried and killed)	5 mg

Six doses of 0.1 ml emulsion were given initially (four intramuscularly and two subcutaneously and after 2 or 3 weeks 5 doses of 0.1 ml IGPS diluted in about 1 ml of 0.154 M saline were given by slow intravneous injection at two day intervals and the animals bled 1 week after the last dose.

(c) Rabbit anti- (guinea pig γ-globulins)

For the preparation of rabbit anti-(guinea pig γ_2-globulin) sera, R 100 and R 101, guinea pig γ_2-globulin isolated directly from whole serum on a DEAE cellulose column was used. Although little contamination was visible on immunoelectrophoresis, these antisera contained antibodies to several β globulins, as shown in the results section. Rabbit anti -(guinea pig γ_2-globulin) sera R 106, R 107 and R 108 were made against guinea pig γ_2-globulin prepared by the two stage process to be described later.

The immunization schedules were:

R 100 and R 101. 1.5 mg of guinea pig γ_2-globulin emulsified in Difco complete Freund's adjuvant was injected in 6 x 0.1 ml doses (four I.M. and two S.C.). After two weeks six injections of 1.0 mg of γ_2-globulin in 0.154M saline were given intravenously at weekly or half weekly intervals and the animals bled periodically.

R 106, R 107 and R 108. 2 to 4 mg of guinea pig γ_2-globulin emulsified in Difco complete Freund's adjuvant was given initially in

2 x 0.5 ml doses I.M. After 5 weeks a similar dose was given in Difco incomplete Freund's adjuvant and after another three weeks 1 mg guinea pig γ_2-globulin in 0.154 M saline was given S.C. followed, after one week by 5 x 1 mg doses in saline I.V. at two day intervals. The rabbits were bled 1 week after the last dose.

Rabbit anti- (guinea pig γ_1-globulin) sera were prepared using guinea pig γ_1-globulin obtained by fractionating on a DEAE cellulose column the slower part of the total γ-globulin separated by preparative electrophoresis (see below). This fraction was contaminated with γ_2-globulin but not with β-globulins and antisera against it contained no anti-β-globulins (see results section) . The immunization schedules were:

<u>R 98 and R 99</u>. The initial dose was 1.0 mg guinea pig γ_1-globulin in Difco complete Freund's adjuvant (6 x 0.1 ml, four I.M. and two S.C.). After two weeks 1.0 mg guinea pig γ_1-globulin in 0.154M saline was given S.C. and after another week six 1.0 mg doses in 0.154 M

saline were given by slow I.V. injection at intervals of 3 to 6 days and the animals bled one week after the last dose.

R 102 and R 103. These rabbits were given guinea pig γ_1-globulin according to the same schedule as described for R 100 and R101.

(c) Guinea pig anti-ovalbumin sera

Batches GP 16 - 18 and GP 19 - 21 were given three injections of 5 mg OA in 0.5 ml complete Freund's adjuvant, of the Weybridge type, at weekly intervals, the first two being I.M., the third S.C. After 6 weeks aluminium hydroxide adsorbed ovalbumin was given I.P. every two days for 4 doses, increasing from 0.2 ml to 1.0 ml. The animals were bled one week after the last injection.

Batch 62/A was prepared by immunization over a long period. 100 mg crude OA in 0.154 M saline S.C. and I.P. was given initially, then 0.5 mg OA in 0.154 M saline S.C. after two weeks, and 10 mg OA in incomplete Freund's adjuvant six weeks later. After 8 months a course of 9 doses of aluminium hydroxide adsorbed OA, 0.2 ml increasing to 1.0 ml, was given I.P. over six weeks, and the animals bled after one more week.

Batches 22, 23 and 25 were immunized accordingly to a schedule similar to that used by Benacerraf et al. (1963). The initial dose was 2 mg OA in Difco complete Freund's adjuvant (0.5mg in 0.1 ml emulsion was injected into each foot pad). This represents a dose of 100µg M. butyricum per animal. (White et al. 1963, have shown that the type of Mycobacterium used influences the sorts of antibody produced.) After two weeks, repeated doses of OA (0.1 mg in 0.1 ml of 0.154 M saline) were given intradermally at weekly intervals until Arthus reactions developed. The animals were bled one week after the last injection.

Batch 26 were immunized according to a similar schedule except the Freund's adjuvant used contained M. tuberculosis human type H 37 Rv (streptomycin sensitive, formalin killed), in a base of the same composition as used for Weybridge type Freund's adjuvant, in such a concentration that the dose of mycobacteria was 400 µg dry weight per animal. The M. tuberculosis H 37 Rv were kindly supplied by Dr. A. Wallace, City Hospital, Edinburgh.

Batch 27, was prepared in the same way as batch 26, except that Weybridge type Freund's adjuvant was used with mycobacteria in such a concentration that each animal received 400 µg dry weight.

(d) Miscellaneous antisera

Various commercially available antisera were used. These are referred to in the results section. Goat anti- (rabbit serum) and anti- (rabbit γ-globulin) sera were kindly supplied by Dr. J.H. Humphrey (National Institute for Medical Research, Mill Hill, London N.W.7.)

Concentration of Protein Solutions

Large volumes of protein solutions were concentrated by pressure dialysis. Smaller volumes were concentrated osmotically. In early experiments a dialysis sac containing the solution to be concentrated was surrounded by polyethyleneglycol (M.W. 6000, or M.W. 20,000, Lights and Co. Ltd., Colnbrook). In all later experiments a highly substituted carboxymethyl cellulose preparation ("Biodryex") was used instead of polyethylene glycol as this has been shown not to penetrate dialysis tubing (Palmstierna 1960).

Microlitre quantities were concentrated by mixing with a small amount of Sephadex G25 in a small centrifuge tube and collecting the supernatant after allowing the Sephadex to swell.

Table 1

Some values of the concentration (c_1 ,mg/ml) of protein solutions with an optical density of 1.0 at 280 mμ with 1 cm path length.

sal. = saline, glob. = globulin, alb. = albumin

Protein	Solvent	c_1	Reference
Rabbit γ-globulin	0.154M sal.	0.76*	J.H. Humphrey (Personal Comm.)
Rabbit γ-globulin	0.1 M NaOH	0.715	
Rabbit γ-globulin	0.154M sal.	0.72	Colquhoun (single determination)
Rabbit γ-globulin	0.1 M NaOH	0.67	
Rabbit γ-globulin	0.1 M NaOH	0.62+	Eisen, 1948
G.Pig γ_1 and γ_2-globulin	0.154M sal.	0.68 +	Colquhoun (unpub.)
Human γ-globulin	Water	0.65	Gitlin, 1949
Human γ-globulin	0.1 MNaOH	0.62	
Human γ-glob.(II-1,2)	0.15 M sal.pH7.0	0.68	Smith and Coy 1946
Human γ-glob.(II-3)	0.15 M sal.pH7.0	0.67	
Human γ-glob.	not specified	0.69	Gurd. Cited by Isliker and Antoniades, 1955
Horse γ-globulin	0.15 M sal.pH7.0	0.73	Smith and Coy, 1946
Horse T-globulin	0.15 M sal.pH7.0	0.76	
Bovine γ-globulin	0.15 M sal.pH7.0	0.77	
Bovine T-globulin	0.15 M sal.pH7.0	0.99	
Human serum alb.	0.1 M NaOH	1.63	Gitlin, 1949
Human serum alb.	Water?	1.89	Cohn et al.,1947
Bovine serum alb.	Water?	1.5.	Cohn et al., 1947
Bovine serum alb.	Not specified	1.50‡	Sogami and Foster, 1962
Bovine serum alb.	Water	1.50‡	Foster and Sterman, 1956
Bovine serum alb.	0.154 M sal.	1.54*	Colquhoun (unpub.)
Bovine serum alb.	0.1 M NaOH	1.35*	
Ovalbumin	0.154 M sal.	1.32*	Colquhoun (unpub)
Ovalbumin	0.1 M NaOH	1.36*	Colquhoun (unpub)

* Values used routinely + assuming 16% N ‡ 279 mμ

ANALYTICAL METHODS

(1) PROTEIN CONCENTRATION

Optical density at 280 mμ was used for many routine determinations of protein concentration. Table 1 shows various values of the concentration of protein producing an optical density of 1.0. The values marked * were used in all calculations. Confidence limits for the slope of the optical density against concentration curves are not given in the table as they are only indirectly related to the error in inferring a value of the independent variable (concentration) from a reading of the dependent variable as is done when the curve is used in practice, and anyway systematic errors are likely to be at least as important as random errors.

Kabat (1961) gives references on nitrogen content, optical density, optical rotation, refractive index increment etc. of various proteins.

Folin method. Proteins were assayed by the modification of the Folin method described by Lowry et. al. (1951) using rabbit γ-globulin (Pentex Inc., Kankakee, Illinois) as standard (see results section for justification of this procedure in the case of guinea pig γ_1- and γ_2-globulins). The results were corrected for the moisture content of the standard protein determined by the loss of weight on heating samples to dryness at 105°C in vacuo and cooling over phosphorus pentoxide in vacuo.

Micro Kjeldahl method. Estimations of protein nitrogen were performed on guinea pig γ_1- and γ_2-globulins using a Markham still and technique similar to that described by Kabat (1961). Standard ammonium sulphate solutions were analysed in parallel to determine the yield of ammonia.

(2) INULIN AND SUCROSE CONCENTRATIONS

Inulin and sucrose were estimated by the method of Fisher and Gilbert (unpublished). Samples of lung tissue were washed quantitatively into a ground glass homogenizer tube (Type TTH/20 Loughborough Glass Co. Ltd., Loughborough, Leicestershire) and homogenized for 2 minutes. The homogenate was washed quantitatively into a 10 ml volumetric flask with distilled water, protein precipitated by adding 1.0 ml of 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 inulin estimated in the supernatant . In the determinations of inulin space, standard solutions of nominal concentration 15, 30 and 45 μg/ml were made from the supernatant inulin solution which had been incubated with the lung tissue. These solutions were diluted with distilled water and metaphosphoric acid added so that the final concentration was the same as in the unknown solutions (i.e. 2%). The colour reaction was performed using a Technicon Autoanalyser. A sample cup containing distilled water was placed between each solution to be assayed and samples run at the rate of 60 per hour. The tubes used in the proportioning pump were:

Sample 0.056" internal diameter
(code colour, yellow)
reagent 0.065" internal diameter
(code colour, blue)
and 0.110" internal diameter
(code colour, purple and clear).

After mixing, the samples pass through a 98°C heating coil for 5 minutes and then through a mixing and cooling coil to the colorimeter which was fitted with 480 mμ reference and sample filters and size 3 aperture, and the transmittance recorded. The reaction is for fructose so, as expected, about twice the concentration of sucrose is required to produce the same optical density as a given concentration of inulin (on a weight basis). The reagent was made as follows:

Stock solution. Ferric chloride 0.082 g
(anhydrous)
or 0.1356 g
(hexahydrate)
Concentrated hydrochloric acid
(S.G. = 1.18) to 1 l.

600 ml of this solution is made up to one litre with distilled water containing 1.0 g resorcinol, so that the final concentration of resorcinol is 0.10%.

A method of inulin assay giving even more accurate results has recently been developed (Fisher, personal communication). The only differences from the method described above are that an air tube (code colour white and white) is included as well as the sample and reagent input tubes, and that 40, rather than 60, samples are analysed per hour.

(3) PAPER ELECTROPHORESIS

In some of the earlier experiments proteins were tested on paper electrophoresis using Whatman 3MM paper strips and barbitone buffer as described, for example by Smith (1960).

(4) MICROIMMUNOELECTROPHORESIS (MIE)

Immunoelectrophoresis was performed on microscope slides as described by Scheidegger (1955) using 1% Ionagar No 2 (Oxoid Ltd.) in 0.05 M pH 8.6 barbitone - acetate buffer. The results were photographed (on Ilford N-40 plates with D-163 developer) using dark ground illumination, at various times after the addition of the antisera. Sometimes the slides were stained also. They were washed in saline, then water and dried, as described by Heremans (1960), then stained for 10 minutes in 0.5% thiazine red (c.f. Crowle 1958) which is better for photography than Amido-black. After washing with 1% acetic acid in equal volumes of methanol and water, and then with running water, the slides were immersed for 10 minutes in 1% aqueous glycerol and then dried at 37°C.

(5) GEL DOUBLE DIFFUSION

Experiments were performed on microscope slides as described by Hartmann and Toilliez (1957) in an agar gel of the same composition as used for MIE. The peripheral wells had a capacity of about 4 µl and the central well either 4 µl or 16 µl. The results were photographed in the same way as MIE.

(6) QUANTITATIVE MEASUREMENTS OF SPECIFIC PROTEINS

Preliminary measurements were made using a method similar to that described by Darcy (1960), but using microscope slides as described above. The 4 µl volumes of solution were measured using an Agla micrometer syringe (Burroughs Wellcome and Co., London). The precision of the results obtained should be capable of improvements and attempts to do this are in progress. It may be noted that the method for calculation of the confidence limits of the estimated concentration given by Darcy (1960) is incorrect.

(7) QUANTITATIVE PRECIPITIN REACTION

Analyses were performed by a method similar to that described by Kabat (1961). The total volume of the reaction mixture was 1.0 ml. The washed precipitates were dissolved in 0.1M sodium hydroxide and their protein content estimated spectrophotometrically at 280 mµ.

(8) HAEMAGGLUTINATION TESTS

The approximate relative antibody content of isolated guinea pig γ-globulin fractions was estimated by the ability of two fold serial dilutions of the fractions to agglutinate ovalbumin coated tanned formalinized sheep erythrocytes. The cells were formalinized by the method of Csizmas (1960) and coated with ovalbumin using a method described by W.J. Herbert (personal communication. To be described in the tenth edition of Mackie and McCartney, Handbook of Practical Bacteriology Ed. R. Cruickshank). I am grateful to Mr. W.J. Herbert for several batches of cells.

STORAGE OF MATERIALS

Sera were shell frozen using acetone-solid carbon dioxide mixture and either freeze dried or stored in a deep freeze.

Purified γ-globulins were filtered through a cellulose acetate membrane filter (Oxoid Ltd., London) in a glass assembly (Type FD-440. Gallenkamp Ltd., Technico House, Christopher Street, London E.C.2.) into a closed glass chamber with a side arm connected, via a cotton wool filter, to a water pump, and an outlet at the bottom to which was connected a hypodermic needle. The whole filter assembly was sterilized before use by autoclaving for

30 min. at 10 lbs in $^{-2}$. After filtering the solution into the lower glass chamber it was run out through the hypodermic needle into sterilized glass ampoules which were sealed by fusion of the glass and stored at 4°C. Some comments on the efficacy of this method of storage are made in the results section.

PURIFICATION OF PROTEINS

(1) Preparative electrophoresis

Preparative electrophoresis was performed using polyvinyl chloride - polyvinyl acetate co-polymer particles (Pevikon C-870, Guest Industrials Ltd., 81 Gravechurch Street, London E.C.3) (Müller -Eberhard 1960).

After washing the particles with barbitone buffer (pH 8.6, 0.09M), a suspension in this buffer was poured into a perspex frame lined with thin polythene sheet. The block was 6.5 cm above the level of the buffer in the electrode baths. Connections were made with three thicknesses of lint. In the earlier experiments the serum sample, dialysed against barbitone buffer (pH 8.6, 0.09M), was applied directly to the block, but later it was always concentrated first. Usually 20 ml of serum were concentrated to 5-8 ml using Biodryex. After cooling the block in a cold room at 1°C the sample was applied by cutting a slot about 4 mm wide (or 10 mm for 20 ml samples) 5 to 7 cm from the cathode end of the block. The slot ended about 2 cm from each edge of the frame and a short distance from the bottom. The material removed from the slot was blotted on lint and mixed with the serum to form a slurry which was poured slowly and gently back into the slot. After smoothing the surface with a palette knife it was covered with polythene and electrophoresis was performed using a potential gradient of 3.5 to 4.5 V cm^{-1}.

For separation of total γ- globulins a block 36 x 19 x 1.5 cm was used with a load of up to 20 ml of serum and electrophoresis continued until the β-globulins approached the anode end. Haemoglobin is a convenient marker for the β-globulin region. If all serum components were required a larger block, 50 x 24.5 x 1.5 cm, was used. When the ambient temperature in the cold room was 1°C, the steady state temperature in the smaller block was about 11°C. At the end of the run the block was cut into 1 cm wide sections each of which was suspended in 10 ml of 0.154 M saline and the eluate separated on a sintered glass filter (porosity 1 or 2) leading to a container with a side arm which was attached to a water pump. The particles were resuspended and the process was repeated until 30 ml of eluate had been collected. A control experiment showed that the first three elutions removed about 96% of the total amount of protein removed by four elutions even when the total volume for the first three was rather less than 30 ml. The eluates from each section were analysed for protein concentration by the Folin method and concentrated by pressure dialysis at 1°C.

All sections cathodal to the first coloured sections were taken as "total γ-globulin". In most experiments the eluates from the cathodal part of this region, and those from the anodal part were kept separate. The most anodal $\frac{1}{3}$ or $\frac{1}{2}$ is referred to as "fast total γ-globulin", the remainder as "slow total γ-globulin". The pooled concentrated eluates were filtered through cellulose acetate membrane filters in an all glass assembly to remove very fine particles of Pevikon which were not sedimented by several hours centrifugation at 2500 g. It was often necessary to use more than one filter as they clogged rapidly.

(2) DEAE cellulose chromatography

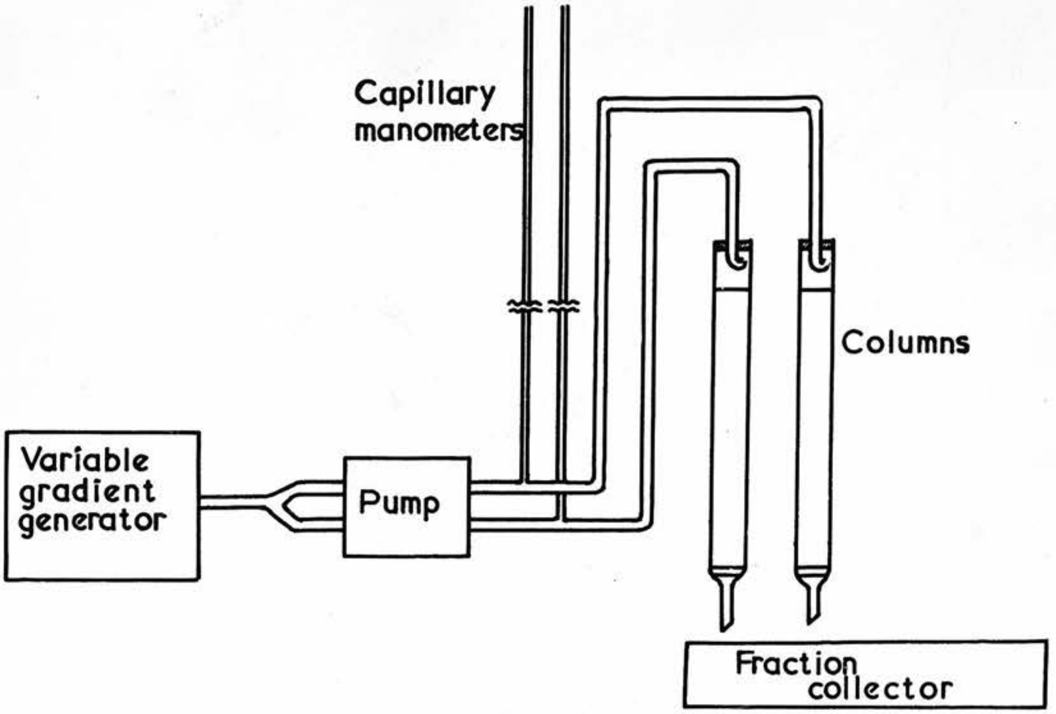
Columns were made from diethylaminoethyl (DEAE) cellulose prepared using 100-200 mesh Solka Floc cellulose according to Peterson and Sober (1956), or bought from Kodak Ltd. (Kirkby Trading Estate, Liverpool). All columns and samples were equilibrated with 0.01M pH 8.2 sodium phosphate buffer. For the preparation of γ_2-globulins up to 20 ml of equilibrated serum was applied to a column of 2.8 cm diameter and 40 to 45 cm height and elution carried out with the same buffer as was used to equilibrate the column.

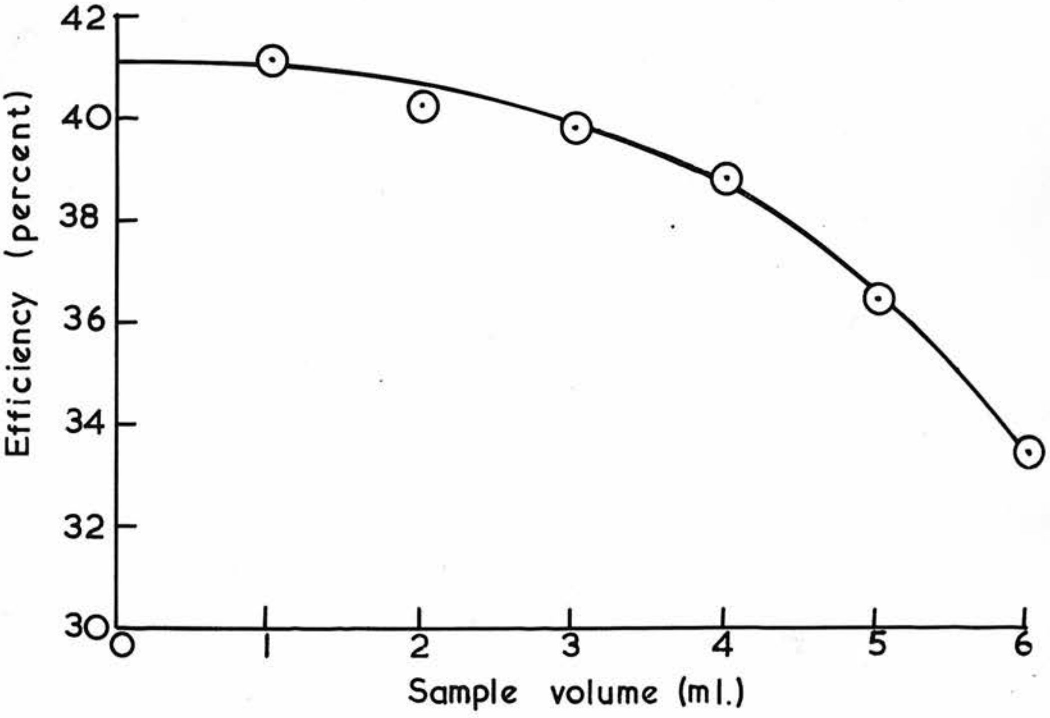
To separate the total γ-globulins obtained by preparative electrophoresis the samples were concentrated to a volume of 3 to 5 ml using Biodryex, and then dialysed against 0.01M pH 8.2 sodium phosphate. The concentrated samples were applied to columns 1.5 cm diameter and 35 to 40 cm height. Usually two or more columns were run simultaneously with different electrophoretic fractions of the total γ-globulin on each. The arrangement used is illustrated in Fig. (1). For most of the experiments the buffer flow was divided after

the manometer, but the arrangement shown, with a separate manometer for each column, was later found to be more satisfactory. After the first protein peak had been eluted, a gradient of rising ionic strength was started using a device of the sort described by Peterson and Sober (1959). The initial buffer was that used for equilibration of the sample and column, and the final buffer was the same with the addition of sodium chloride to a total concentration of 0.30M. Intermediate chambers contained molarities linearly interpolated between the initial and final molarities to make a suitable total volume of buffer.
Buffer was pumped from the gradient generator at about 40 ml/hour for each column. Fractions of about 10 ml were collected and scanned for protein concentration by measuring their optical density at 280 mμ, and an index of their electrolyte concentration obtained by measuring their conductance using a Philips Model GM 4249 conductivity bridge. Fractions were concentrated by pressure dialysis at 1°C against 0.154 M saline.

Fig. 1 Diagram of chromatography apparatus used for running two or more columns of DEAE cellulose simultaneously.

Fig. 2 Efficiency of well-type scintillation counter as function of sample volume. EHT voltage = 1200 V, discriminator voltage = 5V, dead time = 50 μsec. Sample, iodine -131.


Capillary manometers
Columns
Variable gradient generator
Pump
Fraction collector


Efficiency (percent)
42
40
38
36
34
32
30
0
1
2
3
4
5
6
Sample volume (ml.)

LABELLING OF PROTEINS

Proteins were labelled with ^{131} Iodine. Two methods were tried.

(1) Method of Francis et. al. (1951). In this method potassium iodate is added to a mixture of ^{131}I and carrier iodide to oxidise the thiosulphate in the ^{131}I solution, and the iodide to iodine which is then used to iodinate the protein. As 6×10^{-4}mM sodium thiosulphate per mc. is stated to be added to the IBS-1 and IBS-2 iodine-131 preparations (Radiochemical Centre, Amersham, Bucks.), and the rate of radiative decomposition of thiosulphate has been determined, it is possible to derive an equation for the amount of thiosulphate per mc iodine-131 present at any time (see Appendix 1 for derivation) and from this the necessary amount of iodate was calculated. A trial experiment in which little or no iodine was incorporated suggested that there was more than the calculated amount of

thiosulphate present in the iodine-131 solution and direct titration confirmed this. The reason was that in fact more than the advertised quantity of thiosulphate had been added (E.J. Partington, personal communications) which means that the (thiosulphate)/mc ^{131}I ratio rises faster than calculated (see Appendix 1). In view of this all future iodinations were done by the next method.

(2) Method of McFarlane (1958) (and Dr Freeman, personal communication). γ-globulins were iodinated with iodine monochloride -iodide -131 mixture in glycine buffer without preoxidation of -SH groups. Before radioiodinating albumins free -SH groups were oxidized with free iodine at pH 4 to 4.5 (at which tyrosine is not ionized) until a permanent colour was obtained followed by removal of iodide and excess iodine.

After radioiodination the protein was separated from unbound small molecules. An ion exchange resin was used at first, but later it was found that gel filtration was easier and more efficient for this purpose. When 3 mg of γ-globulin were iodinated in a total volume of about 2 ml satisfactory separation of non-protein bound radioactivity could usually be obtained on column of 1g Sephadex G-50 in 0.154M saline (in a Quickfit column No CR 12/10). Using this method the proportion of radioactivity not precipitable by 12% trichloracetic acid (in the presence of 10% serum to make sure the total protein concentration was high enough for most of the protein present to be precipitated) was frequently less than 1% and nearly always less than 1.5% at the start of the experiment. More details of this are given in the results section. Occasionally a larger Sephadex column had to be used to give adequate separation. A control experiment showed that iodide-131 was not significantly co-precipitated with protein by 12% TCA.

In most experiments the average degree of iodination was less than 1.0 atoms of iodine per molecule γ-globulin and it was always less than 1.5.

Because of a mistake in a duplicated instruction sheet the iodinations in the experiments to be described were done by rapid injection of the protein solution into the ^{131}ICl solution. As McFarlane (1964) points out, it is better, on theoretical grounds, to inject the ^{131}ICl solution rapidly into the protein. However it is shown in the section on adsorption of guinea pig γ-globulins that the results using the two methods of iodination are indistinguishable. Also it has been shown by Freeman (McFarlane, personal communication) that patients do not distinguish metabolically albumins labelled by the two methods. In Figs. 29 and 30 it is shown that there is no correlation of the adsorption constant with the average number of atoms of iodine per molecule of protein, or with specific activity.

The small amounts of protein to be measured necessitated the use of rather high specific activities, usually between 30 and 70 μc/mg protein. The labelled solutions were kept at 4°C and the experiment always completed within 24 hours of labelling. No protective protein was added to avoid possible interference with the adsorption of γ-globulin.

Some comments on autoirradiation damage are made later.

MEASUREMENT OF RADIOACTIVITY

The radioactivity of tissue samples was measured using a well type scintillation counter with a thallium activated sodium iodide crystal. Samples were usually transferred to a clean thin glass sample tube which had been checked for contamination before counting. It is not feasible to check the tubes for long enough to eliminate small degrees of contamination and there is little doubt that some tubes had a counting rate of 6 or even 7 c.p.s. compared with a background rate of about 5 c.p.s. Normally the counting rates in the experiment were high enough (not less than twice the background rate) for this to be insignificant but occasionally it might cause significant error.

Counting error. Apart from uncertainty of the exact background rate there is also the uncertainty due to the random nature of radioactive disintegration. This should never have resulted in a standard deviation of the net count greater than 5% and usually much less. For example if the background rate is counted for 4000 counts (it was usually more than this) and the sample for 4000 counts (sometimes only 2000 counts were taken but usually 4000 or more) then the standard deviation of the net counting rate (total minus background) would be about 4.6% when the total counting rate (sample plus background) was only twice the background rate, and about 2% when the total count was five times the background. (See, for example, Taylor 1957).

Corrections to count. The observed count rate was corrected for background count rate, loss through dead time of scaler (50 μ sec) and for isotope decay (to the nearest hour).

Efficiency of counter. The values of S^2/B, where S = net count rate (i.e. corrected for background and dead time) and B = background count rate, were measured with a standard sample of 131-iodide. This ratio, which is a criterion for the best ratio of count rate to background rate, was measured at various EHT voltages across the photomultiplier tube, and at various settings of the discriminator bias. It rose as the EHT voltage was increased and as the discriminator bias decreased (in spite of the higher background rates with a low discriminator setting). The settings used routinely were EHT = 1200V, discriminator = 5V.

Using a solution of 131-iodide standardized in an ionization chamber, the efficiency of the counter was determined as a function of the volume of the sample, see Fig. 2. Determinations of radioactivity in solution were always done in a volume of 1.0 ml or less, and an efficiency of 41.0% assumed in order to convert the count rates to millicuries. The lung tissue samples all occupied a volume of less than 1.0 ml and as it can be seen that the rate of change of efficiency with volume is very small in this range, it was ignored.

Stability. The observed efficiency with a volume of 1 ml was observed to be 41.06% on 4th September 1961 (plotted in Fig.2). A redetermination of this figure on 1st April 1964 gave an efficiency of 41.08%.

MEASUREMENT OF LUNG SENSITIZATION

The chopped guinea-pig lung preparation of Mongar and Schild (1953) was used. The lungs were perfused and mechanically chopped and washed as described by Brocklehurst et.al. (1961) and the tissue distributed by a method based on that of Mongar and Schild (1953) into covered flat bottomed sample tubes and rocked in 5 ml Tyrode's solution at 37°C for about one hour by which time most of the tissue had sunk, the Tyrode solution was then changed. Five to 15 minutes before antibody was to be added, the samples were drained again and usually 0.96ml of Tyrode's solution added (the extracellular space already contained about 0.04 ml) followed by 1.0 ml of antibody solution of twice the required find concentration (if proteins were in short supply 0.46 and 0.5 ml respectively were used). The treatments, in all experiments, were arranged in complete random blocks (and sometimes, when the number of replicates was equal to the number of treatments, in a Latin square). The blocks were randomized using the table of random numbers given by Fisher and Yates (1957). After incubation with antibody for the required time and temperature the tissue was drained and in some experiments washed one, two or three times with Tyrode's solution, and left in 1 ml Tyrode's solution. When the adsorbed protein was to be measured, the tissue was carefully drained, transferred to a clean tube, and weighed. 0.96 ml Tyrode solution was at once added and the radioactivity counted. The tissue was then rewarmed for 10 min. at 37°C and 1.0 ml of double strength antigen solution added. After 15 min. at 37°C when most of the histamine released would be in the supernatant solution, the supernate was sucked off as described by Mongar and Schild (1953) and heated in a boiling water bath for 5 min. to release their histamine content and enable an estimate of total histamine content of the samples to be made. All solutions were stored frozen until they were assayed.

BIOASSAY OF HISTAMINE

The assays were done against a standard histamine solution and the results expressed as percent histamine release although the unknown solutions contain substances other than histamine which may interfere with the assay, these complications were largely avoided by working at high dilution and were ignored for the purpose of obtaining an index of sensitization. Assays were done on the guinea pig ileum in Tyrode's solution containing 3×10^{-7} M atropine sulphate. Responses to dilutions of each unknown solution were bracketed in between responses to two standard solutions of histamine, and between two and four replicate analyses done on each unknown solution. The results were calculated by linearly interpolating the dose required to produce the response to the unknown solution. The interpolation was done by eye.

In appendix (2) an analysis is given of the errors involved in this simple and rapid method of analysing the results.

It is clear from appendix 2 and the discussion (p 149) that the errors resulting from calculating the results by visual linear interpolation, rather than measuring responses and interpolating logarithmically, are insignificant as long as the ratio of high and low standard doses is reasonably small.

The standard errors or fiducial limits given for percent histamine release are calculated from the actual histamine releases in ng/sample, the limits for which are simply divided by the estimated total histamine content of the tissue as though it were a constant not an experimental variable. This is the proper procedure when, as in the present case, it is the reproducibility of the results which matters, not the absolute values of the percent release.

MEASUREMENT OF EXTRACELLULAR SPACE

The extracellular space was estimated using a 1% solution of inulin (British Drug Houses Ltd.) in Tyrode's solution. The spaces occupied by sucrose and glucose are described in the results section.

Samples of lung tissue were drained and washed once with about 4 ml of inulin-Tyrode, then incubated at 37°C for about 30 min. (1 hour in earlier experiments) with 4 to 5 ml of inulin-Tyrode. At the end of this period a sample of the supernatant inulin solution was taken for use as a standard in the inulin estimation (the exact concentration of inulin need not be known if this is done) and the lung tissue drained and transferred to a clean dry tube for weighing. After weighing the tissue was kept at 4°C until the next day when inulin was extracted as described above.

Five to ten samples of lung tissue which had not been incubated with inulin (i.e. with Tyrode's solution in their extracellular space) were pooled and treated as a single sample for assessment of the inulin equivalent of the tissue to be subtracted from the apparent inulin content.

Six, 8 or 10 replicate determinations of inulin space were included in each experiment, half at the beginning and half at the end.

MEASUREMENT OF ADSORPTION OF PROTEINS

After incubation with ^{131}I-labelled antibody as described above, lung tissue samples were drained (or in some experiments washed with Tyrode's solution), transferred to a clean dry sample tube, weighed and, after addition of 0.96 ml Tyrode's solution, their radioactivity counted. The samples were then rewarmed before addition of antigen. At the end of the incubation with antibody and immediately before draining, samples of the supernatant antibody solutions were taken for use as radioactive standard solutions in order to get the best estimate of the final concentration of antibody in the supernatant solution (and, by assumption, in the extracellular space of the tissue).

ISOSMOTIC SOLUTIONS

The total molarity of Tyrode's solution is 154.9 x 10^{-3} and the total ionic strength is 160.6 x 10^{-3} mM (this involves counting the phosphate content as though it were uni-univalent, but the error from this source must be small). Solutions of lower ionic strength were made by omitting part or all of the 137 mM sodium chloride content and replacing it with an isosmotic amount of a non-electrolyte (sucrose, mannitol, sorbitol, raffinose or glucose) or a dipolar molecule (glycine).

The calculations of the quantities required were based on freezing point depressions assuming these to be additive. Graphs were plotted of the molal freezing point depression constant (K_f), or the freezing point depression (ΔT) against the molality of the solution using the data in Landolt-Börnstein (1936) or International Critical Tables (1928) for sodium chloride, sucrose and mannitol and the required values interpolated from these graphs. It was assumed that sorbitol produced the same molal freezing point depression as mannitol. For other substances a 0.28M solution was assumed to be isosmotic with plasma. The freezing point depression contributed by 137 mM sodium chloride is 0.472°C. The molalities of other components were calculated such that they produced a total freezing point depression of this value. The molal solutions were prepared by making up the Tyrode's solution with its minor components up to its final volume and adding the non-electrolyte (+ sodium chloride if required) to this final solution (this involves neglect of the fact that 1 litre of final solution does not contain exactly 1000 g of water).

Addition of substances to the final solution also means that the molarity (but not the molality) of the minor components (calcium etc.) will be slightly changed due to the volume change on addition of the non-electrolyte. 85.1 g sucrose/1000 g water (i.e. 248.6 millimolal) is required to replace the sodium chloride in Tyrode's solution.

QUANTITATIVE PASSIVE CUTANEOUS ANAPHYLAXIS

Passive skin sensitization was measured in the guinea pig. The abdomens of male albino guinea pigs were shaved and between 6 and 12 doses of antibody injected intradermally. All antibody solutions were in 0.154 M saline and the volume of all intradermal injections was 0.05 ml nominally. At first glass 1 ml tuberculin syringes were used for the injections, but a comparison of these syringes with sterile disposable plastic 1 ml tuberculin syringes, the results of which are given in appendix 3, suggested that the consistency of disposable syringes was very similar to that of glass ones and the heterogeneity of the former was, if anything, lower, so disposable syringes were used routinely for most experiments. Both sorts of syringe delivered, on the average, rather more than the nominal volume, and were significantly heterogeneous. All doses have been calculated as though the nominal volume (0.05 ml) were in fact delivered. Short bevel 26 gauge needles were used.

After the required time (usually 3 to 4 hours) had elapsed following antibody injections, 1.0 ml of a mixture of antigen and dye (1 volume 10% ovalbumin + 2 volumes 5% Pontamine sky blue) was given intravenously. After another 20 min. the animals were killed and the size (longest and shortest dimensions) and intensity (on a 5 point subjective scale) of the blue lesions recorded by transillumination of the skin.

The treatments in each experiment were arranged in complete randomized blocks (blocks corresponding to guinea pigs) and sometimes in a Latin square (with columns corresponding to sites of injection). A significant difference between guinea pigs was only detected in some experiments. Most of the fairly large variability seemed to stem from other sources, e.g. injection technique. The comparison of potencies of different protein solutions was done in the usual way for parallel line biological assay. It was found that the geometric mean of the longest and shortest dimensions of the blue lesions was approximately linearly related to the logarithm of the dose of antibody over a reasonable range of response size. Most of the assays employed three doses of each protein usually in the ratio 1 : 3 : 9.

PART I

THE ADSORPTION OF γ-GLOBULINS BY GUINEA PIG LUNG TISSUE AND PASSIVE SENSITIZATION IN VITRO

Introduction

In attempting to learn about the relationship between the adsorption of antibody onto a tissue and the passive sensitization of tissue it is necessary to define as clearly as possible what is meant by adsorption and by sensitization. The operational definition of adsorption which has usually been used in previous studies is that "protein not removed from the tissue by washing is considered to be adsorbed". It has of course been understood that this was somewhat arbitrary as there was no means of telling how much adsorbed material was removed by the washing process and how much non-adsorbed material was not removed. An attempt has been made to avoid these problems by means of a method of adsorption measurement not involving washing of the tissue, but making allowance for the amount of non-adsorbed γ-globulin in the fluid occupying the extracellular space as estimated by the inulin space. Although this seems a better way of measuring adsorption, in the usual physico-chemical sense of the term, it is not known whether the adsorption so measured is of the right sort (e.g. whether the adsorption is on the right cellular sites) to convey information about sensitization of the cells.

The nature of the bond between cells and protein may be investigated by altering the properties of the environment. For example, calcium concentration (calcium could be involved in protein-cell bonds) and ionic strength (so altering surface potentials of both cells and protein molecules, and activity coefficients of charged molecules).

Measurements of the rate of adsorption, desorption and sensitization might provide information about the average life of adsorbed molecules on the cell surface, the proportion of those colliding with the surface which become adsorbed, the area of the protein molecule involved in combination with the cell surface (making certain assumptions), activation energies of adsorption and desorption and the extent, if any, of rapid adsorption on to free cellular sites compared with the hypothetical slow adsorption by exchange with endogenous non-antibody γ-globulin. Before useful measurements of the rates of adsorption and desorption could be made it was necessary to discover to what extent the rate of adsorption was controlled by diffusion. The experimental results and theoretical calculation in the following sections shows that even in small particles the rate of diffusion largely prevents any inferences being made from observed rates of adsorption or sensitization, at least during the period of 30 or 60 minutes from the first contact with antibody solution.

Fig. 3 MIE(24)4 (30/10/62)

Well.	Rabbit γ-globulin
Trough.	Goat anti (rabbit serum) serum
Well	Normal rabbit serum

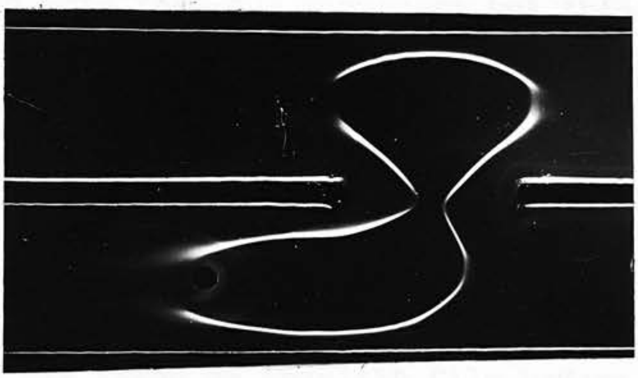
Fig. 4a MIE(37)4 (29/3/63)

Well	Rabbit γ-globulin (batch 52+53)
All troughs	Goat anti (rabbit γ-globulin) serum
Well	Normal rabbit serum

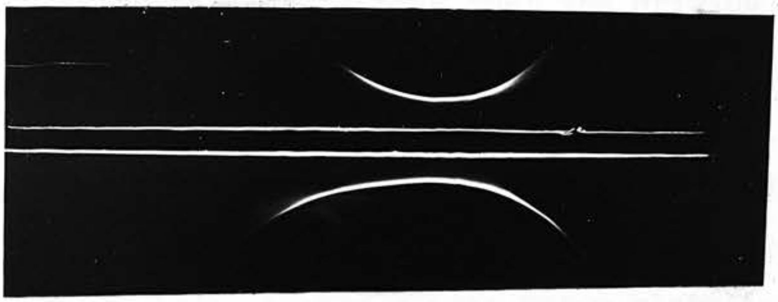
Fig. 4b MIE(37)1 (29/3/63)

Well	Rabbit γ-globulin (batch 52+53)
Trough	Goat anti (rabbit γ-globulin) serum
Well	Normal rabbit serum

Fig 3



F 4a



F 4b

RESULTS

(a) Purification of rabbit anti-BSA γ-globulin

γ-globulin was separated from rabbit anti-BSA sera in one stage on DEAE cellulose columns. Testing by immunoelectrophoresis usually showed one component. The γ-globulin preparations which were run on paper never showed more than one component but this is not a very sensitive method of testing for contamination. Fig. 3 shows a typical immunoelectrophoresis test (considerably more lines were visible in the whole serum on the original slide than show on the photograph, which was taken on Kodalith film before the desirability of using a less contrasty material was realized). No contamination was visible. Fig. 4a shows a reaction of identity between the isolated γ-globulin and that of normal serum (note that the origins are not at the same distance from the ends of the slides) and Fig. 4a and 4b both show that although the mobility of the slowest γ-globulin in whole normal serum is about the same as that in isolated anti-BSA γ-globulin, the isolated material does not contain the serum γ-globulins of highest mobility.

Fig. 5 MIE(64)7 (9/4/64)

Well	Normal rabbit serum
Trough	Goat anti (rabbit γ-globulin)
Well	Rabbit γ-globulin (batch 52+53)

Fig. 6 MIE (19)1

Well	Rabbit γ-globulin (stored unsterilized)
Trough	Goat anti (rabbit serum)
Well	Normal rabbit serum

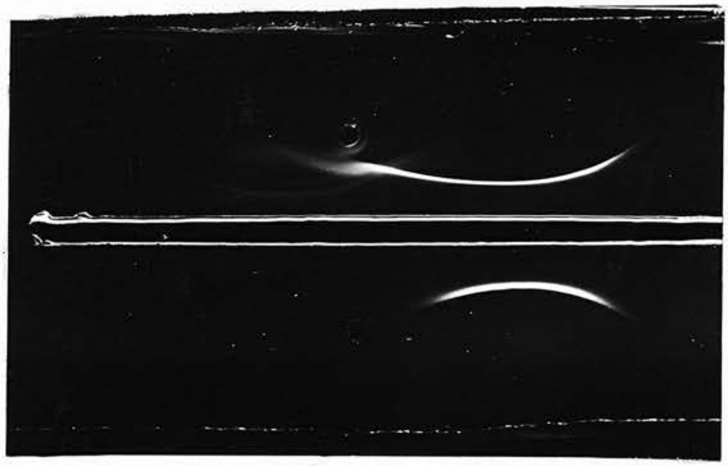
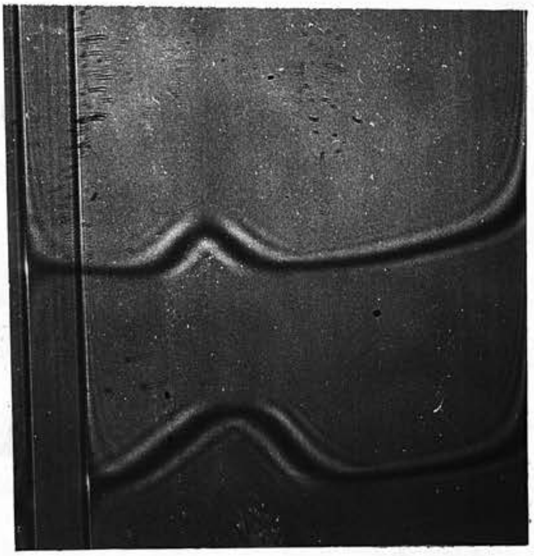


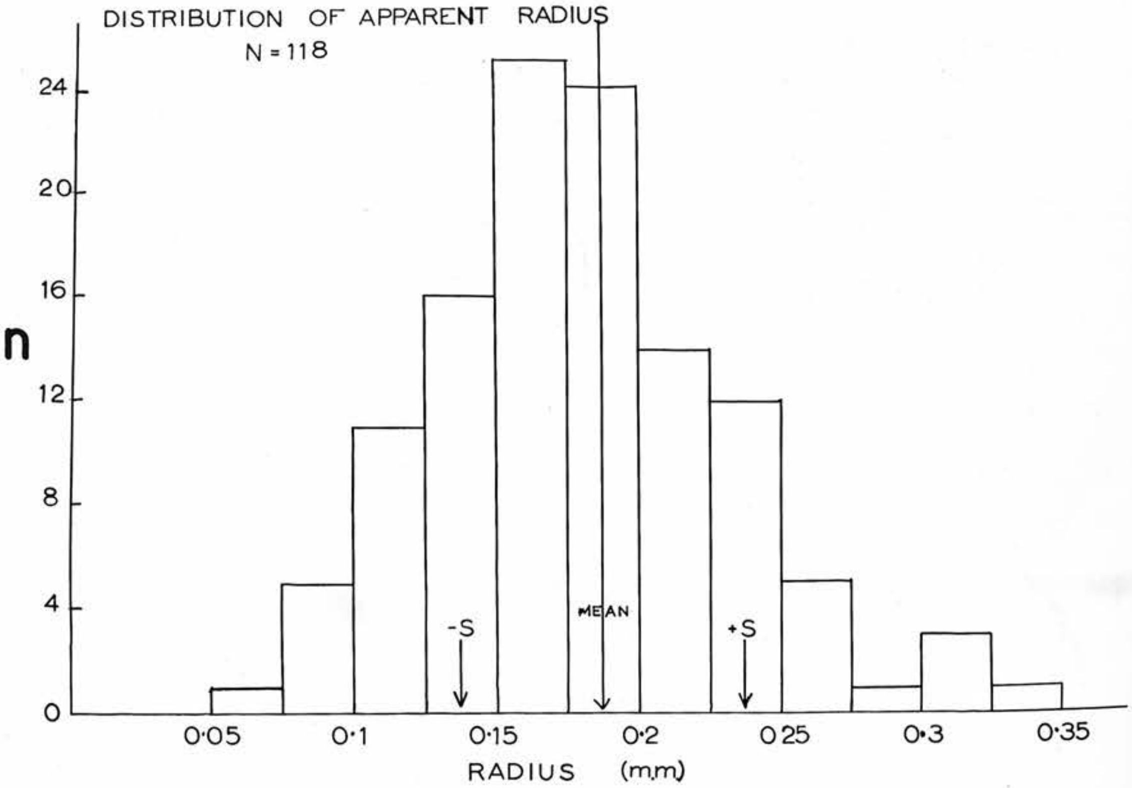
Fig. 7 Ultracentrifugation of rabbit γ-globulin. Spinco E. Photograph of Schlieren pattern after 60 min. at 50,740 r.p.m.

Upper: Freshly prepared RGG. 0.78mg/ml (DEAE 47)

Lower: RGG stored under non-sterile conditions in solution. (DEAE 39-41). 2.4 mg/ml.

Fig. 8 Distribution of radius of lung tissue particles (Ex. 47). Mean and standard deviation marked.





DISTRIBUTION OF APPARENT RADIUS
N = 118
n
0
4
8
12
16
20
24
-S
MEAN
+S
0·05
0·1
0·15
0·2
0 25
0·3
0·35
RADIUS (m.m)

Fig. 5 shows the immunoelectrophoretic pattern of a specimen of rabbit anti-BSA γ-globulin which had been kept in solution for more than 1½ years at 4°C after sterilization by membrane filtration as described earlier. It looks perfectly normal and the solution was completely clear. When the γ-globulin solutions were not sterilized they rapidly became infected with micro-organisms and Fig. 6 shows a double line found with a sample of rabbit γ-globulin kept for 2 months at 4°C in solution without sterilization.

In the ultracentrifuge (Fig. 7) freshly isolated γ-globulin had $s_{20,w} \simeq 7S$ whereas the degraded γ-globulin shown in Fig. 6 gave an asymmetrical peak with $s_{20,w}$ less than 7S and was obviously heterogeneous.

Antibody content. Most of the rabbit anti-BSA γ-globulin preparations were between 30 and 35% precipitable with crystalline BSA. Particular values are given in the text where relevant.

(b) Extracellular space and diffusion measurements in chopped guinea pig lung

(1) The size of lung tissue fragments in the preparation.

The method of preparation of chopped lung would lead one to expect that the fragments would be rectangular parallelepipeds. This shape is not obvious when the particles are seen under the microscope and as diffusion calculations are easier for cylindrical fragments it was considered that it would be an adequate approximation to treat the fragments as cylinders. The diameters of samples of lung fragments were measured using dark ground illumination and a graticule calibrated by means of a haemocytometer. The distribution of radii observed in one such experiment is shown in Fig. 8 (distributions observed in other experiments were very similar). It has a positive skew. The mean radius (calculated from the ungrouped observations) was 1.87 x 10^{-2} cm but in all experiments this was very variable, the coefficient of variation being, in this case, 27.7%. In the example shown

40 of the measurements were made at the start of an experiment when the tissue had been in Tyrode's solution at room temperature for a short while, and 78 measurements after the tissue had been incubated at 37°C for 4 hours. The mean radii were 1.917×10^{-2} cm and 1.848×10^{-2} cm respectively, showing no sign at all of a significant difference ($P \simeq 0.5$).

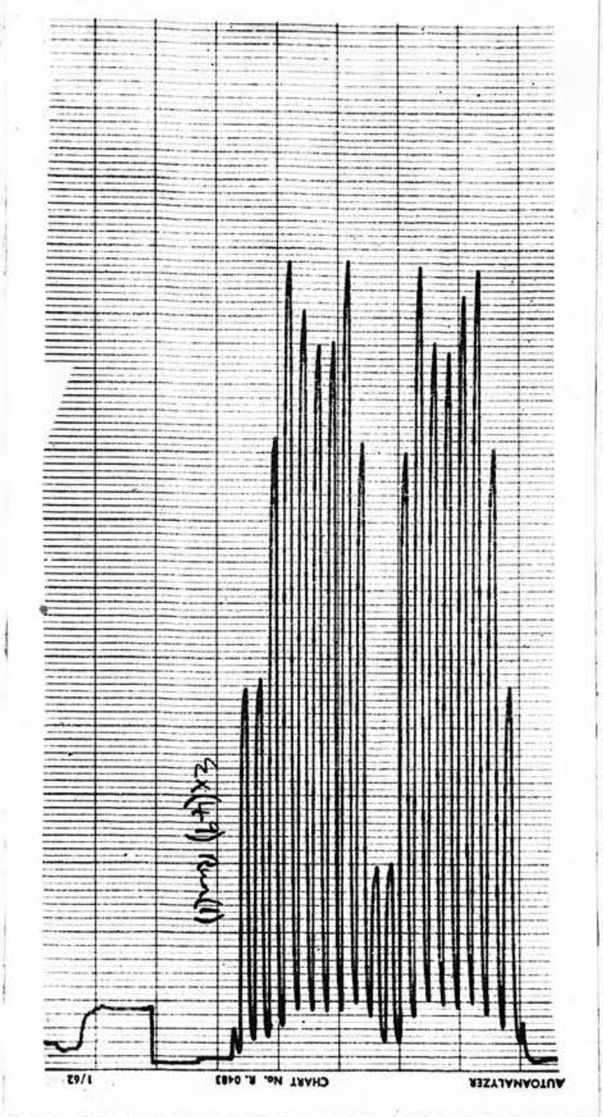
In another experiment different lobes of the same lung were inflated to different extents before chopping. No correlation of the mean radius with degree of inflation was observed. In this experiment a mean radius of 1.811×10^{-2} cm was observed for tissue fragments kept for 3 hours at room temperature, then for 1 hour at 37°C. After 7 hours at 37°C the mean radius was 1.825×10^{-2} cm, again no sign of change.

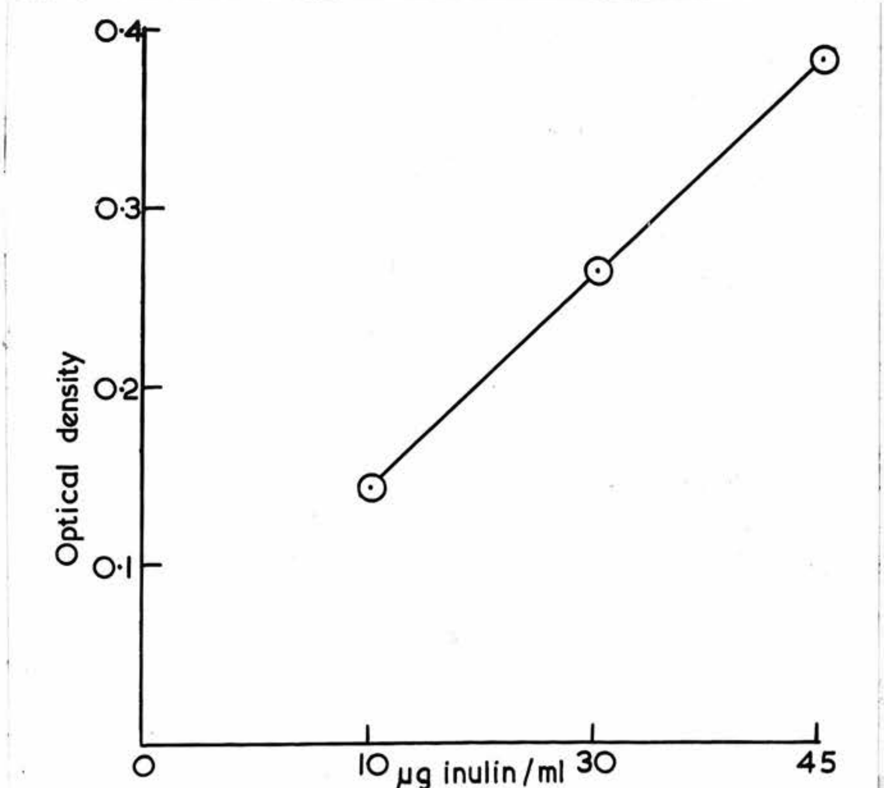
There was a tendency for fragments with large diameters to be longer than those with narrow diameters but this was not investigated in detail. The lengths of most particles were considerably ($\sim$ 20 X) greater than their radii.

A count of 1180 particles indicated that there were roughly 400 fragments per 100 mg wet (drained) weight of chopped lung, i.e. an average weight of roughly 0.25 mg/fragment.

Fig. 9 Record of inulin estimation on Autoanalyser. The first four and last three samples are standards of nominal inulin concentration 15, 30 and 45 µg/ml. Six unknowns are estimated in duplicate arranged symmetrically around the central point. The centre two samples are controls from 7 pooled tissue samples without inulin.

Fig. 10 Standard curve for inulin estimation.


Ex(4+9) Run(1)
AUTOANALYZER
CHART No. R. 0483
1/62


Optical density
0·4
0·3
0·2
0·1
0
10
30
45
μg inulin/ml

(2) The inulin space of chopped lung tissue

The record of transmittance from an inulin assay is shown in Fig. 9 and in Fig. 10 the mean optical densities produced by the three standard solutions are plotted against concentration. The autoanalyser almost always produced a curve departing only very slightly from linearity when two replicate analyses of each solution were done.

In most experiments the supernatant solution from samples of lung tissue was drained off by suction through a nylon brush of the type described by Brocklehurst et al. (1961), and the tissue transferred to a clean tube and weighed. Tissue prepared in this way is hereafter referred to as "drained tissue". In most experiments the mean wet weight of drained tissue samples was between 70 and 90 mg and the coefficient of variation of the drained wet weight between 9 and 13%, so in an average experiment one might expect roughly two samples out of 40 to lie outside the range 62 to 98 mg. The method of preparing the samples is sufficiently variable for it to be necessary for each sample to be weighed if its inulin space is to be estimated from the inulin space per 100 mg wet weight determined on parallel tissue samples.

The inulin space is, of course, not a quantity of physiological significance. It is just an analytical tool for the measurement of adsorption of material onto (or entry into) cells.

In preliminary experiments the tissue samples were blotted with Whatman No. 54 filter paper after incubating with inulin and before weighing. Comparison of the wet and dry weights of blotted samples with later results yields an approximate estimate of the additional extracellular fluid removed by blotting drained samples, viz. 16.0 µl per 100 mg drained wet weight. The inulin space (I.S.) measured by this method is given in Table 2

Table 2

Experiment	n	IS	CV
18	14	42.9 µl/100 mg	8.9%
21	5	46.8	4.1
22	4	48.5	2.9
23	6	44.4	4.3

where n is the number of tissue samples measured, IS the inulin space in µl/100 mg blotted wet weight and CV the coefficient of variation of the estimate of IS on a single tissue sample. These do not look much less than the values found when the tissue was simply drained. This, of course, is because blotting reduces the wet weight almost in proportion to the inulin space. The figure of 16.0 µl/100 mg mentioned above is found by reference to the dry weight of the samples. Blotted tissue is sticky and difficult to handle and it was also clear from preliminary experiments that the tissue was damaged during the blotting process as the spontaneous histamine release of tissue treated in this way was increased and the antigen induced histamine release decreased. Consequently in all further experiments the tissue samples for inulin space and adsorption measurements were drained and not blotted.

Inulin space measurements (in µl/100 mg drained wet weight) on drained tissue gave results exemplified by those in Table 3.

Table 3

Experiment number	n	IS µl/100 mg	CV
25	10	51.5	3.2%
27	7	53.3	5.3
29	4	53.2	5.0
31	11	47.9	3.5
32	8	49.2	4.3
36	5	46.9	3.2
41	4	45.8	4.4
43	15	56.7	10.3
45	4	50.5	6.8
47	13	44.8	4.4

In one experiment three sorts of homogenizer were used to extract inulin from tissue samples. Two propellor type homogenizers gave inulin spaces of 50.8 (mean of 4) and 49.5 (mean of 2) µl/100 mg, and the ground glass type homogenizer used in most of the experiments to be described gave 52.3 µl/100 mg. The differences between these values are slight, especially as an accurate tissue blank was available for only one of the methods.

In later sections it is shown that there is no evidence that the inulin spaces varies with calcium concentration, although the space available to smaller molecules may do.

(3) The rate of uptake of inulin by chopped lung tissue samples

The rate of inulin uptake is shown in Fig. 11. Equilibrium is reached within not more than 10 minutes and, once reached, the equilibrium inulin content of the tissue stays constant over at least an hour. These findings were confirmed in two other experiments. In most experiments half the tissue samples used for inulin space determination were used at the start of the experiment and the other half to 3 hours later at the end. No consistent difference was observed between these at the beginning and those at the end.

(4) Estimation of the effective diffusion coefficient of inulin in lung tissue.

In some cases there is good theoretical reason for believing that diffusion in a medium which contains structures offering obstruction to the diffusing material, can be represented by the same equations as are appropriate for free diffusion in solution but using a reduced value for the diffusion coefficient (the "effective diffusion coefficient" or "diffusion coefficient in the tissue"), and the assumption that this is so is commonly made even when there is no theoretical reason to believe it valid (see, for example, Lauffer 1961), and is made here. No attempt has been made to calculate the effective diffusion coefficient in view of the complex structure of lung tissue, though there are writings extending as far back as Lord Rayleigh in the 19th Century bearing on such calculations. Instead the effective diffusion coefficient of inulin has been estimated empirically from the observed rate of uptake.

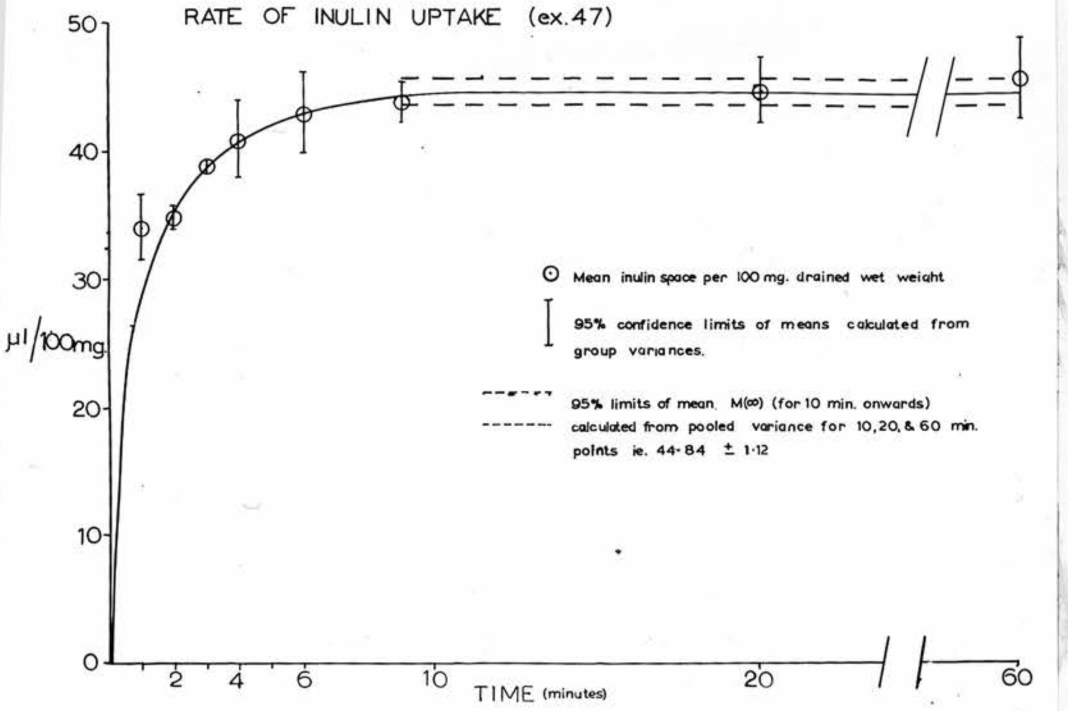
Firstly it is necessary to know the diffusion coefficient of inulin in solution. A determination by the method of Schantz and Lauffer (1962) (see appendix 4) using the same batch of inulin that was used in the experiments with lung tissue, although not wholly satisfactory (see appendix 4), gave a result agreeing sufficiently closely with other figures given in the literature for the present purposes. These are shown in table 4. The values have been corrected (see appendix 4) to 38°C in saline solution ($D_{38,s}$).

Fig. 11 Observed rate of inulin uptake by lung tissue with curve drawn by eye (Ex.47).

Fig. 12 Solutions of diffusion equations

<u>Upper</u> Solution for proportion of final uptake achieved by cylinder surrounded by constant concentration.

<u>Lower</u> Solution for population of cylinders.



Solution of diffusion equation for cylinder of radius a_i

$$f_i(t) = \frac{m_i(t)}{m_i(\infty)} = \left[1 - 4 \sum_{\alpha} \frac{1}{\alpha^2} \cdot e^{-\alpha^2 D t / a^2} \right]$$

Equation for population of cylinders containing proportion p_i of radius a_i

$$\frac{M(t)}{M(\infty)} = \frac{\sum_i f_i(t)\, p_i\, a_i^n}{\sum_i p_i\, a_i^n}$$

where

D is the diffusion coefficient

m is amount of material diffused into cylinder

M is amount diffused into population of cylinders

α are roots of $J_0(x) = 0$

Fig. 13 Inulin content of tissue (percent of final value) as function of time (Ex.47)

Observed points with 95% confidence limits. Dotted lines are confidence limits for final uptake, M (∞)

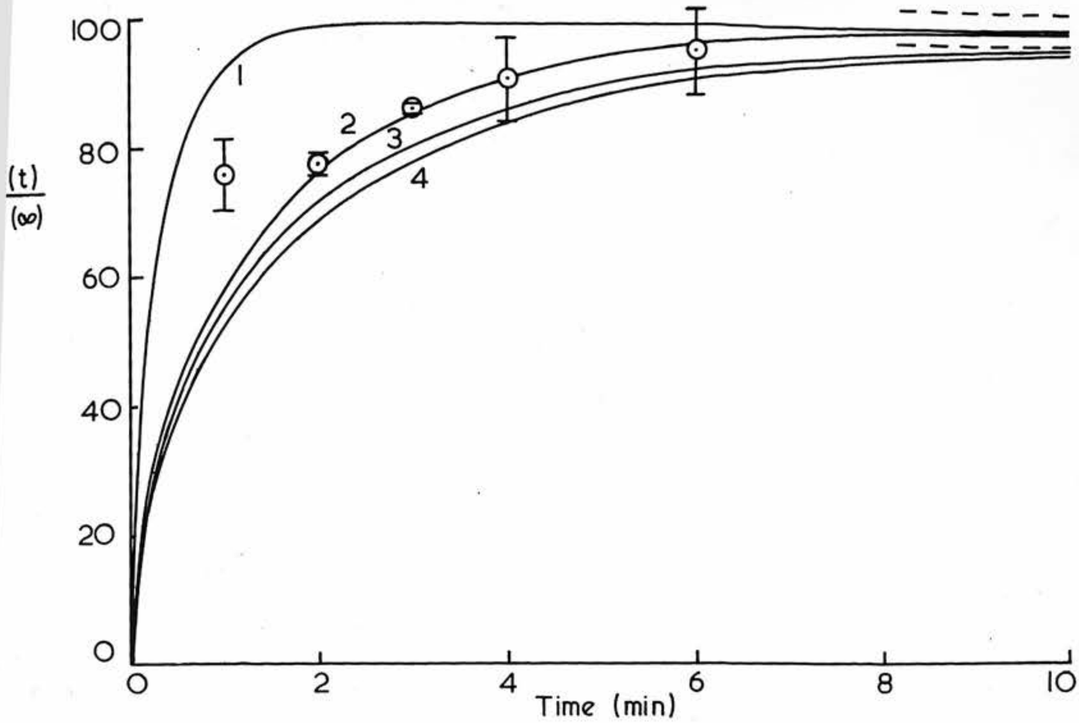
Curve 1. Calculated diffusion curve for cylinders of constant radius (using mean radius) using diffusion coefficient of inulin in solution D.

Curve 2. As curve 1, but using D'= D/4.25

Curve 3. Calculated diffusion curve for population of cylinders of constant length with radius distributed as in Fig. 14 and D'= D/4.25

Curve 4. As curve 3 but assuming length of cylinders proportional to their radius.

Fig. 14 Distribution of radius of cylinders observed in Ex. 47 grouped coursely for diffusion calculations.


100
80
60
40
20
0
(t)
(∞)
1
2
3
4
0
2
4
6
8
10
Time (min)

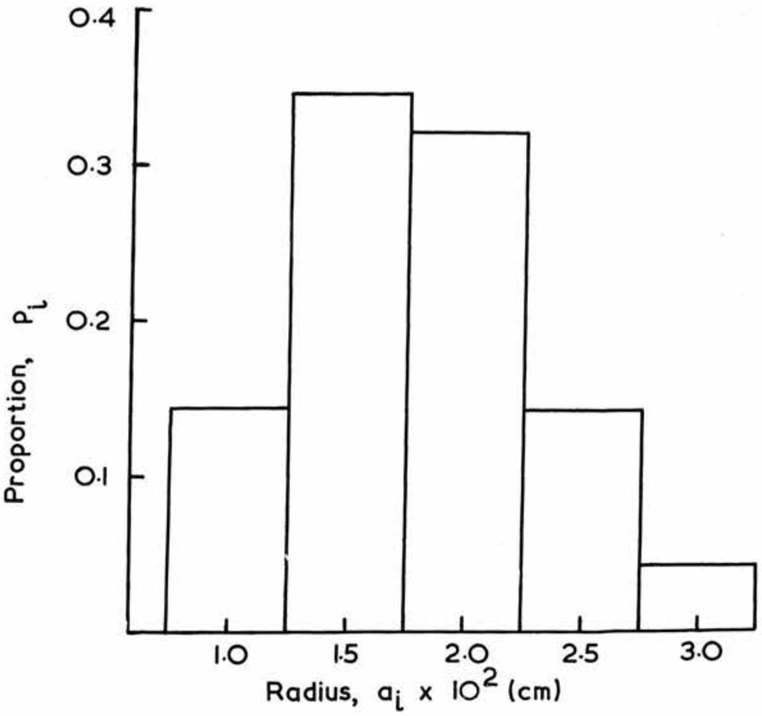

0.4
0.3
0.2
0.1
Proportion, P_i
1.0
1.5
2.0
2.5
3.0
Radius, $a_i \times 10^2$ (cm)

Table 4
Diffusion coefficients of inulin

Plant source	D_{38}, sal. (cm^2 sec^{-1})	Reference
?	2.29×10^{-6}	Appendix 4
Dahlia	1.74 to 2.37×10^{-6}	Bunim, Smith and Smith (1937)
Chicory	2.28 to 2.42×10^{-6}	Bunim, Smith and Smith (1937)
	2.47×10^{-6}	International Critical Tables
	3.22×10^{-6}	Conway and Fitzgerald (1942)

For the purposes of diffusion calculations the lung fragments were treated as infinitely long cylinders surrounded by a constant concentration of inulin. The solution of the diffusion equation,

$$\frac{\partial c}{\partial t} = D \ \text{div grad } c,$$

with the above boundary conditions is given in the upper half of Fig. 12 (see also appendix 4). This solution assumes that the radius, a, is a constant. Theoretical values for the fractional equilibrium inulin content $M(t)/M(\infty)$ against time, calculated from this solution are shown in curve 1 of Fig. 13. These values were calculated using the mean observed radius (1.87×10^{-2} cm) and the diffusion coefficient of inulin in solution (taken as 2.29×10^{-6} cm^2 sec^{-1}) and it is seen that the calculated rate of equilibration is, as expected, much faster than the observed rate. If the diffusion coefficient is reduced by a factor of 4.25 i.e. $D' = D/4.25$ where D' is the effective diffusion coefficient of inulin in the tissue, the resulting calculated curve, again assuming constant radius, (Fig. 13, curve 2) approximately fits the observed points, thus $D/4.25 = 0.539 \times 10^{-6}$ cm^2 sec^{-1} might be taken as the effective diffusion coefficient.

In fact the diffusion is being observed in a population of cylinders the radii of which are far from constant and it is of interest to see to what extent this affects the results. It has proved possible to solve the appropriate equations for diffusion in a population of cylinders with normally distributed radii, but all the calculations given here are based on an approximate discontinuous distribution of radii (the normal distribution is probably not correct either, as the distribution of radii has a positive skew). It is shown in appendix 4 that the lower equation in Fig. 12 represents diffusion in a population of infinite cylinders containing a proportion p_i of radius a_i. The exponent n takes the value 2 if the cylinders are all of the same length, and 3 if the length of each cylinder is proportional to its radius, (assuming, of course, that the length is sufficiently greater than the radius for the neglect of diffusion through the ends of the cylinders to be justified). Fig. 14 shows the distribution of radii used for subsequent calculations on the results in Fig. 13. The rather coarse grouping means that the average radius of the grouped distribution deviates noticeably from that of the original observations. It has been shown that arbitrary adjustment of the proportions to produce a distribution with the same mean radius as the original observations does not drastically alter the estimate of D' (by about 8 per cent). It is very probable that the uncertainty in the experimental results produces a greater uncertainty in D' than does the grouping of the distribution.

When the rate of equilibration is calculated using the distribution of radii in Fig. 14 and the same effective diffusion coefficient as before ($D' = D/4.25$), the results (Fig. 13, curves 3 and 4 for n = 2 and n = 3 respectively) show that the predicted rate is considerably slower than when the mean radius is used, and that the shape of the curve is different, there being more slowing at longer times (for example comparing curves 2 and 3 shows that the time for $M(t)/M(\infty) = 0.50$ is about 10 per cent greater on the latter, but for $M(t)/M(\infty) = 0.95$ it is about 50 per cent greater. If the diffusion coefficient only changes this proportion is the same at all uptakes . The effect is even greater when the cylinders have lengths proportional to their radii (curve 4). These results are qualitatively what would be expected as the larger cylinders, which equilibrate slowly, make a large contribution to the uptake (for a given number of cylinders) than those with smaller radius, which is why the weighted mean in Fig. 13 involves not only p_i but also a_i. Fig. 15 shows the curves calculated using the distribution of radii in Fig. 14 for free diffusion ($D' = D$) and for diffusion with an effective diffusion coefficient $D' = D/3.25$ i.e. 0.705×10^{-6} cm^2 sec^{-1}. The latter is seen to fit all observed points but the first very well, so the estimate of the effective diffusion coefficient may be taken as D/3.25. These curves are all for cylinders of constant length. The effect of making explicit allowance for the variability of the radius of the tissue fragments has been to increase the estimate of the effective diffusion coefficient by 31 per cent.

The calculated curves do not go through the one minute point in Figs. 13 and 15, but this point was not so far out of line in other experiments. The technical difficulties involved in trying to make measurements over short periods are considerable, e.g., the exact time at which the end of contact with external inulin solution should be considered to take place has a considerable uncertainty which becomes important at short time intervals. It may also be that the model adopted is not a good approximation to the true situation. For instance it might be assumed that the 16.0 µl/100 mg drained wet weight of extracellular fluid removed by blotting the tissue is lying between, rather than within, the tissue fragments.

Although it doesn't seem probable that it all is, at least part of it might be. This part of the extracellular space should then be assumed to equilibrate instantaneously, and the remainder by diffusion into the cylindrical tissue fragments. The results of such a calculation are shown in Fig. 16. If this model were assumed then the effective diffusion coefficient would appear smaller than found above - about D/4.25 as shown. The experimental results are not sufficiently precise to distinguish between these two models, but the difference between them, when they are applied to the results on uptake of γ-globulin, is not large.

The accuracy of the above and all subsequent results depend on the assumption that inulin is not appreciably adsorbed.

Fig. 15 Observations as in Fig. 13

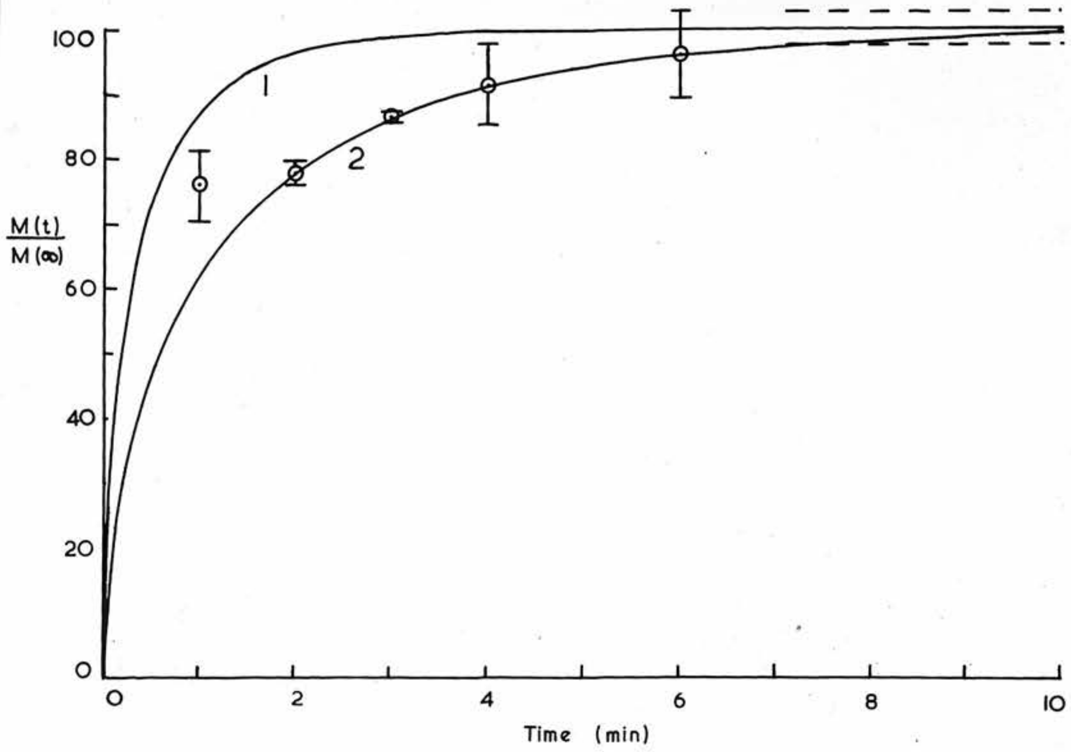
Curve 1. Calculated curve using distribution of radius shown in Fig. 14, diffusion coefficient in solution (D) and assuming cylinders of constant length.

Curve 2. As curve 1 but using an effective diffusion coefficient D'=D/3.25.

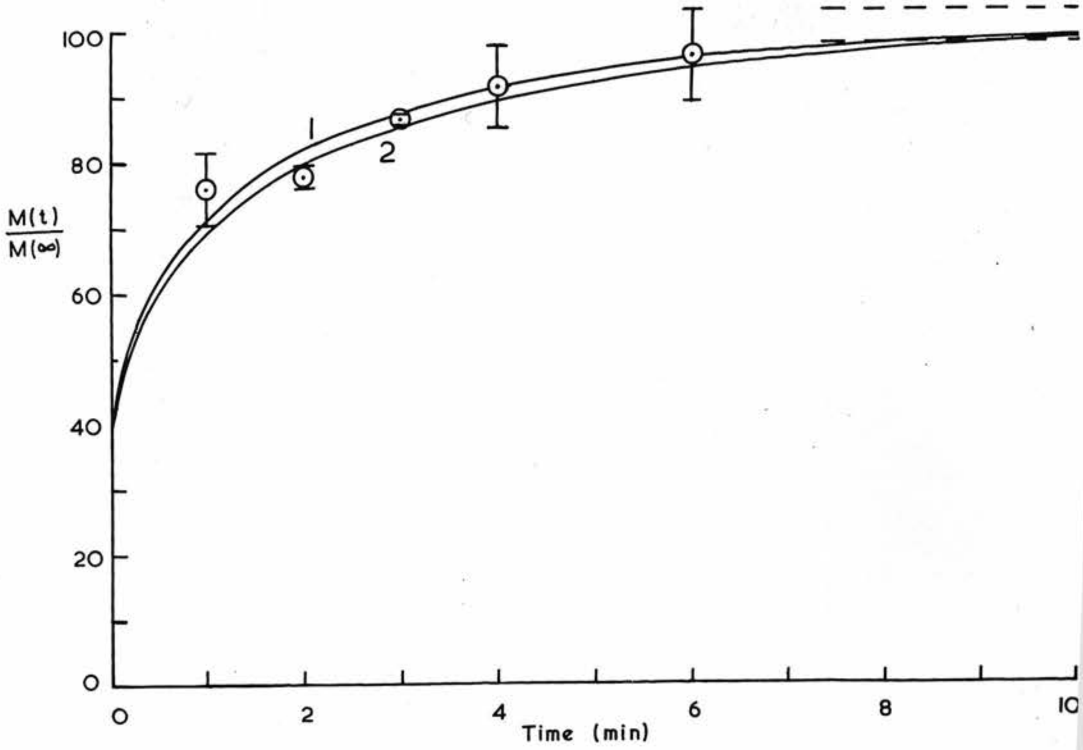
Fig. 16 Observations as in Fig. 15 with calculated curves assuming instantaneous equilibration of 16.0 µl/100mg of interparticle fluid.

Curve 1 D'= D/4.25

Curve 2 D'= D/5.0



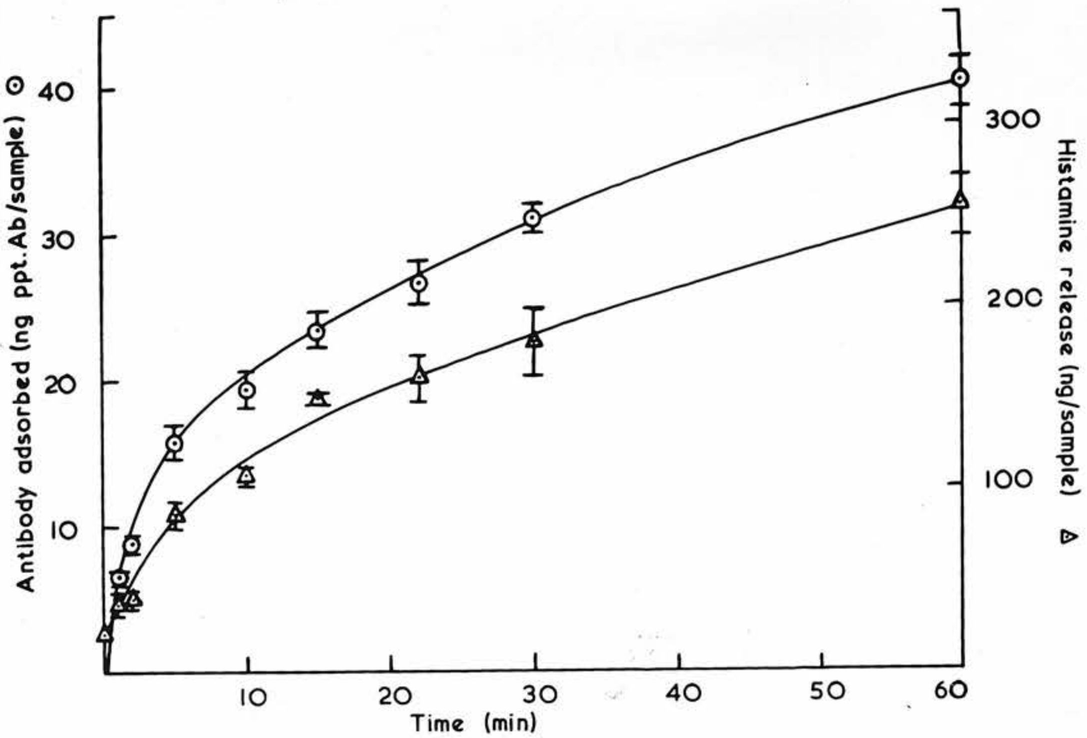
M(t)/M(∞)
100
80
60
40
20
0
1
2
0
2
4
6
8
10
Time (min)



M(t)/M(∞)
100
80
60
40
20
0
1
2
0
2
4
6
8
10
Time (min)

Fig. 17 Adsorption and sensitization as functions of time (Ex.10).
Adsorption measured by wash-off method (3 washes) ± standard error.
Histamine release ± standard error.

Fig. 18 Correlation between adsorption and sensitization plotted from results in Fig. 17. Standard errors are shown by bars.


Antibody adsorbed (ng ppt.Ab/sample) ⊙
Histamine release (ng/sample) △
10
20
30
40
100
200
300
10
20
30
40
50
60
Time (min)

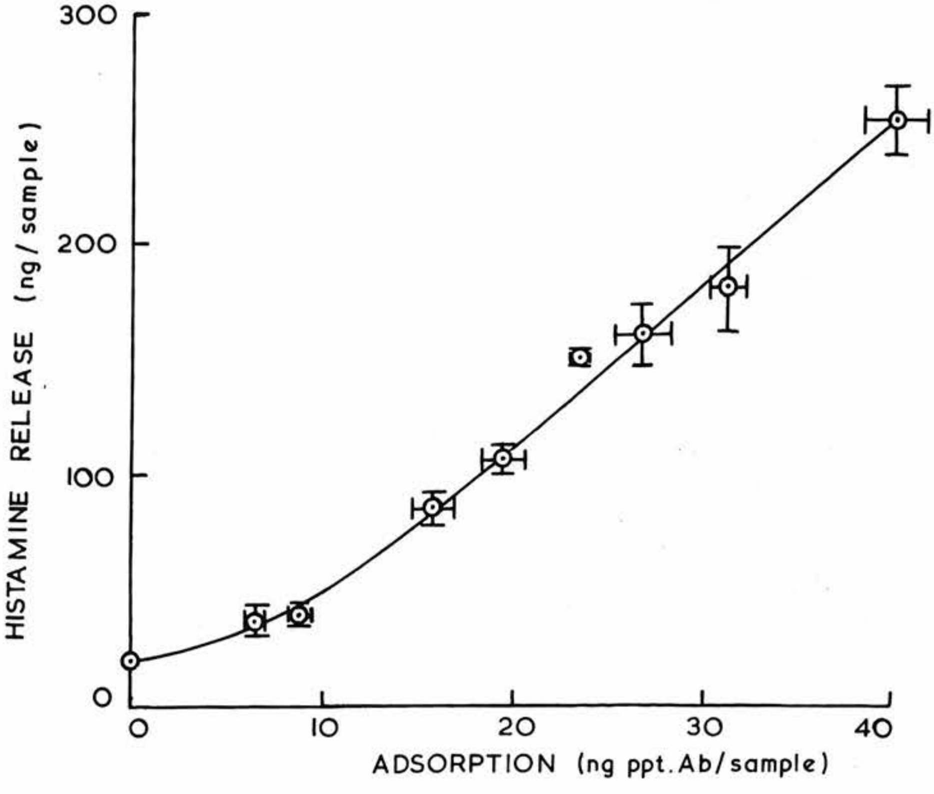
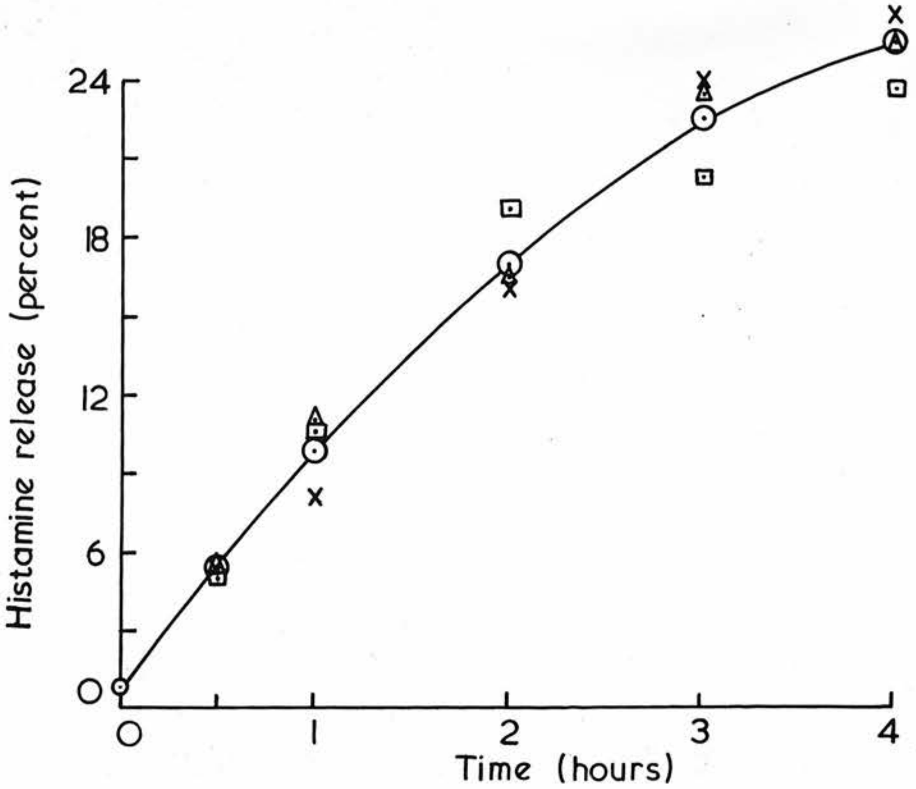
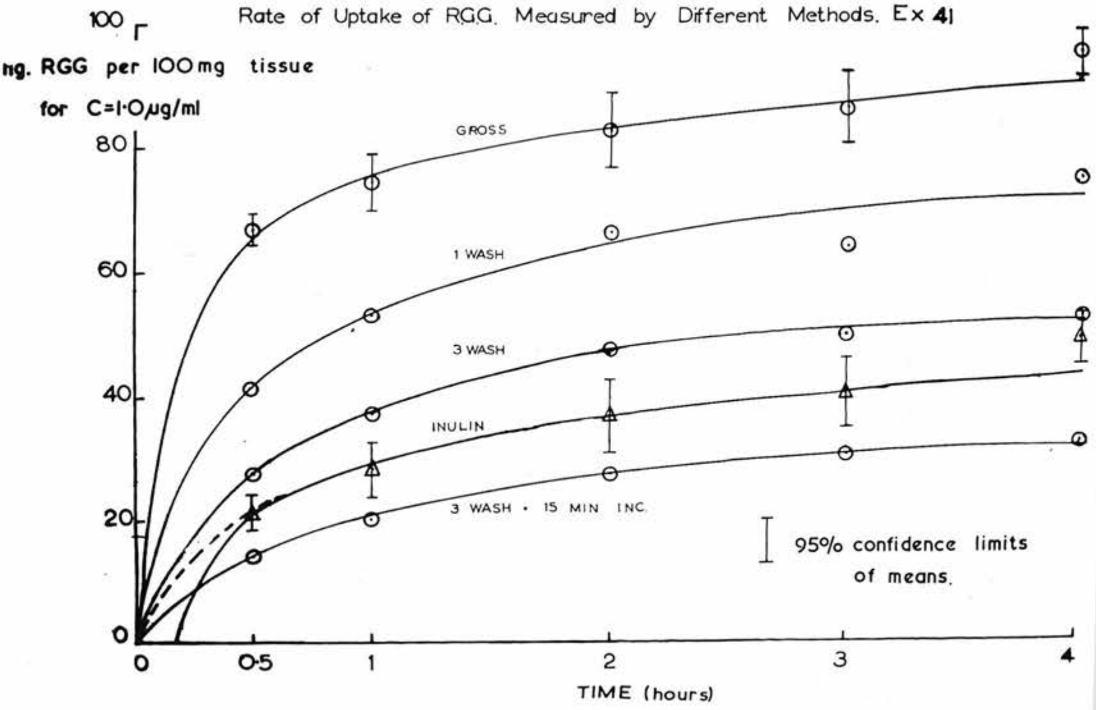

HISTAMINE RELEASE (ng / sample)
0
100
200
300
0
10
20
30
40
ADSORPTION (ng ppt. Ab/sample)

Fig. 19 Histamine release after treatment of lung tissue by different methods (Ex. 36).

- △ One wash with Tyrode
- x Three washes with Tyrode
- ⊡ Three washes + 15 min. incubation with Tyrode
- ⊙ Mean of all results

Fig. 20 Results of adsorption measurements with rabbit γ-globulin (RGG) by different methods. The top curve (marked gross) is the total γ-globulin content of drained tissue (Ex. 41).




Rate of Uptake of RGG. Measured by Different Methods. Ex 41

(c) The rate of adsorption of rabbit γ-globulin onto lung tissue and of sensitization

(1) Adsorption measurements at times of at least 30 minutes.

Two experiments in which lung tissue samples were incubated with rabbit anti-BSA γ-globulin (32% precipitable with BSA) in a concentration 1.0 μg precipitating Ab per ml showed that at this antibody concentration, adsorption of antibody (measured in these experiments by the wash-off method) and sensitization of the tissue run roughly parallel in time and that both are still increasing after one hour. The results of one of these are shown in Figs. 17 and 18.

It has been mentioned previously that blotting the lung tissue reduces the amount of histamine which subsequently can be released from it by antigen. However no consistent evidence has been found that the washing procedures alter the degree of sensitization. Fig. 19 shows the results of an experiment in which the tissue was washed once or three times, or washed three times and then incubated for 15 minutes in 3 to 4 ml of Tyrode's solution, after incubation with antibody. No consistent difference between the treatments is discernible. (A similar result is shown in Fig. 28). The sensitization is still increasing after four hours incubation with antibody, as was always observed.

It was found that the adsorption of γ-globulin measured by the inulin method was rather less than the amount which remains in the tissues after 3 washes with Tyrode's solution, though more than remains after 3 washes plus 15 minutes incubation in 3 or 4 ml of Tyrode's solution. A comparison of the results of adsorption measurement by different methods is shown in Fig. 20. It can be seen that when the time of incubation with antibody is less than about 30 minutes, the inulin method clearly gives the wrong answer if it is assumed that the concentration of γ-globulin in the extracellular space (e.c.s.) is the same as in the supernatant solution and it may therefore be inferred that roughly this length of time is necessary for equilibration of the e.c.s. The calculations and results in the next section confirm this idea.

(2) Adsorption and sensitization at low times. Equilibration of the extracellular space.

If it is assumed that the diffusion coefficient of γ-globulin in lung tissue is reduced by the same factor as that of inulin, the rate at which the extracellular space equilibrates with γ-globulin can be predicted. This is reasonable as all theories of the "obstruction effect" in diffusion (or in electrical conductivity which is formally the same problem) predict that the reduction in diffusion coefficient should depend only on the nature of the obstruction, not on the nature of the solute. There is however, in the case of γ-globulin, the additional complication of concurrent adsorption. This will tend to slow down diffusion to an extent depending on the rates of adsorption and desorption. As it is not possible to estimate these two rate constants a simpler model must be adopted.

If, for example, adsorption is a rapid process compared with the rate of diffusion so that it can be assumed that there is always adsorption equilibrium at every point in the tissue, and the isotherm is linear (as it is over the concentration range involved) then

$$s(r,t) = Kc(r,t)$$

where s is the amount adsorbed and c the concentration in the extracellular space at distance r from the centre of the cylinder and time t. The equilibrium constant for adsorption, K, must be dimensionless, and it is necessary to use the same dimensions for s and c in order to get a numerical value of K (see appendix 5). It can easily be shown (see, for example, Crank 1956) that the form of the adsorption equation appropriate to the model just described, i.e.

$$\frac{\partial c}{\partial t} = D' \text{ div grad } c - \frac{\partial s}{\partial t}$$

reduces to

$$\frac{\partial c}{\partial t} = \frac{D'}{K+1} \text{ div grad } c$$

The effect of adsorption is thus to further reduce the effective diffusion coefficient from D' to $D'/(K + 1)$. This model predicts that the amount of γ-globulin adsorbed will rise at exactly the same rate as the concentration in the extracellular space rises, and will be referred to as model 1.

Model 2. The other extreme assumption is that adsorption is a very slow process compared with diffusion, so that the extracellular space equilibrates with the external solution before any appreciable amount of adsorption has taken place. In this case adsorption can have no effect on diffusion rate, and the effective diffusion, D', is appropriate.

The diffusion coefficient of rabbit γ-globulin is given by Cammack (1962) as

$$D_{2\,0,w} = (4.66-1.21c) \times 10^{-7}\,\mathrm{cm}^2\ \mathrm{sec}^{-1}$$

where c is the concentration of γ-globulin in g/100ml. Correction to the conditions used in adsorption experiments, as described in appendix 4, gives

$$D_{3\,8,\mathrm{sal}} = 7.20 \times 10^{-7}\,\mathrm{cm}^2\ \mathrm{sec}^{-1}$$

which is effectively independent of concentration in the range of concentrations needed for sensitization.

The effective diffusion coefficient in lung tissue can now be found using the factor of 1/3.25 inferred from the experiments with inulin already described.

$$D' = D/3.25 = 2.22 \times 10^{-7}\,\mathrm{cm}^2\ \mathrm{sec}^{-1}$$

This is the appropriate diffusion coefficient for model 2.

For model 1 an estimate of the adsorption equilibrium constant K, is needed. It is shown in appendix 5 that K has a value of about 1.0. This would correspond to the adsorption at equilibrium of 50 ng γ-globulin per 100 mg tissue when the bulk concentration was 1.0μg/ml and the inulin space 50 μl/100 mg(so that the free concentration of γ-globulin is 50 ng/100mg tissue also). The actual values inferred from different experiments vary slightly of course, and equilibrium is probably never really reached anyway, but K = 1.0 is a fairly representative value. The effect diffusion coefficient for model 1 is therefore

$$D'' = \frac{D'}{K+1} = \frac{2.22 \times 10^{-7}}{2} = 1.11 \times 10^{-7} \text{cm}^2 \text{ sec}^{-1}$$

It can be seen from the solution of the diffusion equation in Fig. 13 that if the diffusion coefficient is reduced by a given factor, the time taken to reach any given $M(t)/M(\infty)$ is increased by the same factor. This is true whether or not the distribution of radii is taken into account.

Fig. 21 Calculated rate of equilibration of extracellular space with γ-globulin using observed distribution of radius of cylinders.

Curve 1. Model 2. $D' = D/3.25 = 2.22 \times 10^{-7}$ cm^2 sec^{-1}. Cylinders of constant length

Curve 2. As curve 1 but for cylinders with length proportional to radius.

Curve 3. Model 1. $D'' = 1.11 \times 10^{-7}$ cm^2 sec^{-1}. Cylinders of constant length.

Curve 4. As curve 3 but for cylinders with length proportional to radius.

Fig. 22 Curve 1. Observed total rabbit γ-globulin content of drained tissue (± standard error) as function of time. (for 1.0 μg/ml supernatant conc.)

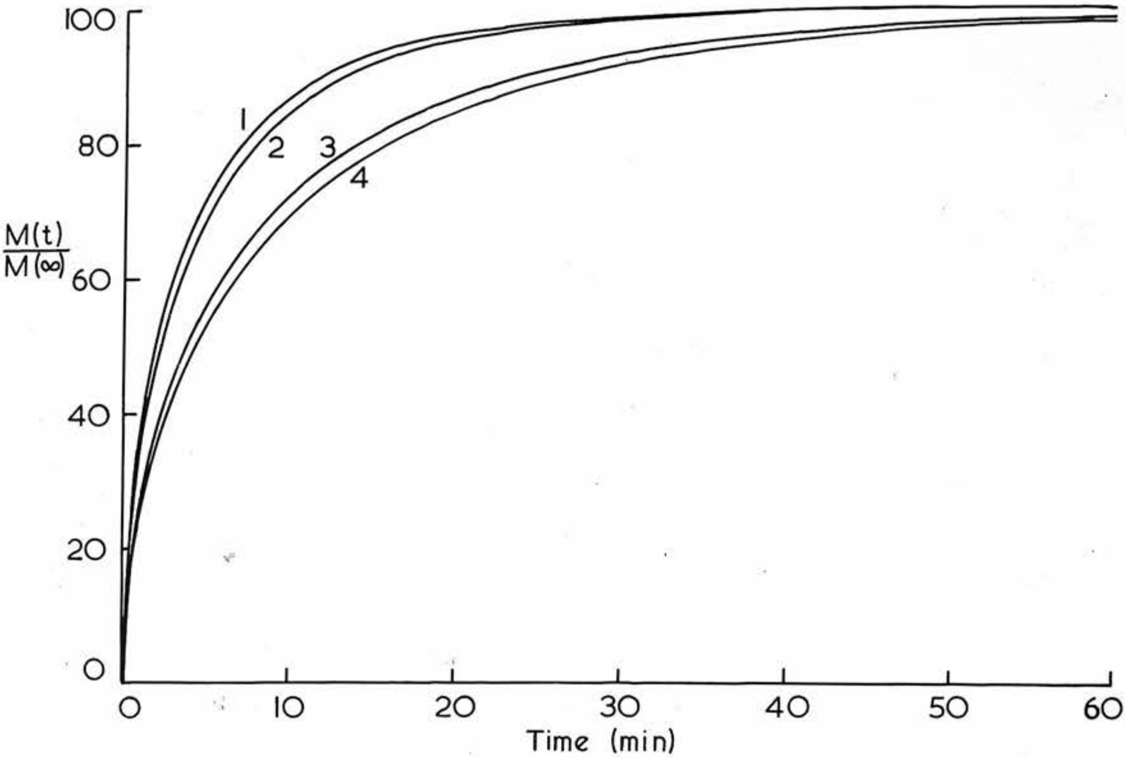
Curves 2 and 3

Calculated γ-globulin content of extracellular space M(t). Derived from curves 1 and 3 of Fig 21 with M(∞)=50.5ng/100 mg, numerically the same as the inulin space observed in this experiment (Ex 45)

Curve 4 Net adsorption (ng/100mg)

⊙ Curve 1 minus curve 3

✗ Curve 1 minus curve 2


100
80
60
40
20
0
M(t) / M(∞)
1
2
3
4
0
10
20
30
40
50
60
Time (min)

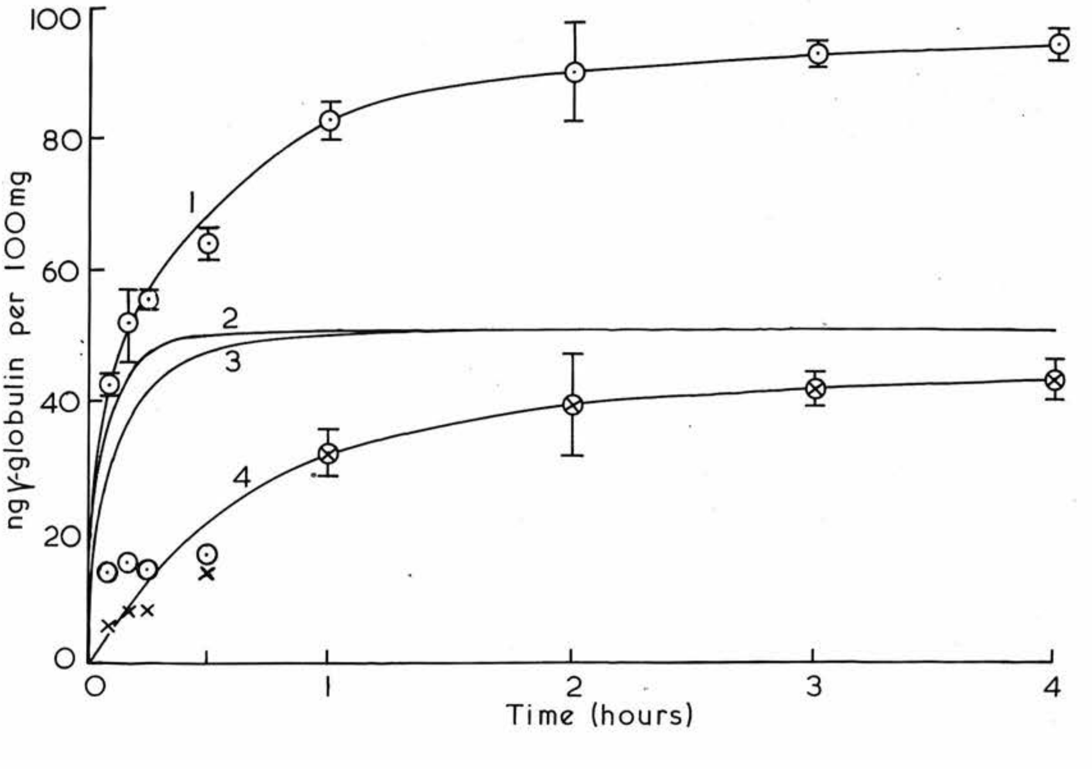
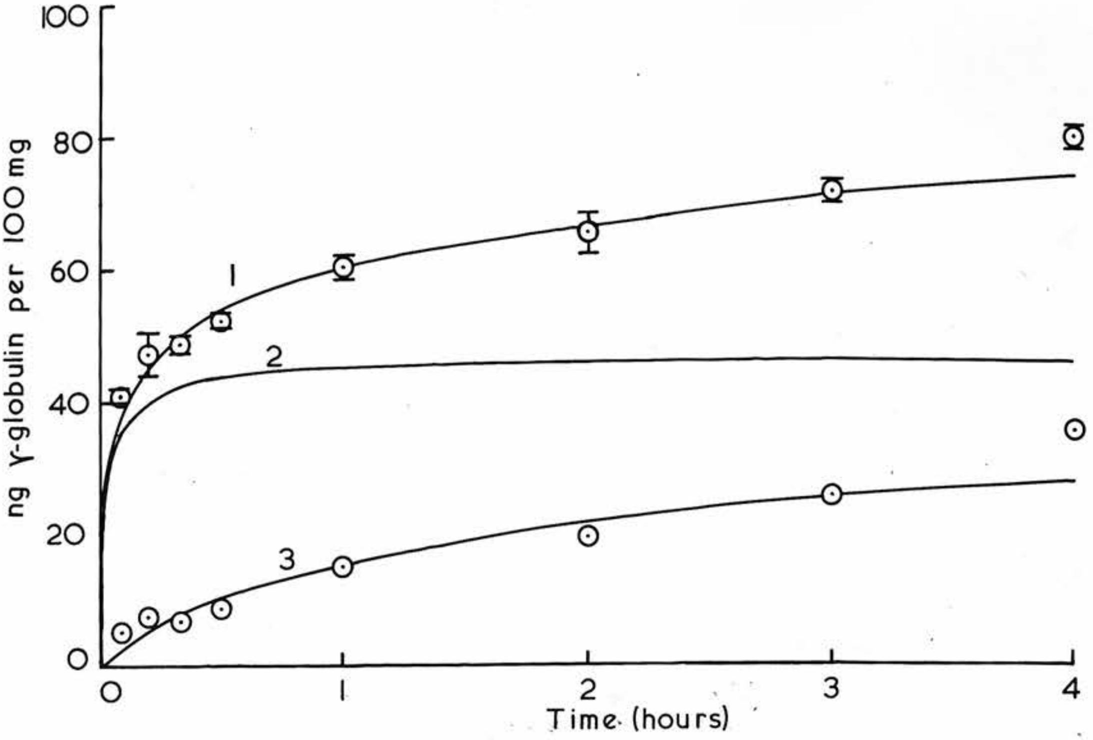

100
80
60
40
20
0
ng γ-globulin per 100mg
1
2
3
4
0
1
2
3
4
Time (hours)

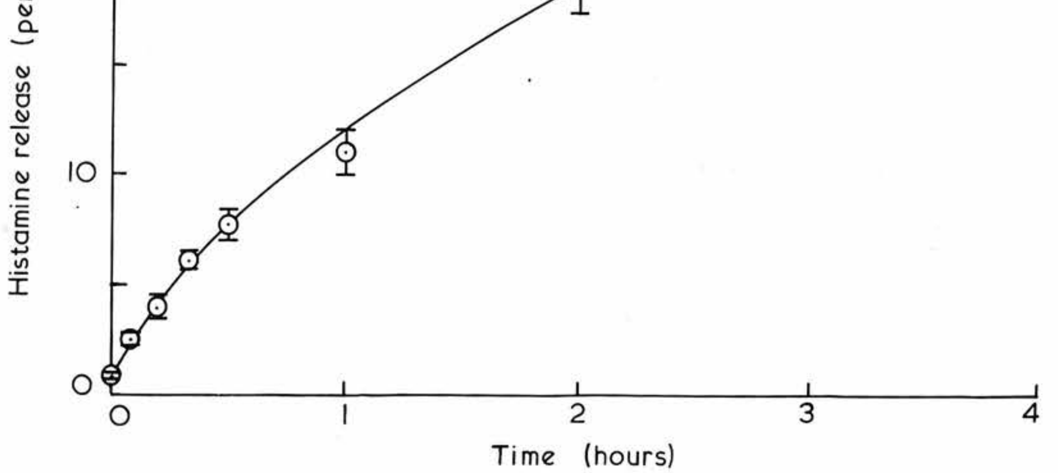
Fig. 23 Curve 1. Observed total rabbit γ-globulin content of drained tissue ($\pm$ standard error) as function of time. ng/100mg for c=1.0 μg/ml (Ex. 48).

Curve 2. Calculated γ-globulin content of extracellular space assuming 16.0 μl/100mg interparticle fluid equilibrates instantaneously. Model 1 with $K = 1.0$ and $D' = D/4.25$ so $D'' = D/8.5$. Cylinders of constant length.

Curve 3. Net adsorption, i.e. Curve 1 minus Curve 2.

Fig. 24 Histamine release as a function of time (Ex. 48). Vertical bars are standard errors.


100
80
60
40
20
0
ng γ-globulin per 100 mg
1
2
3
0
1
2
3
4
Time (hours)


25
20
10
0
Histamine release (percent)
0
1
2
3
4
Time (hours)

The results of calculations based on these diffusion coefficients are shown in Fig. 21. It is predicted that about 93% of the equilibrium γ-globulin content of the extracellular space is reached in about 30 minutes (model 1, fast adsorption) or 15 minutes (model 2, slow adsorption). The difference between assuming cylinders of constant length and of length proportional to radius is not large and the former model is used in subsequent calculations.

The curves in Fig. 21 can be used to calculate the amount of γ-globulin, M(t), expected in the extracellular space at any time t, and by subtraction of this from the observed total γ-globulin content the adsorption inferred. Fig. 22 shows the result of such a calculation. Again the predicted points seem to be in the right region though no distinction between the models can be made. As both the total γ-globulin curves and the calculated curves are rising fast when $t \angle 30$ minutes, the small difference between them is subject to very considerable errors of estimation.

It was shown in the section on inulin uptake that it could plausibly be assumed that 16.0 μl/100 mg wet weight of the extracellular fluid equilibrated instantaneously. This model resulted in an estimate of the effective diffusion coefficient $D' = D/4.25$. Application of this model to γ-globulin adsorption results is shown in the calculated curve in Fig. 23, and the net adsorption thus inferred again seems very reasonable.

The time course of sensitization over 4 hours is illustrated in Fig. 24. The adsorption measured in the same experiment is that shown in Fig. 23. This result is typical of those in several other experiments. In five experiments the sensitization was seen to be still increasing at 4 hours as it is in Fig. 24. The adsorption is usually still increasing at this time also (this point is discussed further on), but the sensitization often appeared to be increasing a good deal faster.

The rate of loss of γ-globulin when the tissue is washed when, or before, it has reached equilibrium with the extracellular space is discussed in a later section. This bears on the results found by washing methods.

(4) The effect of the slow continuous uptake of of γ-globulin on the validity of adsorption measurements

In Fig. 23 it can be seen that γ-globulin was still being taken up slowly at a roughly constant rate between 1 and 4 hours. This rate was rather variable, being small in Fig. 22, but rather larger than that in Fig. 23 in some other experiments. The fastest rate observed was about 16.4 ng/100mg per hour. It may be that this slow uptake is due to exchange of labelled antibody for autologous γ-globulin as the latter spontaneously desorbs, but whatever the mechanism it is clear that while γ-globulin is still being taken up by the tissue there must still be a concentration gradient between the outside and inside of the tissue fragments and therefore the mean concentrations in the extracellular space must be less than that in the external fluid. The question to be answered is whether the error thereby introduced into the inulin method of adsorption measurement is large enough to be serious.

Although the mechanism of the slow uptake is not known for certain, an approximation to the answer required can be obtained if it is assumed that it is the result of removal of γ-globulin by a first order reaction. When a steady state is attained there will be a concentration gradient through the tissue (and, as the reaction proceeds at a rate proportional to concentration, the reaction rate will also vary throughout the tissue), and material will be taken up at a constant rate. The equation to be solved is

$$\frac{\partial c}{\partial t} = D' \text{ div grad } c - kc$$

where D' is the effective diffusion coefficient, c the concentration and k the rate constant of the first order reaction. It can be shown (see appendix 5) that the steady state solution of this equation under the same conditions as before is

$$\frac{c(r,\infty)}{c_0} = \frac{I_0(r\sqrt{k/D'})}{I_0(a\sqrt{k/D'})}$$

where $c(r,\infty)$ is the concentration of γ-globulin at a distance r from the centre of the cylinder as time → ∞ (i.e. in the steady state), c_o is the concentration in the external solution, a is the radius of the cylinders and I_o is a modified Bessel function of the first kind of zero order.

It is shown in appendix 5 that this implies:

$$\frac{M(\infty)}{M_o} = \frac{2}{a\sqrt{k/D'}} \quad \frac{I_1(a\sqrt{k/D'})}{I_o(a\sqrt{k/D'})}$$

where $M(\infty)$ is the amount of γ-globulin in the extracellular space in the steady state, M_o is the amount that would be present if there were no slow uptake, i.e. k = o, and I_1 is a modified Bessel function of the first kind of first order defined by $I_m(x) = i^{-m} J_m(ix)$ where $i = \sqrt{-1}$.

It is not, of course, possible to obtain the rate constant, k, in the equations above directly from the observed rate of slow uptake, since the concentration (which the rate is proportional to) varies throughout the tissue, but the relationship between the observed rate of uptake and the rate constant can be plotted from the theoretical relationship between them, viz.

$$F(\infty) = \frac{2c_o\sqrt{kD'}}{a} \quad \frac{I_1(a\sqrt{k/D'})}{I_o(a\sqrt{k/D'})}$$

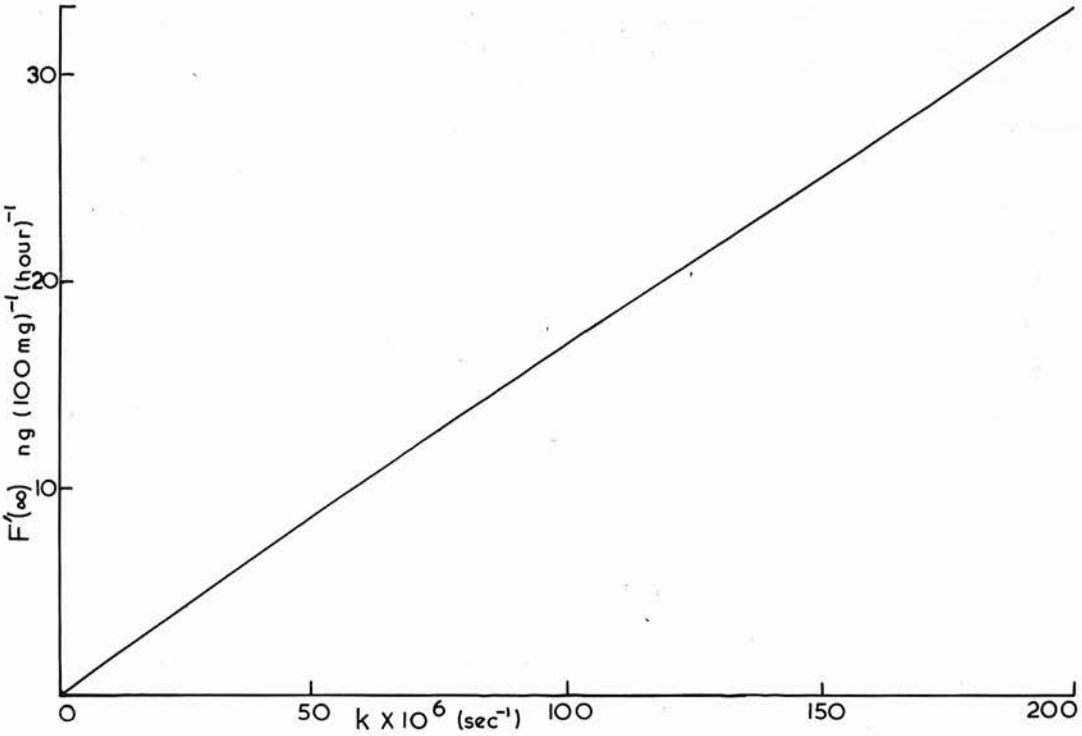
where $F(\infty)$ is the observed steady state rate of uptake of γ-globulin the dimensions of which depend on the dimensions used for c_o and k; see appendix 5) This relationship is shown in Fig.25. Over the range which concerns the present experiments the relation is almost linear, with slope c_o (when appropriate units are used), which implies that the steady state concentration in the extracellular space is not far from c_o. This is seen to be the case when the percentage error in ignoring the slow uptake i.e. $100(1-M(\infty)/M_o)$ is plotted directly against the rate of uptake in Fig. 26. It can be seen that in the example given (inulin space = 48μl/100mg, D'=D/3, distribution of radii ignored), when $F(\infty)$ is 16.4 ng γ-globulin per 100 mg of tissue per hour (which was about the fastest rate of steady uptake seen), the rate constant is $k = 9.77 \times 10^{-5}$ sec^{-1} and from Fig. 26 the steady state quantity of γ-globulin is 1.8% below that calculated assuming equilibrium.

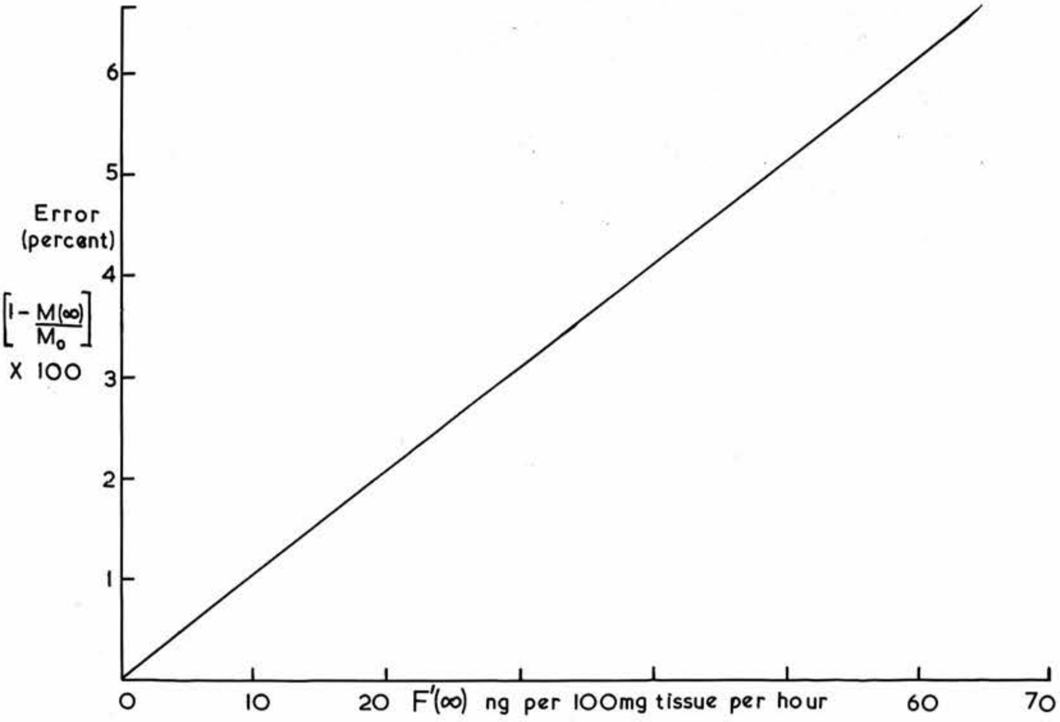
Inserting the value of k into the second equation in this section shows that the concentration at the centre of the cylinder, $c(o, \infty)$, would be about 96.5% of the external concentration in these circumstances.

Since, even with the fastest rate of steady uptake seen, the concentration (and hence reaction rate) vary only slightly throughout the cylinder it would have made little difference if the reaction had been assumed to be zero order (as an exchange reaction might be). The erroneous assumption that equilibrium is reached thus contributes very little to the error of measuring the adsorption of γ-globulin.

Fig. 25 Diffusion of γ-globulin plus first order chemical reaction. Plot of observed rate of uptake (ng per 100mg per hour) in the steady state against 1st order rate constant, k. Calculated for the case $D'=D/3$, constant radius $= 1.87 \times 10^{-2}$ cm, supernatant concentration 1 μg/ml, inulin space 48 μl/100mg.

Fig. 26 Conditions as in Fig. 25. Plot of percentage by which steady state content of extracellular space in presence of slow first order reaction is less than the content which would be found if there were no such reaction, against observed steady rate of uptake.


30
20
10
$F'(\infty)$ ng $(100\text{ mg})^{-1}(\text{hour})^{-1}$
0
50
$k \times 10^6$ (sec^{-1})
100
150
200


6
5
4
3
2
1
Error
(percent)
$\left[1 - \frac{M(\infty)}{M_0}\right]$
X 100
0
10
20
$F'(\infty)$ ng per 100mg tissue per hour
60
70

(d) The measurement of adsorption at equilibrium

(1) Calculation of the extent of adsorption and the adsorption constant

The following definitions will be used:

n'	count rate of drained tissue. c.p.s./100 mg
n	count rate of supernatant solution in equilibrium with tissue. c.p.s./µl
c	concentration of supernatant solution. µg/ml
A	amount adsorbed. ng/100 mg
E	extracellular space. µl/100 mg.
K'	adsorption constant expressed as the adsorption per 100 mg tissue for a supernatant concentration of 1.0 µg/ml. This has dimensions of µl/100 mg and must be distinguished from the dimensionless adsorption equilibrium constant K (see appendix 5).

(a) Inulin method If the equilibrium were reached the concentration in the extracellular space would be c. Therefore

$$A = (n' \times \frac{c}{n}) - Ec = c(\frac{n'}{n} - E)$$

where c/n is the factor (ng/cps) for conversion of count rate to ng γ-globulin. The concentration c may be assumed to be the nominal value but in fact may deviate from this and in most experiments it was inferred from the observed count rate of the supernatant solution using a factor derived from accurate standards done on stock solutions at the beginning of the experiment. When the adsorption isotherm is linear (as it is over the range of concentrations used in the experiments to be described) its slope is a measure of the affinity of the adsorbate for the tissue and is estimated by

$$K' = \frac{\underline{A}}{c}$$

$$= \frac{\underline{n}'}{n} - E$$

It is seen that concentration does not enter into this expression which involves only the ratio of the observed count rates of the tissue and of the supernatant solution, and the volume of the extracellular space. The actual concentration need not be known unless absolute values for the amount adsorbed are required. The amount adsorbed if the supernatant concentrations were any given value can be inferred directly from K' without knowledge of what the concentration actually was. K' does however depend on the weight of tissue and volume unit chosen as standard. It has the dimensions µl/100 mg wet weight. It is shown in appendix 5 that the true (dimensionless) equilibrium constant for adsorption when the isotherm is linear, is

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

Thus

$$K=\frac{n'}{nE} -1$$

which is obviously independent of the weight of tissue to which n' and E are referred (and of volume units when the isotherm is linear). Since nE is the count rate per 100 mg resulting from γ-globulin in the extracellular space it follows that if K=1, i.e. n'=2nE, then the amount of γ-globulin in the extracellular space would be the same as the amount adsorbed.

(b) Wash-off method. The adsorption is estimated by

$$A= n' \times \frac{c}{n}$$

so the adsorption constant will be

$$K' = \frac{n'}{n}$$

when the isotherm is linear.

If this method really measured adsorption, the equilibrium constant would be found as above.

$$K = \frac{K'}{E} = \frac{n'}{nE}$$

(2) Sources of error in adsorption measurement

All methods assume that γ-globulin does not penetrate cells.

The wash off method assumes that the extracellular space is completely emptied of γ-globulin whereas none of the adsorbed γ-globulin is removed.

The inulin method assumes that

(a) the inulin space is the same as the "γ-globulin space" which would be measured if γ-globulin were not adsorbed. In that large molecules usually give a smaller estimate of the extracellular space than small ones, the inulin space might, if anything, be on the large side and therefore the calculated adsorptions too small. This subsumes the usual assumption that inulin is not itself adsorbed.

(b) the concentration of γ-globulin in the extracellular space is the same as in the supernatant solution. This point is dealt with extensively in later sections and there is reason to think that at 60 min. incubation it will not be in great error, though any deviation would again lead to a calculated value of the adsorption which was too small.

It is assumed that antibody γ-globulin is (1) labelled and (2) adsorbed to the same extent as γ-globulin which is not antibody against the antigen used. It has been shown that the former of these two assumptions is true in the case of rabbit anti-ovalbumin (Askonas et al. 1960, Farthing). In that the part of the molecule not containing the antibody sites is the only part which is an effective antigen for reversed passive cutaneous anaphylaxis (Ovary and Karush 1961) it might be expected that the latter assumption was also valid but there does not appear to be any direct evidence on this matter, except results given in the section on adsorption of guinea pig γ-globulins indicating that antibody and "non-antibody" γ_2-globulin are adsorbed to the same extent.

Errors owing to the fact that not all of the ^{131}I is attached to γ-globulin are ignored. The dissociation of the ^{131}I label from γ-globulin has been reported by several workers (e.g. Margan and Tarver, 1957 and Brocklehurst et, al. 1961). The latter authors felt that this might be due to catabolism of protein by the cells. In several experiments the percentage of the radioactivity not precipitable with trichloracetic acid was measured again after the experiment. The solution which had been at 38°C or at room temperature on the day of the experiment was kept at 3°C overnight and analysed 12 hours later. In one such experiment with rabbit anti-BSA γ-globulin which contained 0.36% non-precipitable ^{131}I on the morning of the experiment, this figure had risen to 0.76% non-precipitable after a 4.5 μg/ml dilution of the original had been kept in Pyrex cylinder for 10 hours at room temperature and then about 12 hours at 3°C. The same dilution when kept in a polythene container for 7 hours at 38°C and then 12 hours at 3°C had risen to 2.3% non-precipitable.

A similar experiment with rabbit anti-BSA showed rises from an original value of 0.69% to 1.1% when kept either undiluted (264 μg/ml) or diluted (4.5 μg/ml) in Pyrex vessels at room temperature, and to 2.7% when kept diluted at 38°C for 10 hours in a polythene vessel. In an experiment on the uptake of this particular antibody on chopped lung, there was a noticeable decline in the count rates of the standard solutions taken from the supernatant of each tube as the experiment proceeded, and also of the count rates of the samples and in fact the count rate of the antibody kept at 38°C in polythene was only 70% of the nominal value on the day after the experiment, whereas the count rate of the same diluted antibody kept in Pyrex at room temperature was 98% of its nominal value and the undiluted antibody similarly stored was 100%. Inspection of the polythene tube showed that it had a count rate equivalent to the loss of about 16% of the counts in the solution it contained, and that these counts were not removable even by very prolonged washing. The use of polythene vessels was abandoned after this as it had been found that glass tubes only remove a small proportion of the radioactivity from the solution they contain.

In an experiment with guinea pig γ_1- and γ_2-globulins the preparations were 1.9% and 1.0% non-precipitable respectively at the start of the experiment (the former preparation contained the highest percentage which was ever used). The percentage of precipitable radioactivity was determined in the supernatant solutions which had been incubated with lung tissue for 2 hours at 38°C (and stored in Pyrex at 37° for 2 to 4 hours before this), then kept for a few hours at room temperature and 3°C overnight. Determinations were also done on solutions which had been kept at 38°C in Pyrex without being incubated with tissue and from samples which had been preadsorbed with chopped lung tissue for 1 hour at 38°C. The changes in non-precipitable ^{131}I are shown in Table 5.

Table 5

Percentage of radioactivity not precipitable with TCA

γ_1-globulin (initially 1.9%)	Preadsorbed	38°C	3.4 %
		38°C + 2hr. with lung	8.6 %
	Not preadsorbed	38°C	3.9 %
		38°C + 2hr. with lung	5.1 %
γ_2-globulin (initially 1.0%)	Preadsorbed	38°C	3.9%
		38°C + 2hr. with lung	0 %
	Not preadsorbed	38°C	4.0 %
		38°C + 2hr. with lung	6.3 %

The experimental error in these figures is probably rather large as they had to be determined by difference from the radioactivity of the precipitates because of the low count rate involved. They nevertheless clearly show an increase in non-precipitable radioactivity in dilute solutions of labelled γ globulin and that the increase is larger when incubated with lung than when kept in Pyrex at the same temperature.

It should be pointed out that the proportion of non-precipitable radioactivity would not be expected to be as large as the figures given above indicate at the actual time of the experiment, and that results presented in the section on adsorption of guinea pig γ-globulins show that no difference in apparent adsorption is detectable between preparations with quite widely differing proportions of non-precipitable radioactivity at the start of the experiment, suggesting that this factor is not a very important source of error.

Errors due to loss by adsorption onto tissue should be largely eliminated by the use of standard solutions taken from the supernatant antibody solutions at the end of the incubation period with lung. This was standard procedure in all but the earliest experiments.

In a few experiments the proportion of non-precipitable radioactivity was unacceptably large. In these cases the preparation was put through another Sephadex G-50 column. In several experiments with rabbit γ-globulin this appeared to reduce the specific activity of the protein by much more than could be accounted for by removal of non-precipitable radioactivity. In one case the specific activity fell from 56.1 μc/mg to 42.6μc/mg (i.e. 76%) after running on a 2nd column. The biggest change ever observed was seen with a preparation the specific activity of which was higher than usual, 71 μc/mg falling to 47.6 μc/mg (i.e. 67%). In two experiments with guinea pig γ-globulins the change was not so large. In one case the specific activity of a γ_2-globulin preparation fell from 27.3 μc/mg to 21.2 μc/mg (i.e. 75%), but in another the specific activity of a γ_1-globulin did not fall at all but changed from 24.9 μc/mg to 26.0 μc/mg. This 4% rise is probably within the limits of experimental error as only one determination of protein concentration and count rate was done on each preparation. The reason for this phenomenon was not investigated, but it seems to imply either that the column can distinguish between molecules labelled to different extents (c.f. chromatographic differences between native and ^{131}I labelled human serum albumin reported by Fahey et al. 1958) or that part of the label comes off the protein during the time it is rechromatographed. It is possible that excessive heterogeneity of labelling might result in heavily labelled molecules which remained on the column.

(3) Self-irradiation of labelled protein

The possibility also exists that labelled γ-globulin was altered by self-irradiation. In a solution of small volume most of the radiation dose will be due to β rays which are mostly absorbed in the solution. Only a small proportion of γ radiation will be absorbed. To avoid complications in the interpretation of experiments no protective protein was added to labelled γ-globulin preparations.

The labelled protein hardly ever had a specific activity greater than 60 μc/mg, or was more concentrated than 0.4 mg/ml. The maximum concentration of radioactivity was therefore about 24 μc/ml. The solution was kept not more than 15 hours before starting the experiment.

The dose of radiation can be estimated from the formula given by Yalow (1956), viz.,

Dose (rads) = 120 (μc ^{131}I decayed per ml).

The proportion of ^{131}I decaying 15 hours is 5.24%, i.e. 1.26 $\mu c/ml$ in the above example. The maximum dose of self irradiation is therefore about 151 rad.

Another formula is given by Veall and Vetter (1958), viz.,

Dose rate (rad/hour) = 2.25 x c($\mu c/ml$) x mean energy of β rays

= 0.45 c

taking the mean energy of β rays as 0.2 MeV (Strominger 1958). As c varies with time this must be integrated to get the total dose with the result

$$\text{Dose (rad)} = 0.45\, c_o \left(\frac{1-e^{-kt}}{k}\right)$$

where c_o is the initial concentration ($\mu c/ml$) and $k = 3.5922 \times 10^{-3}$ hours $^{-1}$ is the time constant for the decay of ^{131}I. In the above example this formula estimates the self-irradiation dose to be 158 rad, not much different from Yalow's formula. The difference arises as the constant 120 in Yalow's formula is replaced by $0.45/k$ = 125.2.

The results given by Yalow and Berson (1957) and by Bloom et.al. (1958) enable an estimate of the damage expected from this amount of radiation (if it is assumed that γ-globulin behaves in respect of radiation damage like human serum albumin with which most experiments have been done). According to the results of these authors radiation damage can be measured in terms of a single parameter, the number of mg of protein per ml damaged (as measured by rapid metabolism) per rad. This is independent of protein concentration. For human serum albumin values from 1.2×10^{-6} to 9.0×10^{-6} mg ml^{-1} rad^{-1} have been reported for different preparations. Taking 10^{-5} mg ml^{-1} rad^{-1} as a maximum value implies that not more than 1.5×10^{-3} mg/ml of protein is damaged by 150 rad, the maximum dose received in any of the present experiments. If the protein concentration were 0.2 mg/ml, the maximum value for the percentage damaged by self-irradiation would be

$$\frac{1.5 \times 10^{-3}}{0.2} \times 100 = 0.75\%$$

and in most experiments the figure would be less than this.

Yalow and Berson (1957) also found that human serum albumin retained most of its ^{131}I label after 2.0 k rad and the label was still 94 - 98% precipitable after 45 k rad, so the loss of label cannot be attributed to self-irradiation.

Fig. 27 Adsorption isotherms for rabbit γ-globulin. (Ex 29).

⊙ Inulin method with calculated regression line constrained to go through origin and 95% confidence limits (broken lines) for its slope.

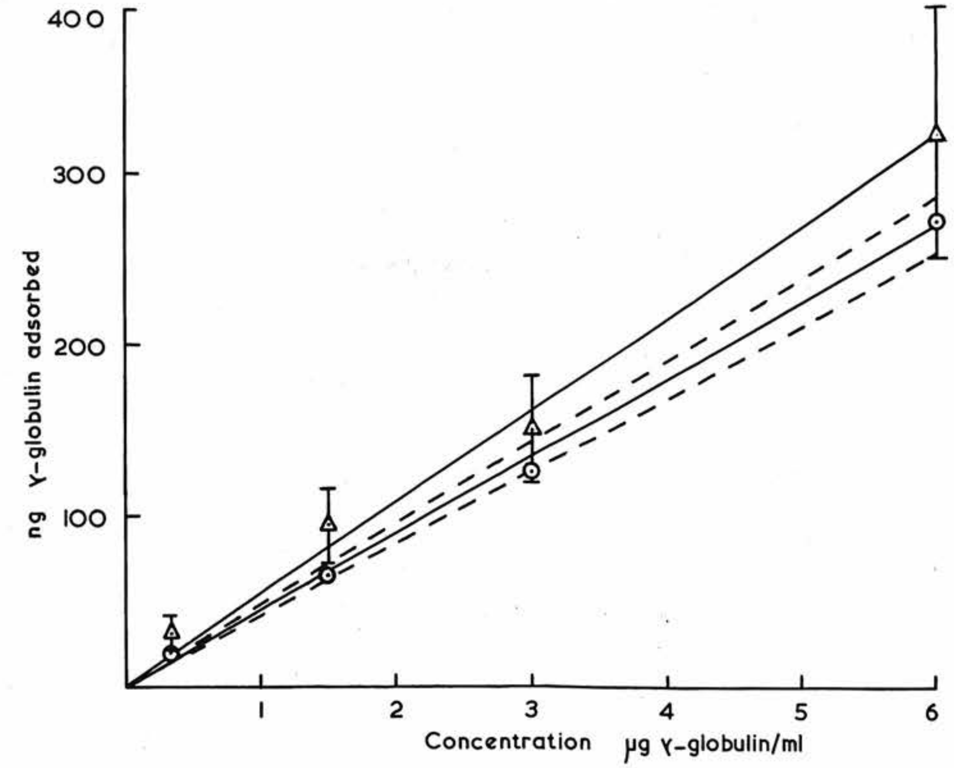
◬ Wash-off method with 95% confidence limits for means (vertical bars).

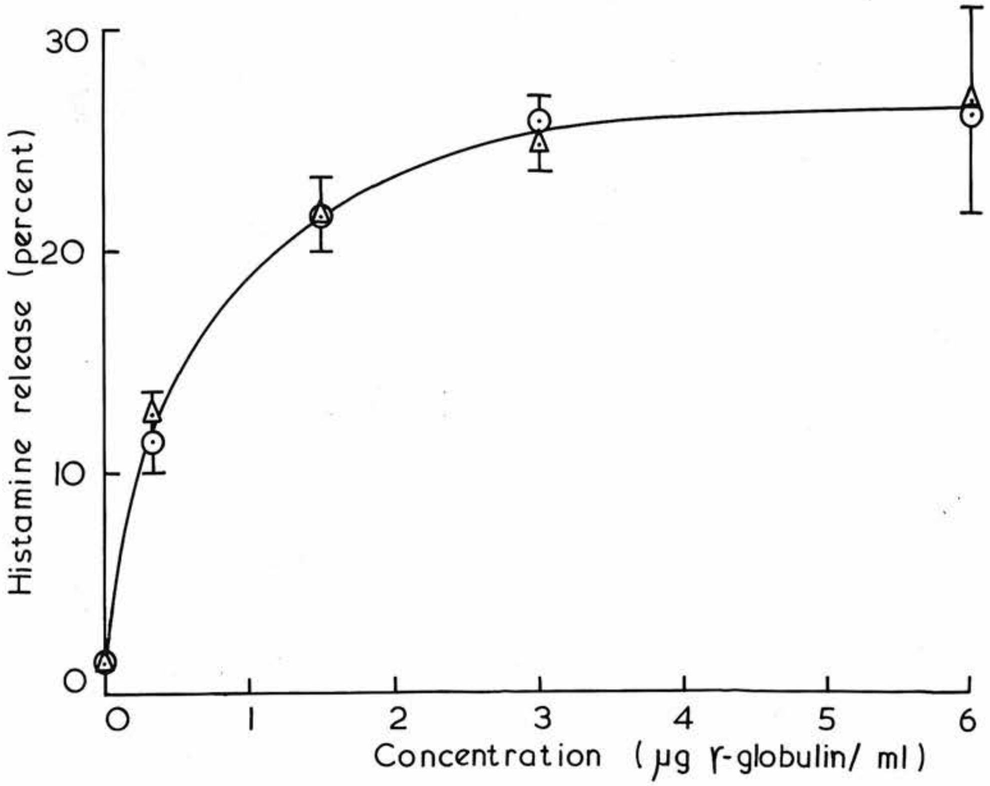
Fig. 28 Histamine release with different treatments of tissue at end of incubation with antibody, for experiment shown in Fig. 27 (Ex 29).

⊙ Drained tissue (no washes)

◬ Three washes with Tyrode

Vertical bars are 95% confidence limits for combined mean.


400
300
200
100
ng γ-globulin adsorbed
1
2
3
4
5
6
Concentration μg γ-globulin/ml


30
20
10
0
Histamine release (percent)
0
1
2
3
4
5
6
Concentration (μg γ-globulin/ ml)

(e) The adsorption isotherm

Adsorption of Rabbit γ-globulin

Adsorption and sensitization were measured over a range of antibody concentrations which produced graded sensitization. Most experiments were done with 1 or 2 hours incubation with antibody. As it was found in later experiments that adsorption is still not complete even at 4 hours most of the curves obtained are not proper isotherms as equilibrium was not attained. Two hours incubation was adopted as a reasonable compromise between possible deterioration of the tissue and achievement of conditions near equilibrium.

No consistent evidence was obtained for other than a linear isotherm, though looking back over the experiments done suggested that possibly there might be a plateau in the isotherm at the point at which sensitization was becoming maximal. There is not enough evidence to establish this, however, and as similar shapes were seen for BSA adsorption isotherms there is no justification at the moment for any other inference than that the deviations from linearity are simply due to sampling error. Statistical analysis of regression confirms this but does not rule out the possibility that a plateau might be detectable with more precise results.

Fig. 27 shows adsorption as a function of antibody concentration. No significant deviations of the isotherm from linearity are detectable by either method of adsorption measurement. The analysis of variance for the wash-off method results, which show the greatest deviation, is given. The regression line has been constrained to pass through the origin as the intercept did not, in this and most other experiments, differ appreciably from zero. The inulin method, as usual, gave lower results (though in this experiment the difference between methods is less than average).

Source	d.f.	M.S.	F	P
Linear reg.	1	62275	652.1	≪0.001
Deviations from linearity	2	1273.7	1.24	>0.2
Between concentrations	3			
Error	16	1030.9		
Total about origin	19			

Fig. 28 shows the sensitization observed in the experiment illustrated in Fig. 27. The rabbit anti-BSA γ-globulin used in this experiment was about 30% precipitable with BSA. The time of incubation with antibody in this experiment was one hour which is not long enough for the surface to reach equilibrium (it has been shown that the e.c.s. would be very close to equilibrium at this time). If the adsorption process were rapid compared with diffusion so that adsorption was diffusion controlled then it is apparent from the equations given that if the isotherm were linear at equilibrium it would also be linear (but with smaller slope) at shorter times.

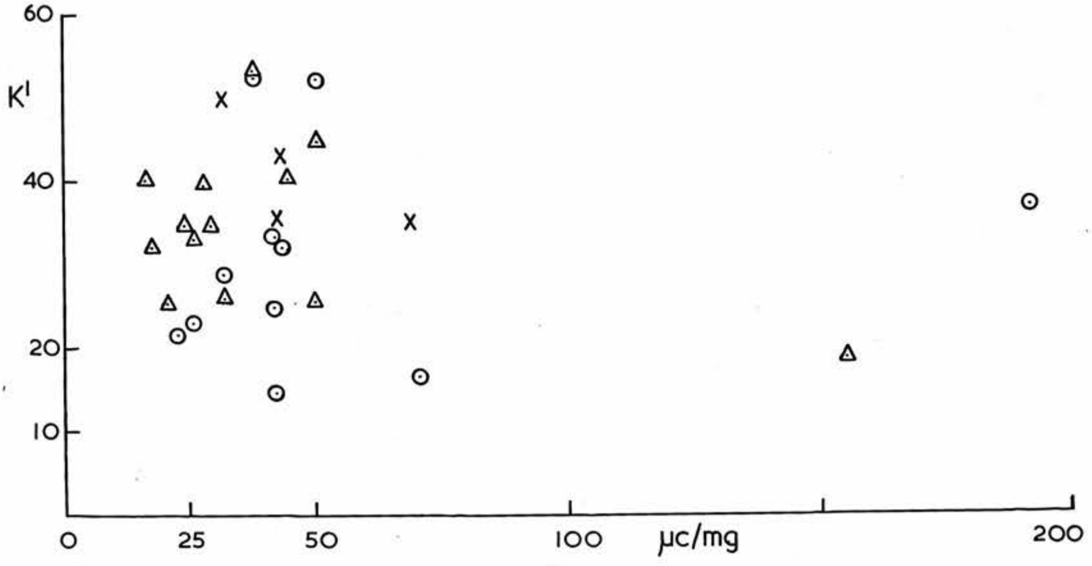
Fig. 29 Adsorption constant, K', for rabbit γ-globulin preparations with different specific activities

△ Wash method. 1 hour incubation. Adsorption per sample.

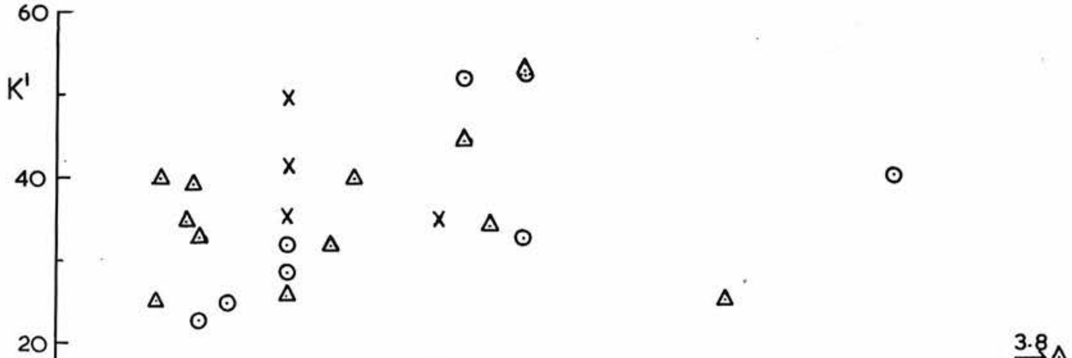
⊙ Inulin method. 1 hour incubation. Adsorption per 100 mg.

× Inulin method. 4 hour incubation. Adsorption per 100 mg.

Fig. 30 As Fig. 29 except that abscissa is average number of atoms of iodine per molecule of γ-globulin.



60
K'
40
20
10
0
25
50
100
μc/mg
200



60
K'
40
20
0
0.5
1.0
ATOMS/MOLE
2.0
3.0
3.8

Values of the adsorption constant (ng RGG per sample, or per 100 mg wet weight for a bulk concentration of 1.0 μg/ml) obtained in different experiments at 1 hour and at 4 hours are shown plotted against the specific activity in Fig. 29 and the number of atoms of iodine per molecule of γ-globulin in Fig. 30. Neither of these experimental variables appears to be correlated with the affinity of the protein for the tissue.

At one hour most of the values obtained by the inulin method fall between 20 and 35ng/100mg (for c=1 μg/ml). Two exceptionally low values (14.5 and 16.6 at one hour) both came from experiments in which the protein had been run through a second Sephadex column to remove excess non-precipitable radioactivity though there is no real reason to believe that this is responsible for the unusually small adsorptions. At 4 hours, when equilibrium has been much more nearly attained, values lying between 35 and 50 ng/100mg for c=1.0 μg/ml were observed.

Results given later for guinea pig γ-globulins confirm the linearity of the adsorption isotherm.

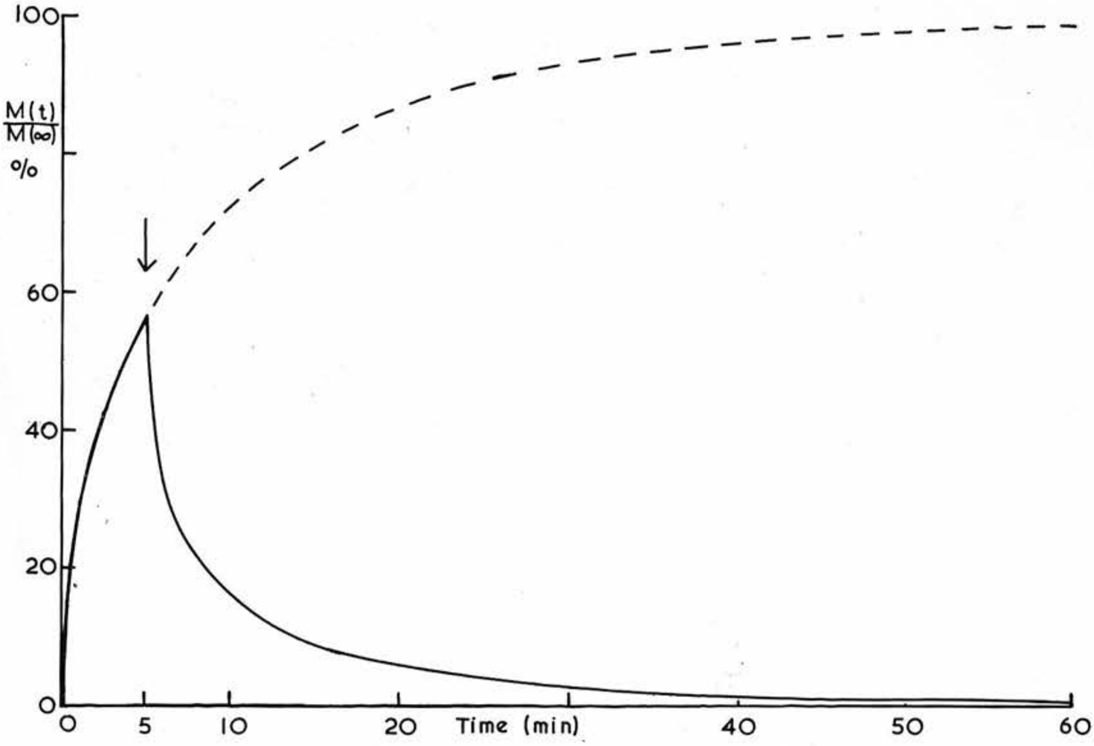
Adsorption of Bovine serum albumin

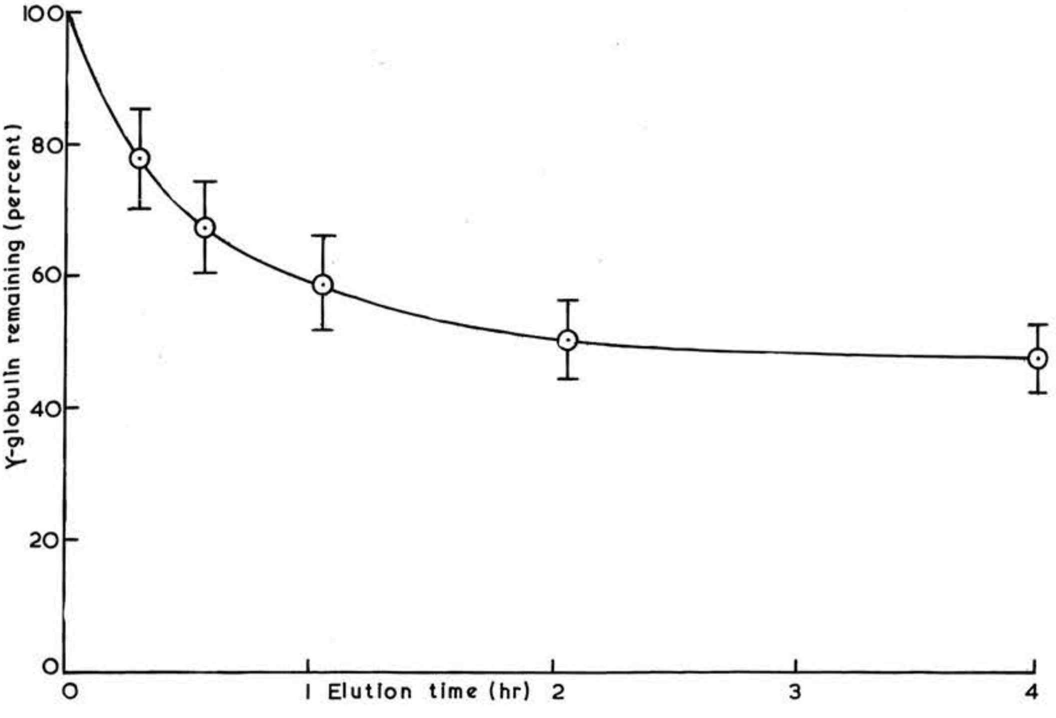
The adsorption of BSA was measured in several experiments. In three experiments by the wash-off method, the adsorption constant at 1 hour appeared to be less than that of γ-globulin - 8.3, 5.6 and 8.0 ng/sample for c= 1μg/ml. However in two later experiments higher values were found viz. 23.4 ng/sample by the wash-off method and 8.3 ng/100mg by the inulin method in one experiment, and in another 50.3 ng/sample by the wash-off method and 40.4 ng/100mg by the inulin method. The affinity in this last experiment was most of those observed for γ-globulin, though there was no obvious reason for the disagreement with other experiments. The BSA used in the experiment giving the highest result had a specific activity of 27.7 μc/mg and 0.91 atoms iodine/molecule protein, less than in all but one of the other experiments, though it is possible that variability in the heterogeneity of labelling between experiments might make the average number of atoms of iodine per molecule misleading.

These results certainly indicate that BSA is adsorbed to some extent but are not in satisfactory quantitative agreement. It would be interesting to repeat them using guinea pig albumin.

Fig. 31 Calculated percent equilibration of extracellular space with γ-globulin (Model 1; based on Curve 3 of Fig. 21) when concentration external to cylinders is reduced to zero after 5 min. The dotted line is the calculated curve if the external concentration is maintained at c.

Fig. 32 Amount of γ-globulin remaining in tissue after various periods of elution (following 2 hour period of adsorption) expressed as percentage of net adsorption at end of adsorption period. Means with 95% confidence limits.


100
M(t)/M(∞) %
60
40
20
0
0
5
10
20
Time (min)
40
50
60


100
80
60
40
20
0
γ-globulin remaining (percent)
0
1
Elution time (hr)
2
3
4

(f) The desorption of rabbit γ-globulin from lung tissue

It can be shown that the calculated curves in Fig 21, as well as representing the filling of the extracellular space from an external constant concentration, also predict the rate at which the extracellular space, if initially at a uniform concentration, would wash out if the external concentration were suddenly reduced to zero. The ordinate is simply interpreted as the percentage of γ-globulin washed out, i.e. the proportion remaining in the tissue is $1-M(t)/M(\infty)$ where $M(t)/M(\infty)$ are the figures plotted in Fig. 21. In fact the linear nature of the differential equations involved makes the more general statement true, that if the external concentration is reduced to zero even before equilibrium has been reached, the curve representing the amount of material present in the extracellular space can simply be found by subtracting from the rising curve an exactly similar curve descending from zero (i.e. $-M(t)/M(\infty)$) starting at the time the external concentration is made zero. The predicted curve (based on curve 3 of Fig. 21) for the wash-out of the extracellular space after only 5 min. contact with γ-globulin solution is shown in Fig. 31. The curve is unsymmetrical and relatively slow (because diffusion will still be taking place inwards near the centre of the cylinders while material is being lost from the outer part).

It is evident from Fig. 21 and Fig. 31 that the usual washing procedures cannot be expected to empty the extracellular space whether or not it has equilibrated with the external solution. Three standard washes with Tyrode's solution do not usually occupy more than three or four minutes. If the surface and the extracellular space had equilibrated with the external solution, the percentage of extracellular γ-globulin washed out during 3½ minutes can be predicted from Fig. 21.

On model 2 (slow adsorption equilibrium compared with diffusion), curve 1 of Fig. 21 predicts that 65% of the extracellular γ-globulin and none of the adsorbed γ-globulin (ex hypothesi) would be removed in 3½ minutes. If K = 1.0 so that the amount of γ-globulin adsorbed is equal to the amount in the extracellular space, then loss of 65% of the latter will mean that 100 x (2-0.65)/2 = 67.5% of the total γ-globulin content of the drained tissue should remain after washing, or rather less if, as is usual, the adsorbed γ-globulin makes up a rather smaller proportion of the total.

After 3½ minutes washing, according to model 1 (rapid adsorption equilibrium) (Fig. 21, curve 3), only 48% of the extracellular γ-globulin would be removed, but 48% of the adsorbed γ-globulin would be also, so that 52% of the total γ-globulin would remain after washing. This is quite close (by cancellation of errors) to the 40 or 50% which would remain if all the non-adsorbed, and none of the adsorbed material were removed.

The experimental results, as expected, fell in between the predictions of the two theoretical models. For example the results in Fig. 20 show that the proportion of the total γ-globulin remaining in the tissue after three washes was 55 to 60% at the 2, 3 and 4 hour points. In the elution experiment referred to below, 3 washes of drained tissue which had been equilibrated for two hours, left about 66% of its γ-globulin content unremoved.

The predictions of the two theoretical models are too similar for results of this sort to distinguish between them.

The elution of γ-globulin from the tissue was investigated in one experiment with labelled rabbit γ-globulin. The tissue was incubated for 2 hours with rabbit anti-BSA γ-globulin (2.25 μg protein/ml), then drained, weighed, counted, washed 3 times in the standard way and incubated for various times in 5 ml of Tyrode's solution. The net amount adsorbed at two hours, was measured by the inulin method (K' = 30.5, i.e. within the usual range). The proportion remaining after various elution periods is shown in Fig. 32. About half of the adsorbed material seems to be removed within an hour but the rate of loss between one and four hours was very slow. There is, of course, no way of telling how much, if any, of the remaining γ-globulin was actually inside the cells. The sensitization in this experiment was rather erratic but certainly declined after prolonged elution. No great loss of ability to release histamine with time has been observed in experiments of similar length sithout prolonged elution.

Since three washes alone gave a value of K'=52.2, the concentration of γ-globulin in the 5 ml solution used for elution could not have been more than 10 ng/ml (per 100 mg tissue for an initial bulk concentration of 1 μg/ml) which, at equilibrium, would result in the presence of about 0.8 ng γ-globulin /100mg in drained tissue. This figure corresponds to about 2.6% on the ordinate of Fig. 32, and therefore shows that the flattening of the elution curve cannot be attributed to the fact that the volume of eluting fluid is not infinite.

It should be possible to test the hypothesis that γ-globulin becomes more strongly adsorbed with time by looking at the proportion eluted after incubation for various times with γ-globulin. In two experiments in which tissue samples were incubated for periods of ½, 1, 2, 3 and 4 hours with rabbit anti-BSA γ-globulin (2.25 μg protein/ml) and then washed three times and eluted for 15 minutes in 5 ml Tyrode's solution as above, the proportion of the <u>net</u> amount of γ-globulin adsorbed (measured by the inulin method) remaining after this elution, was similar (about 70%) at each time as shown in Table 6 . There is therefore no evidence for increasing firmness of adsorption with time, and this might also be interpreted as evidence against penetration of the label into cells. The proportion of the <u>total</u> γ-globulin content of drained tissue (absorbed and extracellular) remaining after elution did increase with time, but it is easily shown that if the proportion of adsorbed material remaining after elution is constant, this must inevitably be the case while adsorption is still increasing.

Table 6

Proportion of rabbit γ-globulin remaining on on lung tissue after 3 washes and 15 min. incubation in Tyrode's solution, following different periods incubation with labelled γ-globulin.

(a) amount remaining as percentage of amount of γ-globulin adsorbed (measured by the inulin method)

(b) amount remaining as percentage of total amount of γ-globulin (adsorbed and free) present in drained tissue.

Experiment 36			Experiment 41	
Incubation time (hours)	(a)	(b)	(a)	(b)
½	70.1	30.9	67.3	21.3
1	67.4	32.9	69.8	26.9
2	70.2	38.5	74.2	33.3
3	68.9	41.7	75.0	35.3
4	75.6	48.1	66.4	34.6

In neither of these experiments was there any detectable loss of sensitization during elution, though 30% of the adsorbed γ-globulin was removed. The results for experiment 36 have been shown in Fig. 19.

(g) Lack of effect of changing calcium concentration during sensitization.

In two experiments lung tissue was incubated for one hour with labelled rabbit anti-BSA γ-globulin (1.0 μg protein/ml) in Tyrode's solution containing various concentrations of calcium. The tissue was washed with the solution of appropriate calcium concentration and drained before starting the incubation with antibody. Solutions of inulin in the same low calcium media, incubated with tissue for 1 hour, were used for determining the inulin space. In the experiment quoted below the calcium concentration was returned to normal (i.e. 1.8 mM) about 15 min. before addition of antigen. It is quite probable that the calcium concentrations are not very accurate at the lower levels, owing to the incomplete removal of calcium from the tissue before placing in the low calcium solution, but the concentrations must nevertheless be grossly different from normal.

It was shown (Table 7) that the inulin space measured after 1 hour equilibration did not vary with calcium concentration and it was therefore inferred that the adsorption could be determined by the inulin method.

Table 7

Inulin space at various calcium concentrations.

Nominal calcium conc. mM.	Inulin space µl/100mg observations	mean
0	52.1 52.2	52.2
0.0083	50.2 50.1	50.1
0.05	50.9 48.4	49.7
0.3	54.0 50.4	52.2
1.8	52.9 50.9	51.9

The results of adsorption measurements at different calcium concentrations, and the ensuing sensitization (measured in 1.8 mM calcium) are shown in Table 8 .

Table 8

Adsorption and sensitization with various calcium concentrations during the adsorption stage. Means of 5 replicates.

Nominal calcium conc. mM.	Adsorption constant K' µl/100mg	Histamine release ng/sample
0.000	22.9	152
0.0083	21.9	160
0.05	26.9	175
0.3	25.9	145
1.8	27.4	163

Analysis of variance of these results confirms that there is no evidence at all for any difference between the extents of adsorption or sensitization observed at different calcium levels.

It is relevant to point out that unless chelating agents are used there is probably a considerable molar excess of calcium relative to γ-globulin, as the concentration of the latter is only about 6.5×10^{-6} mM. This is even more important when it is remembered that whatever calcium is present will tend to localize on negatively charged cell surfaces to a far greater extent than monovalent ions, and it is in this position that the calcium might conceivably play a role in the bonding of antibody to the surface. No experiments have been performed with chelating agents.

(h) Adsorption and sensitization at low temperature

One experiment was performed with ^{131}I labelled rabbit γ-globulin incubated with lung tissue in a bath containing ice. The results show that adsorption proceeded slowly and roughly paralleled sensitization. After 52 hours the tissue was evidently not in good condition as shown by the fall in histamine release and apparent adsorption and also by a rise in the inulin space at this time. The values of K' are exceptionally high but the significance of this has not been investigated.

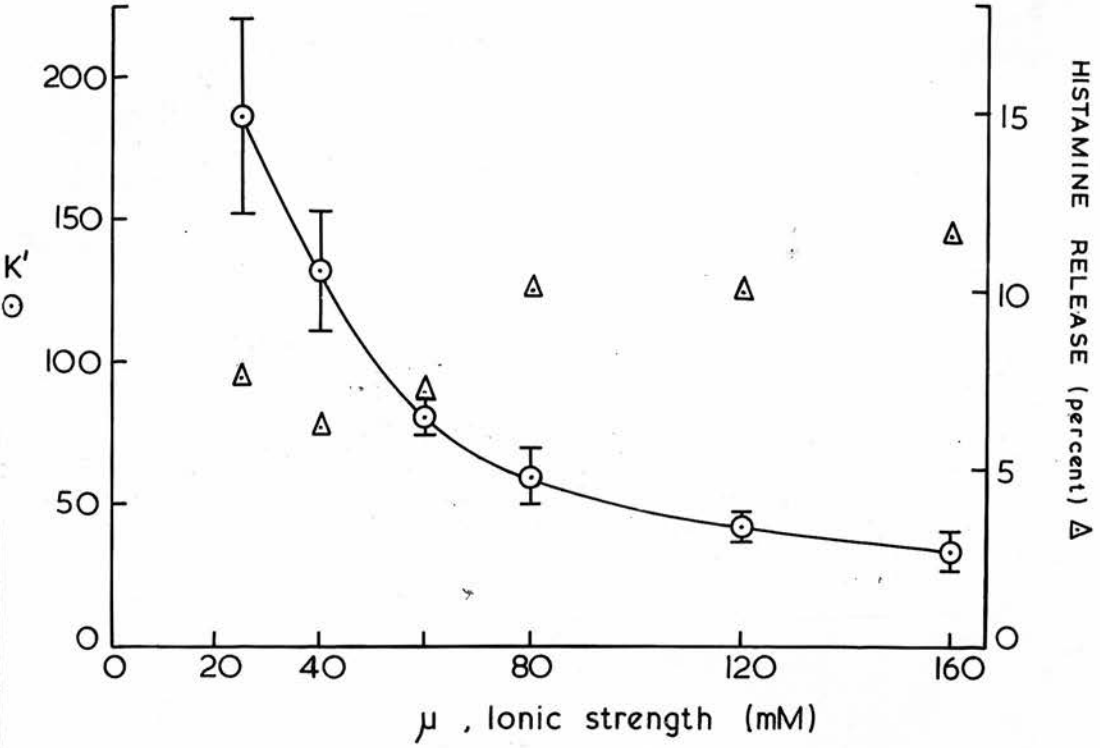
Time (hours)	Adsorption (K')	Histamine release (percent)
1	47.2	2.0
4	94.3	4.0
10	101.6	12.7
20	104.5	20.1
35	85.5	24.5
52	77.0	13.3
Control		1.8

The impression from previous experiments that the sensitization may still rise quite steeply when the adsorption has become almost constant is confirmed.

Fig. 33 Effect of ionic strength on adsorption of labelled rabbit γ-globulin. Ex. 32. Tyrode's solution with varying proportions of sodium chloride replaced by an isosmotic quantity of sucrose. Nominal protein concentration, 1.5 μg/ml. Label; 14.6 μc/mg, 0.61 atoms I/mole. Incubated 1 hour with antibody. Ionic strength restored to normal (indicated by arrow) before adding antigen.

⏀ Adsorption constant K' with 95% confidence limits

△ Histamine release (percent).


K'
⊙
200
150
100
50
0
0
20
40
80
120
160
μ , Ionic strength (mM)
HISTAMINE RELEASE (percent) Δ
15
10
5
0

(i) 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. The results of an experiment in which part or all the sodium chloride of Tyrode's solution containing labelled rabbit anti-BSA γ-globulin was replaced by an isosmotic amount of sucrose are shown in Fig. 33. The adsorption is seen to rise sharply at low ionic strengths. The form of the curve is roughly what would be expected on physicochemical grounds. It can also be seen that the histamine release on challenge with antigen (carried out at normal ionic strength i.e. μ= 160.6mM) did not increase in parallel with the increased adsorption of antibody, in fact tended to be somewhat lower and erratic when the ionic strength of the antibody solution used for sensitization had been low. It was found, as might be expected, that the concentration of antibody in the supernatant solution at the end of incubation declined as the ionic strength was reduced. This is corrected by using standard solutions from the supernatant, but means that the actual amounts of antibody adsorbed do not rise quite as steeply as the adsorption constants when ionic strength is reduced.

Similar results were seen in 7 other experiments. The adsorption of antibody was greatly increased in solutions in which part or all of the electrolytes were replaced by the non-electrolytes, sucrose, glucose, mannitol, sorbitol or raffinose, or by the dipolar ion glycine. When the solution containing antibody had no electrolyte at all added to it the adsorption was increased still further, by up to 15 times. For example the adsorption constants (K') with 95% confidence limits (as % of mean) found in an experiment with 1 hour incubation were

Normal Tyrode $K' = 40.7$ (±12.6%)
NaCl-free Tyrode + glycine $K' = 261.3$ (±14.3%)
Isosmotic glycine $K' = 586.5$ (± 6.7%)
Isosmotic buffered sucrose $K' = 623.3$ (± 7.2%)

The ionic strengths quoted are probably not accurate. The conductivity of isotonic buffered sucrose solution which had been in contact with tissue was considerably larger than that of the original solution suggesting that ions incompletely removed from the extracellular space or escaping from the cells were present.

The histamine release in this, as in all other experiments in which ionic strength was varied did not increase to any significant extent, in spite of the large amount of antibody adsorbed. Only in isosmotic glycine was there any sign of increased sensitization and this was not a large effect.

It seemed possible that the tissue was damaged in some way by incubation at low ionic strength. In a control experiment actively sensitized tissue was incubated in various low ionic strength solutions and then challenged in normal Tyrode. It was found that one hour incubation in isosmotic buffered sucrose and probably also isosmotic buffered glucose caused reduction in histamine release ($P = 0.01-0.001$ and $P < 0.05$ respectively). The total tissue histamine content was probably also reduced.

Other results, though not fully investigated, suggest the possibility that the tissue was not in good condition. For example tissue samples incubated in isosmotic glycine and isosmotic buffered sucrose were heavier than those incubated in Tyrode suggesting swelling. However those incubated in NaCl-free Tyrode and glycine were normal in weight but still showed no increased histamine release.

The inulin space of the tissue also appeared to be larger in low ionic strength solutions than in Tyrode. For example in isosmotic glycine the mean inulin space of tissue was 55.4 µl/100mg (95% conf. limits ± 4.6%) compared with 44.6 µl/100mg (95% conf. limits ± 5.6%) in normal Tyrode in the same experiment. The difference is statistically significant ($P < 0.001$). In another experiment the mean inulin space in isosmotic buffered glucose was found to be 58.1 µl/100mg (95% conf. limits ± 6.5%) compared with the inulin space in Tyrode of 44.8 µl/100mg (95% conf. limits ± 6.0%). Again a significant difference ($P < 0.001$). In this experiment the mean glucose space was 79.1 µl/100mg.

The sucrose space was, as might be expected, rather higher than the inulin space. When determined with NaCl-free Tyrode containing 84.7 g sucrose/kg, the sucrose space was 61.7 µl/100mg (95% conf. limits ± 2.4%) compared with an inulin space of 48.4 µl/100 mg (95% conf. limits ± 8.6%) in the same experiment. When determined with 1% sucrose in Tyrode (with an isosmotic amount of NaCl omitted) the sucrose space was certainly no lower, being 66.2 µl/100mg (95% conf. limits ± 2.1%) compared with an inulin space of 46.9 µl/100mg (95% conf. limits 3.5%) in the same experiment. However in one experiment in which the sucrose space was determined with isosmotic buffered sucrose (which contains no calcium, magnesium, potassium, phosphate or glucose) the sucrose appeared to penetrate the whole tissue water. The sucrose space was 89.1 µl/100mg (95% conf. limits ± 3.5%) and the weight of solids in the tissue, after making a quite large correction for the weight of sucrose in the dried tissue, 11.2 mg/100mg (95% conf. limits ± 6.2%). The total, taking the density of the tissue fluid as 1.0, is 100.3 mg per 100 mg wet weight.

Although at physiological ionic strength the γ-globulin content of tissue is similar to that which would be found for a non-adsorbed material which penetrated cells, at low ionic strengths it is much greater.

PART II

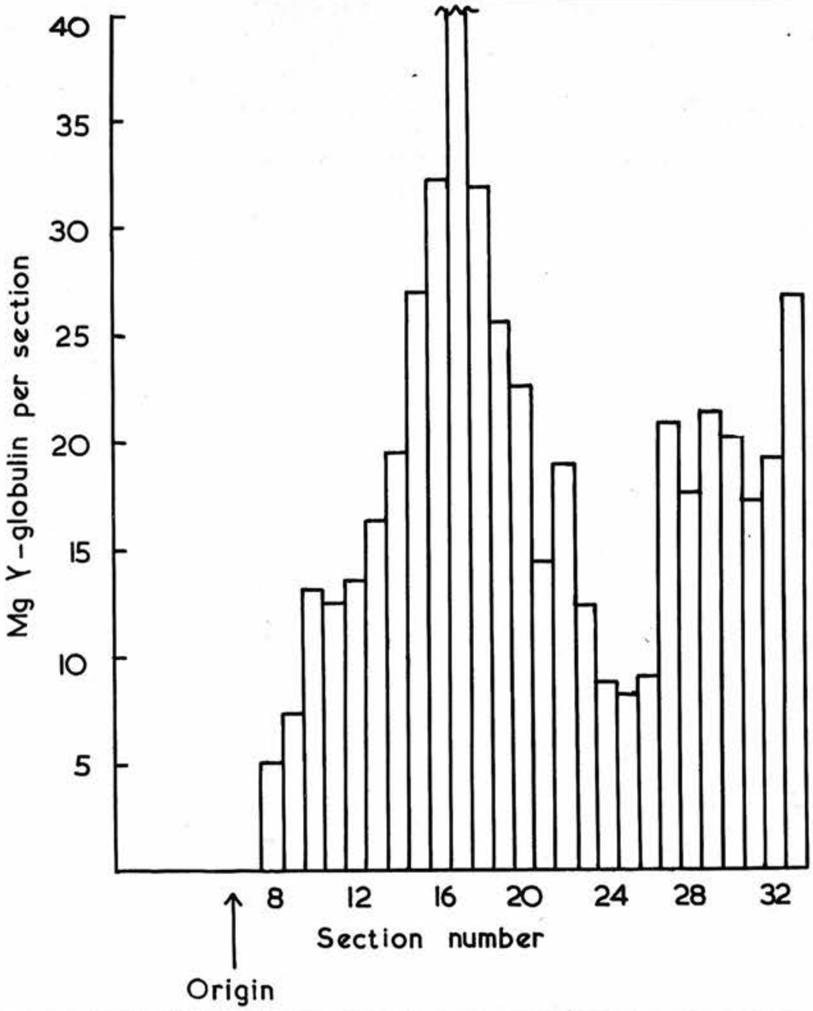
THE SEPARATION AND PROPERTIES OF TWO GUINEA PIG IMMUNOGLOBULINS

Introduction

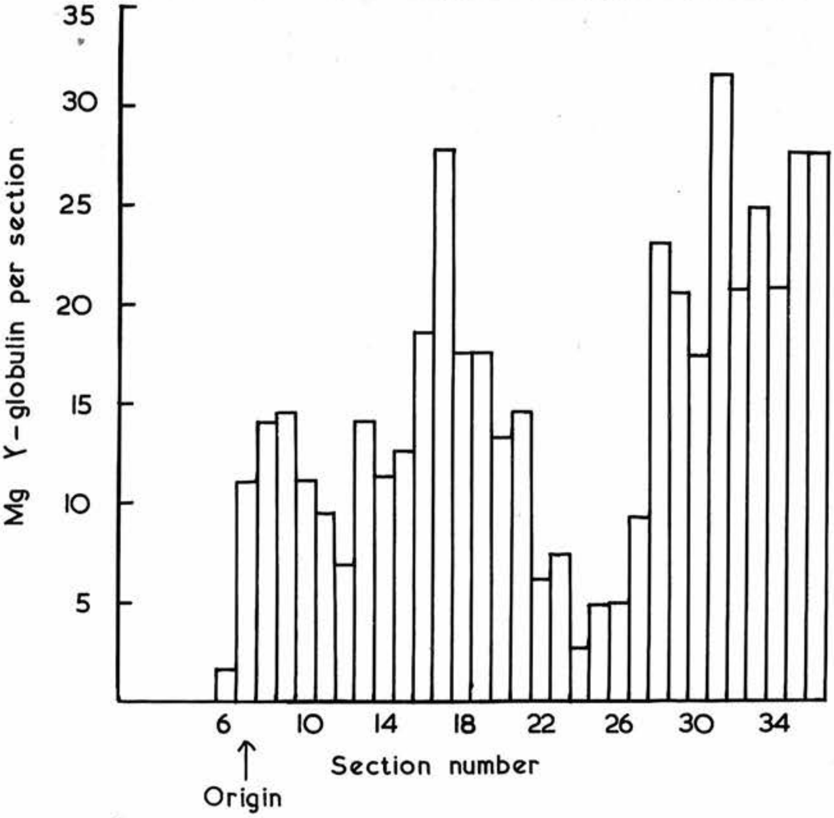
All the work so far described was done with rabbit γ-globulin. This was isolated by DEAE cellulose chromatography and the first peak, which was eluted at low ionic strength, used for sensitization and adsorption experiments. This protein, as expected by analogy with the behaviour of human, guinea pig and mouse immunoglobulins on DEAE cellulose, had the mobility of a γ_2-globulin. It sensitizes the skin and lung of the guinea pig in quite low concentrations. Nevertheless, if the mechanism of anaphylaxis in the guinea pig is being investigated it seems preferable to use guinea pig γ-globulins. At the time the work to be described was started little was known about these proteins, so an investigation was started using similar methods to those used by other workers for the fractionation of human immunoglobulins.

Fig. 34 Elution diagram of preparative electrophoresis (Pev. 18) 1 cm sections. Pevikon block 36 x 19 x 1.5 cm. pH 8.6 0.09M barbitone. 20 ml guinea pig anti-ovalbumin serum GP 19–21 concentrated threefold. Slow γ-globulin sections 7 to 15. Fast γ-globulin sections 16 to 20.

Fig. 35 Block and buffer as for Fig. 34. 14.5 ml guinea pig anti-OA serum GP 26. Slow γ-globulin - sections 6 to 13. Fast γ-globulin sections 14 to 20.



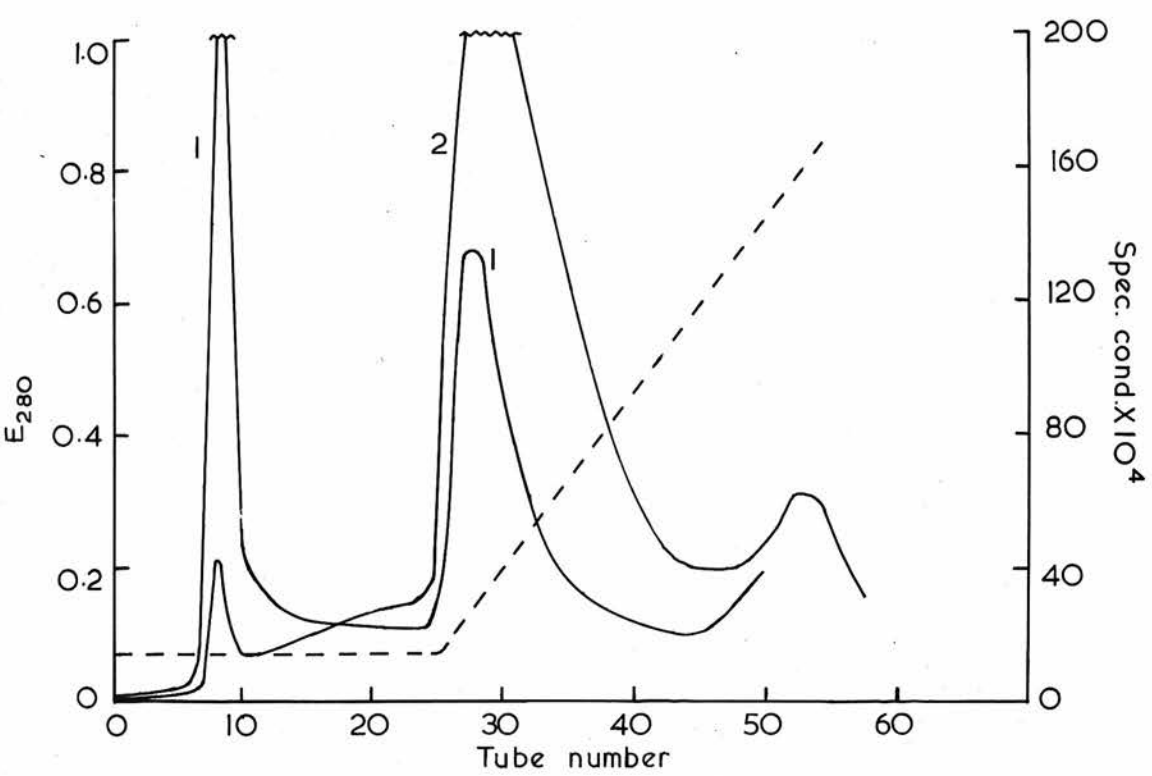
Mg Y-globulin per section
40
35
30
25
20
15
10
5
8
12
16
20
24
28
32
Section number
Origin



Mg Y-globulin per section
35
30
25
20
15
10
5
6
10
14
18
22
26
30
34
Section number
Origin

Fig. 36 Elution pattern from DEAE cellulose columns (94). Ordinate is optical density at 280 mμ. Columns 1 and 2 were loaded with the slow and fast (respectively) total γ-globulin fractions obtained by the preparative electrophoresis shown in Fig. 34. Dotted line represents specific conductivity of the eluate (mho cm^{-1})

Fig. 37 As Fig. 36 but using slow (column 1) and fast (column 2) total γ-globulin from the preparative electrophoresis shown in Fig. 35.


1
2
1
E280
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Tube number
200
160
120
80
40
0
Spec. cond.X 10⁴

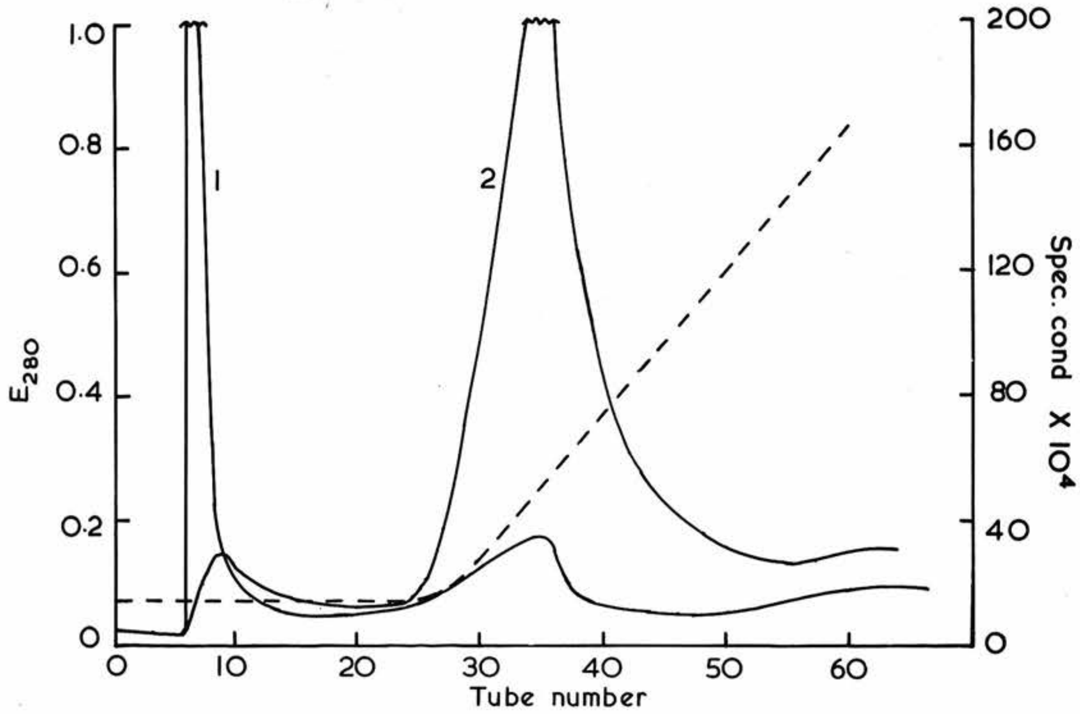
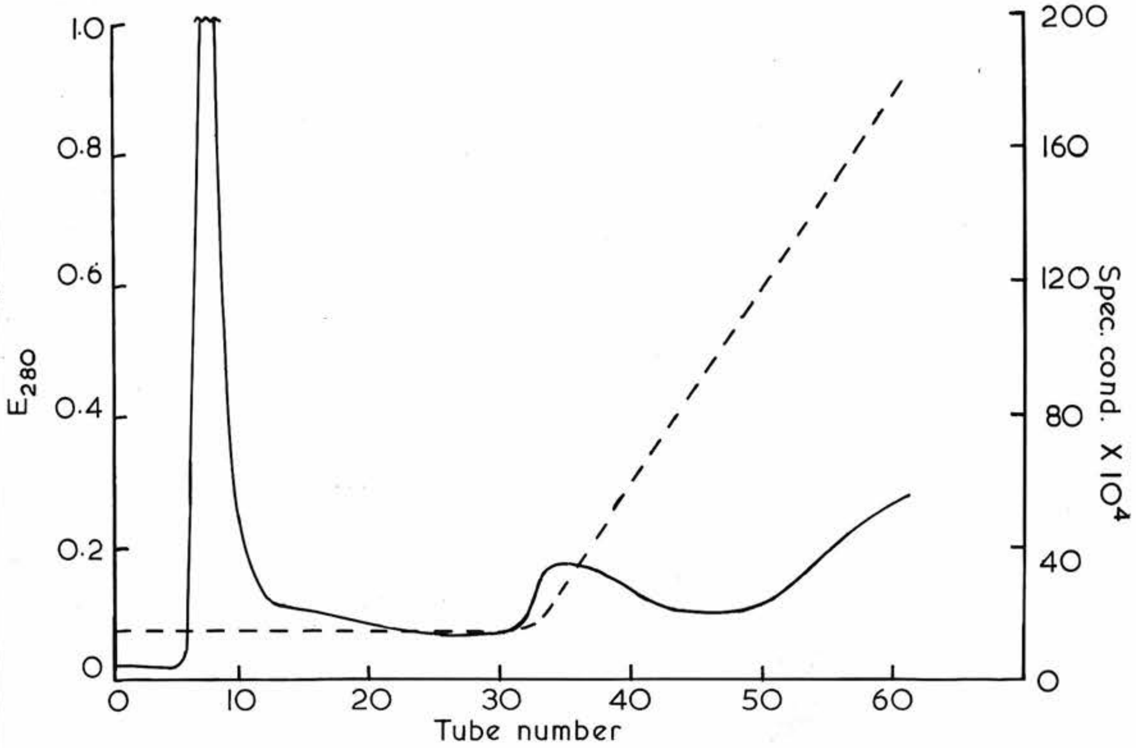

1
2
E280
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Tube number
200
160
120
80
40
0
Spec. cond X 10⁴

Fig. 38 DEAE cellulose column (95) elution pattern of slow and medium mobility total γ-globulin isolated by preparative electrophoresis from normal guinea pig serum.



1.0
0.8
0.6
0.4
0.2
0
E_{280}
200
160
120
80
40
0
Spec. cond. X 10^4
0
10
20
30
40
50
60
Tube number

(a) <u>The separation of guinea pig γ_1- and γ_2-globulins</u>.

Separation of guinea pig sera by preparative electrophoresis to obtain total γ-globulin is illustrated in Figs. 34 and 35. The separation shown in Fig. 34 was of 20 ml bath 19-21 guinea pig anti-OA serum which did not contain much precipitating antibody in the γ_2-globulin. Little or no resolution is visible in the γ-globulin region, which was arbitrarily divided into a fast and slow region, as shown. These fast and slow total γ-globulin fractions were then separated on DEAE cellulose columns as shown in Fig. 36. A sharp first peak is eluted with the initial buffer, pH 8.2 0.01M sodium phosphate, and a second peak of protein starts to come off the column as soon as the increasing gradient of ionic strength is started, with a peak at a specific conductance of between 30 and 40 x 10^{-4} $\text{ohms}^{-1}\,\text{cm}^{-1}$. It is shown later that the first peak contains virtually only γ_2-globulin and the second peak mostly γ_1-globulin. A small third peak was usually seen but its nature was not investigated.

In Fig. 35 the separation of 14.5 ml of batch26 guinea pig anti-OA serum is shown. The γ-globulins are partially resolved into two components. When the fast and slow components were run on DEAE cellulose columns it was found (Fig. 37) that the slow one was mostly γ_2-globulin and the fast one was mostly γ_1-globulin. Immunoelectrophoretic results presented later show that the second peak contains γ_1-globulin (and also fast γ_2-globulin). These results probably cannot be interpreted quantitatively with any accuracy but do suggest that immune sera contain more γ_1-globulin than γ_2-globulin.

In contrast with the results on immune sera Fig. 38 shows the separation on DEAE cellulose of the total γ-globulins from 22ml of normal guinea pig serum (pooled from 3 unimmunized animals). There is seen to be considerably less protein eluted in the γ_1-globulin region than eas observed with immune sera. A small fast fraction (not shown) contained very little protein in the γ_2-globulin region and only a small peak in the γ_1-globulin region. The results of investigations of the purity of these fractions are given later.

Fig. 39 MIE(58)16

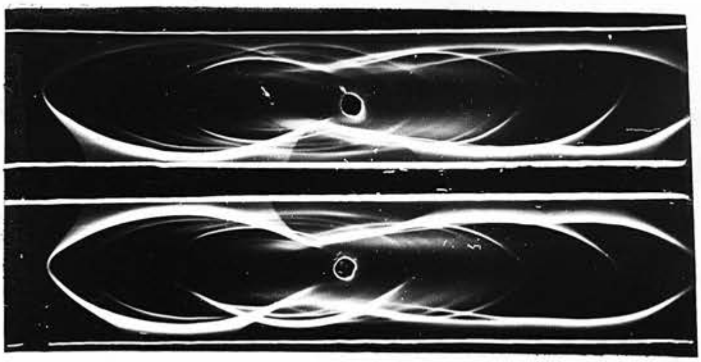
Trough	Mann Res. Labs. Rabbit anti-GPS (RP 9-1862 K-1400)
Well	GP anti-OA serum (23 pooled)
Trough	Rabbit anti-GPS (R94+99)
Well	GP anti-OA serum (23 pooled)
Trough	Wellcome rabbit anti GPS (concentrated x 3)

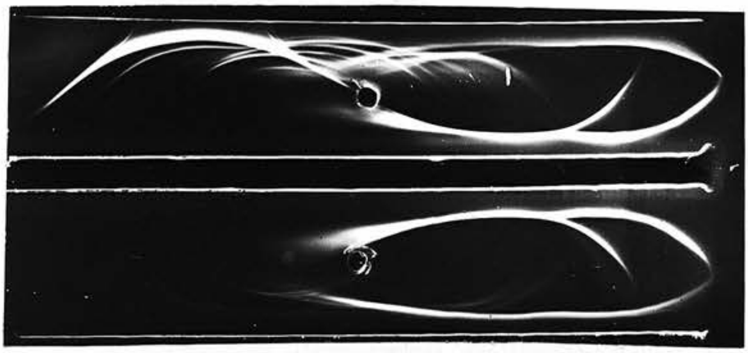
Fig. 40 MIE(58)14

Trough	Rabbit anti-GPS (R94)
Well	GP Anti-OA serum (23 pooled)
Trough	Rabbit anti-GP γ_1-globulin (R99)
Well	GP Anti-OA serum (23 pooled)
Trough	Mann Res. Labs. Rabbit anti-GP γ-globulin serum (RP4 - 1862 J-1872)

Fig. 41 MIE (64)12

Well	GP anti-OA serum (27 pooled)
Trough	Rabbit anti-GPS (R105)





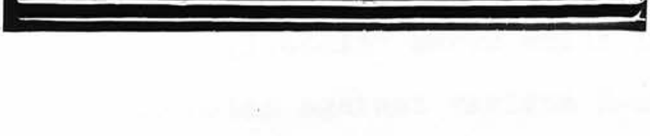


Fig. 41

(b) Immunoelectrophoretic analysis of normal and immune guinea pig sera and purified proteins.

In the earlier stages of this work commercially available rabbit antisera against guinea pig serum were used. Fig. 39 shows the pattern produced by immune guinea pig serum (batch 23) developed with three antisera. The central trough contained a polyvalent mixture of antisera (2 volumes R94 anti GPS + 1 volume R99 anti-GP γ_1-globulin) and clearly shows γ_1- and γ_2-globulin arcs. Neither of the commercial antisera contain a useful concentration of antibodies against the specific antigenic determinants of γ_1-globulin even though the Wellcome antiserum had been concentrated three fold (using Biodryex). Some rabbit anti-GPS sera, e.g. R95 and R97, were weak in antibodies to all sorts of γ-globulin, and all rabbit antisera made against normal guinea pig serum e.g. R90 and R94 were, like the commercial antisera, weak in antibodies against γ_1-globulin. This is shown in Fig. 40. This figure also shows the pattern produced by a commercial "rabbit anti guinea pig γ-globulin" serum which is seen to contain antibodies against various β-and α-globulins, but not against γ_1-globulin. Two antisera, R104 and R105, made against immune guinea pig serum did however show antibodies against γ_1-globulin as shown in Fig. 41. For most of the immunoelectrophoresis patterns one of the following polyvalent antisera was used:

R94 + 99	Anti-normal GPS R94	2 vols.
	Anti-GPγ_1-globulin R99	1 vol.
R90 + 102	Anti-normal GPS R90	2 vols.
	Anti-GPγ_1-globulin R102	1 vol.
R90 + 102 + 106	Anti-normal GPS R90	2 vols.
	Anti-GPγ_1-globulin R102	1 vol.
	Anti-GPγ_2-globulin R106	1 vol.

Normal guinea pig serum characteristically produced the immunoelectrophoretic pattern shown in Fig. 42. At first it was thought that normal serum contained no γ_1-globulin at all on the basis of this pattern. However when normal guinea pig serum is tested with antiserum against γ_1-globulin (R99), it is quite clear (see Fig. 43) that it contains some γ_1-globulin though apparently in a lower concentration than serum from immunized animals. The fact that anti γ_1-globulin forms a strong precipitation arc with both γ_1- and γ_2-globulins,

Fig. 42 MIE(58)13

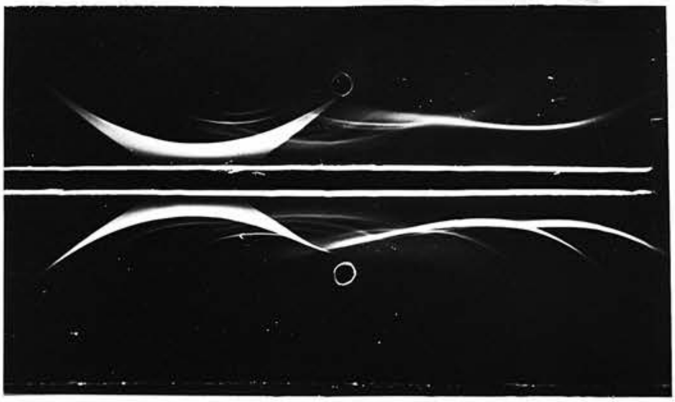
Well	Normal GPS
Trough	Rabbit anti-GPS (R94 + 99)
Well	GP anti-OA serum (23 pooled)

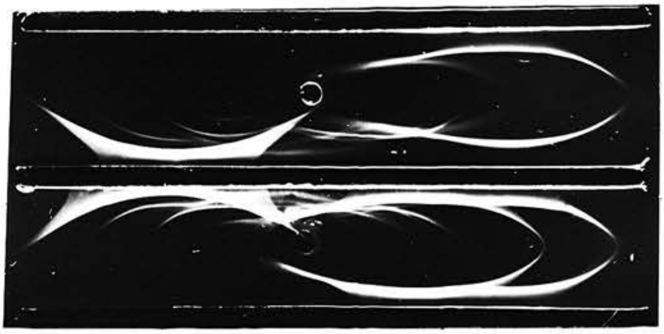
Fig. 43 MIE(61)15

Trough	Rabbit anti-GP γ_1-globulin (R99)
Well	Normal GPS
Trough	Rabbit anti-GPS (R94 + 99)
Well	GP anti-OA serum (26 pooled)
Trough	Rabbit anti-GP γ_1-globulin (R99)

Fig. 44 MIE (68)1

Trough	Specific rabbit anti-GP γ_1-globulin (R102 Abs)
Well	GP anti-OA serum (25 pooled)
Trough	Rabbit anti-GPS (R90 + 102)
Well	Normal GPS (pooled)
Trough	Specific rabbit anti-GP-γ_1 globulin (R102 Abs)





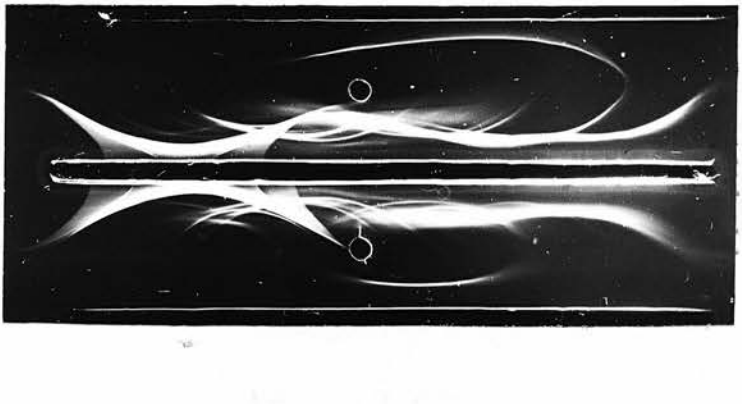


Fig. 45 MIE(68)2

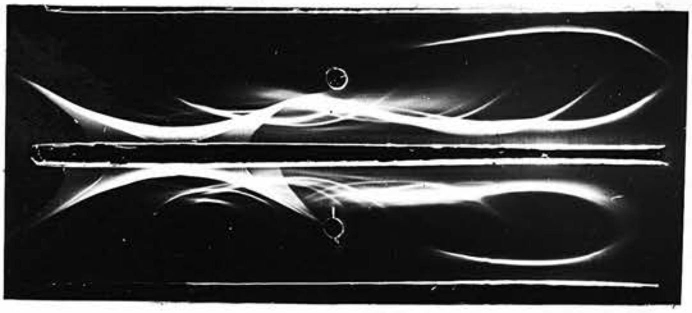
Trough	Specific rabbit anti GP γ_2-globulin (R106 Abs)
Well	GP anti-OA serum (25 pooled)
Trough	Rabbit anti-GPS (R90 + 102)
Well	Normal GPS (pooled)
Trough	Specific rabbit anti GP γ_2-globulin (R106 Abs)

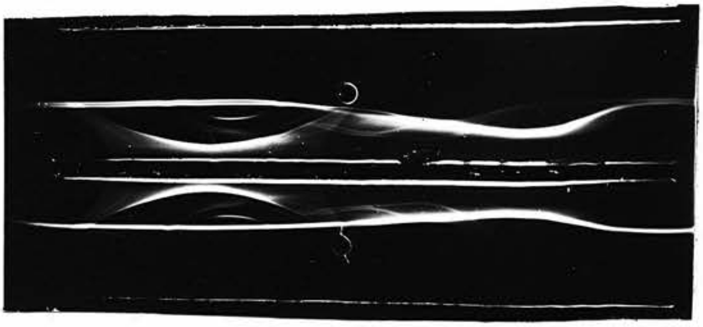
Fig. 46 MIE (68)3

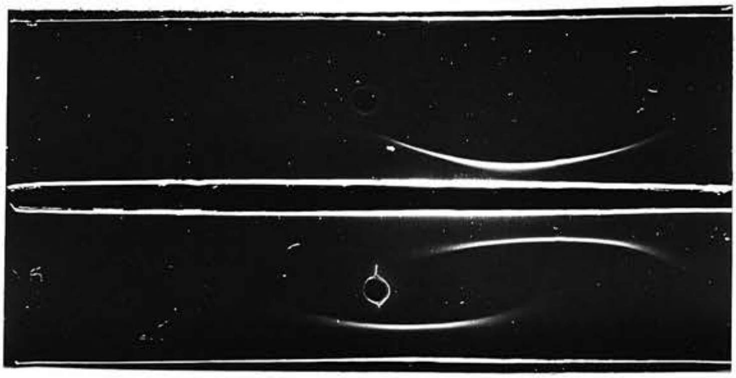
Trough	GP γ_1-globulin from normal GPS (peak 2, Fig. 38)
Well	Normal GPS
Trough	Rabbit anti GPS (R 90+102+106, 1:1:1)
Well	Normal GPS
Trough	GP γ_2-globulin from normal GPS (peak 1 Fig. 38)

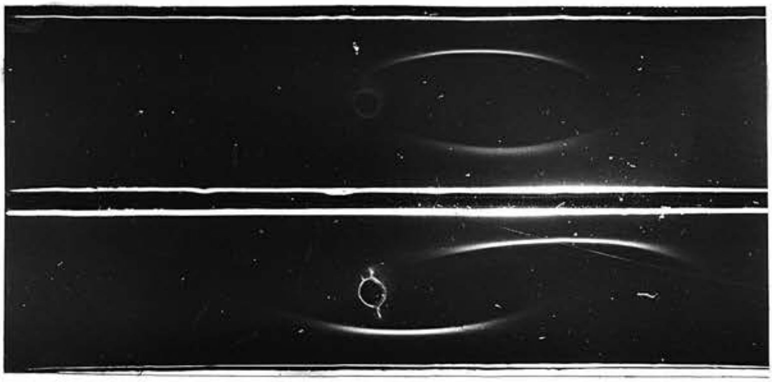
Fig. 47 MIE(69)3

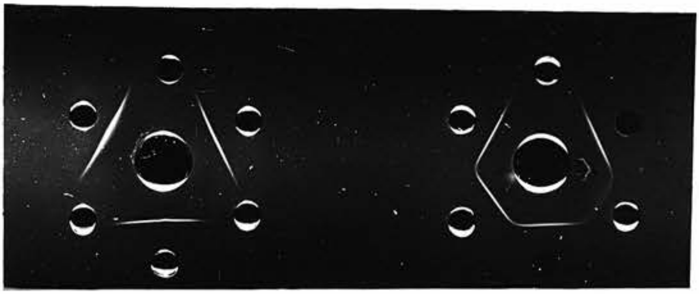
Trough	Specific rabbit anti GP γ_1-globulin (R102 Abs)
Well	GP γ_2-globulin from normal GPS (peak 1 Fig. 38)
Trough	Specific rabbit anti GP γ_2-globulin (R106 Abs)
Well	Normal GPS
Trough	Specific rabbit anti GP γ_1-globulin (R102 Abs)











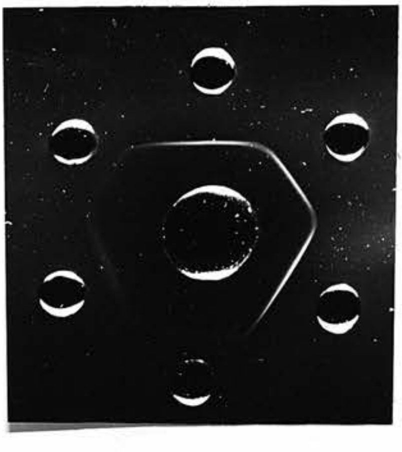


Fig. 48 MIE(69)4

Trough	Specific rabbit anti-GP γ_1-globulin (R102 Abs)
Well	GP γ_1-globulin (peak 2, Fig. 38)
Trough	Specific rabbit anti-GP γ_2-globulin (R106 Abs)
Well	Normal GPS
Trough	Specific rabbit anti GP γ_1-globulin (R102 Abs)

Fig. 49 O(17)1 <u>Left hand side</u>

Centre Well Specific rabbit anti GP γ_1-globulin (R102 Abs)

Outer wells, clockwise starting at top.

(1) GP γ_2-globulin from normal serum (peak 1,Fig 38)6mg/ml
(2) GP γ_1-globulin from normal serum (peak 2,Fig 38)0.5mg/ml
(3) GP γ_2-globulin from normal serum (peak 1,Fig 38)6mg/ml
(4) GP γ_1-globulin from normal serum (peak 2,Fig 38)0.5mg/ml
(5) GP γ_2-globulin from normal serum (peak 1,Fig 38)6mg/ml
(6) GP γ_1-globulin from normal serum (peak 2,Fig 38)0.5mg/ml

<u>Right hand side</u>

Centre Well Specific rabbit anti GP γ_1-globulin (R102 Abs)

Outer wells, clockwise starting at top.

(1) GP γ_2-globulin from normal serum (peak 1, Fig 38)
(2) GP γ_1-globulin from normal serum (peak 2, Fig 38)
(3) Peak III, Fig. 38
(4) Peak 2 from fast total γ-globulin from normal GPS
(5) GP γ_1-globulin from normal GPS (Peak 2, Fig. 38)
(6) Peak 3 from fast total γ-globulin from normal GPS

Fig. 50 O(17)2

Centre Well Specific rabbit anti GP γ_2-globulin (R101 Abs)

Outer wells, clockwise starting at top.

(1) GP γ_2-globulin from normal GPS (peak 1, Fig 38)0.5 mg/ml
(2) GP γ_1-globulin from normal GPS (peak 2, Fig 38) 1 mg/ml
(3) GP γ_2-globulin from normal GPS (peak 1, Fig 38) 0.5mg/ml
(4) Peak 3, Fig. 38
(5) Peak 2, from fast total γ-globulin from normal GPS
(6) Peak 3, from fast total γ-globulin from normal GPS

contaminated with γ_2-globulin. This can be seen clearly in Figs. 47 and 48 in which the same fractions are tested with specific antisera. The isolated γ_2-globulin has similar mobility to that in normal serum. The γ_1-globulin appears to be slower as would be expected since it was isolated from a fairly slow fraction of total γ-globulin (Fig. 38). The γ_2-globulin contaminating the γ_1-globulin preparation (2nd peak, Fig. 38) is clearly visible and has similar mobility to the γ_1-globulin in the same fraction.

The gel diffusion tests shown in Fig. 49 show (a) that the second peak from normal serum (Fig. 38) contained γ_1-globulin whereas the first peak did not contain any detectable quantity of this protein and (b) that the fractions (second peak and after) obtained in small quantities from the electrophoretically fast and slow total γ-globulin also contain γ_1-globulin. The results in Fig. 50 show that, as expected, the first peak contains γ_2-globulin and that the γ_1-globulin preparations are contaminated with γ_2-globulin.

Fig. 51 Upper MIE (59)1

Trough	Rabbit anti-GPS (R94+99)
Well	GP anti-OA serum (16-18 pooled)
Trough	GP γ_2-globulin (82)

Lower MIE (59)2

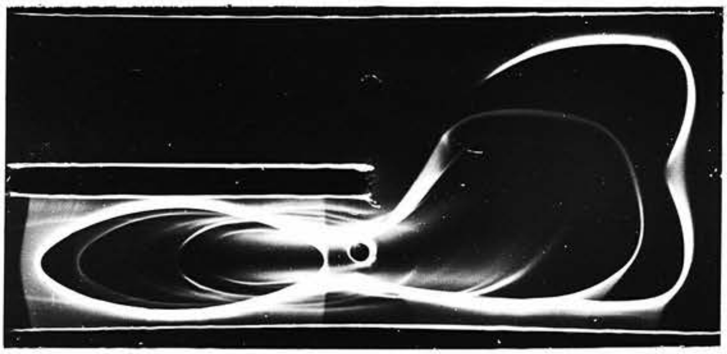
Trough	Rabbit anti GPS (R94+99)
Well	GP Anti-OA serum (16-18 pooled)
Trough	GP γ_1-globulin (82)

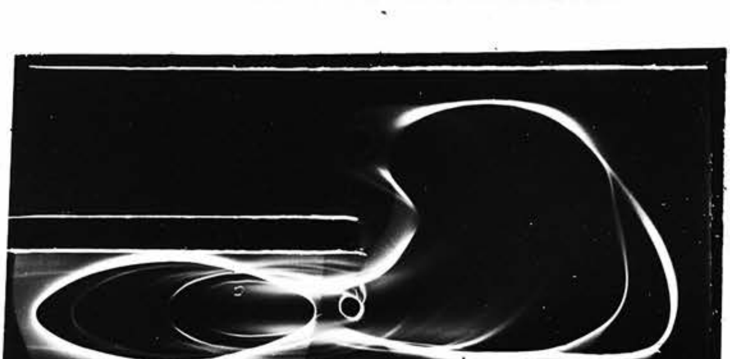
Fig. 52 MIE(58)3

All troughs	Rabbit anti GPS (R94+99)
Well	GP γ_2-globulin (87)
Well	GP Anti-OA serum (23 pooled)

Fig. 53 MIE(58)4

All troughs	Rabbit anti-GPS (R94+99)
Well	GP γ_1-globulin (87)
Well	GP anti-OA serum (23 pooled)





Similar electrophoretic results have been obtained with the fractions (e.g. first and second peaks in Figs. 36 and 37) isolated from immune sera. The identities of the isolated fractions with serum components are shown in Fig. 51 and in Figs. 52 and 53.

The mobility of γ-globulins in isolated fractions and whole sera. The results already described (e.g. Fig. 52) indicate that the slowest γ-globulins have a similar mobility in whole immune serum and in purified γ_2-globulin fractions. Although γ_1-globulin (see Figs. 43 and 44) and to a lesser extent γ_2-globulin (see Fig. 45) do not appear to extend as far (cathodally or anodally) in normal serum as in immune serum, it cannot be decided from the present results whether or not this is simply due to the difference in concentrations of the proteins between normal and immune sera.

The same remarks apply to apparent differences in mobility between isolated fractions and the same proteins in the original serum. In this case, however, the method of isolation would be expected to influence the mobility of the final product. In particular,

Fig. 54 MIE (67)11

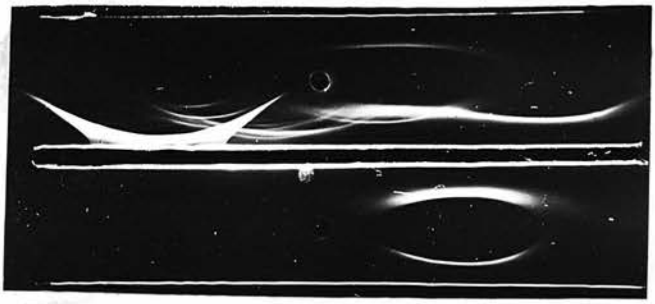
Trough	Specific rabbit anti-GP γ_1-globulin (R102 Abs)
Well	Normal GPS
Trough	Rabbit anti-GPS (R94+99)
Well	GP γ_1-globulin from normal GPS (Peak 2 Fig. 38)
Trough	Specific rabbit anti-GP γ_1-globulin (R102 Abs)

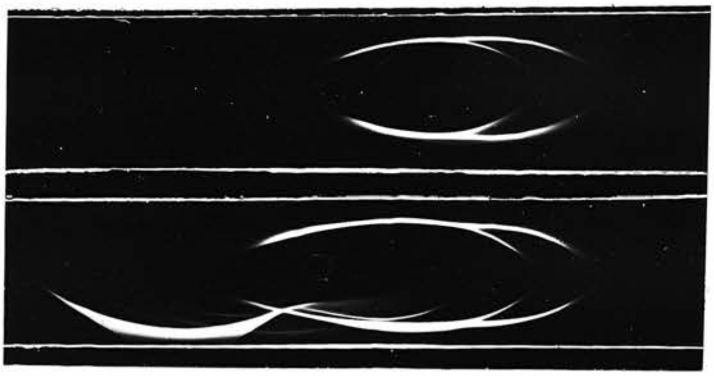
Fig. 55 MIE(59)5

Trough	Rabbit anti-GPS(R94+99)
Well	GP γ_1- and γ_2-globulin. Remixed after isolation. (82)
Trough	Rabbit anti-GP γ_1-globulin (R99)
Well	GP Anti-OA serum (16-18 pooled)
Trough	Rabbit anti-GPS (R94+99)

Fig. 56 MIE(68)5

Trough	Specific rabbit anti-GP γ_1-globulin (R102 Abs)
Well	GP γ_2-globulin (93)
Trough	Specific rabbit anti-GP γ_2-globulin (R106 Abs)
Well	GP anti-OA serum (25 pooled)
Trough	Specific rabbit anti-GP γ_1-globulin (R102 Abs)





the point at which the preparative electrophoretic block is sectioned should control the mobility of the product. It is found, for example, that γ_1-globulin isolated from the slow preparative electrophoretic fraction of total γ-globulin (from normal serum) does not contain γ_1-globulins of the higher mobilities. This is illustrated in Figs. 48 and 54. In other experiments too, there was some evidence for loss of the fastest γ-globulins during the isolation procedure as shown in Fig. 55. This was not always the case, however, and in Figs. 56 and 57 the results of a comparison of the mobilities of isolated fractions with the mobilities of the same proteins in the original serum (by means of specific antisera) show little sign of serious loss during isolation except possibly a small part of the fastest γ_1-globulin. In this case the γ_1-globulin was isolated on DEAE cellulose columns from fast total γ-globulin and γ_2-globulin from slow total γ-globulin.

Fig. 57 MIE(68)6

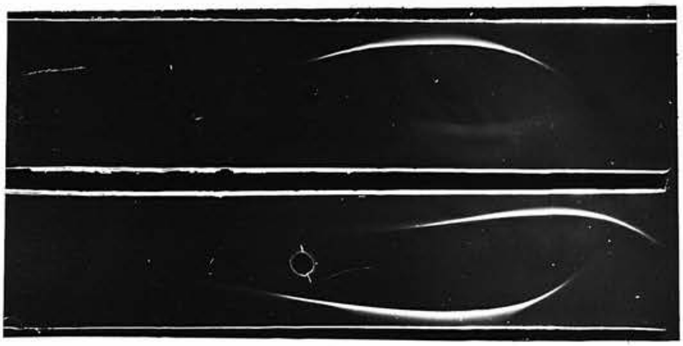
Trough	Specific Rabbit anti GP γ_1-globulin (R102 Abs)
Well	GP γ_1-globulin (from fast total γ-globulin) (93)
Trough	Specific rabbit anti GP γ_2-globulin (R106 Abs)
Well	GP anti-OA serum (25 pooled)
Trough	Specific rabbit anti GP γ_1-globulin (R102 Abs)

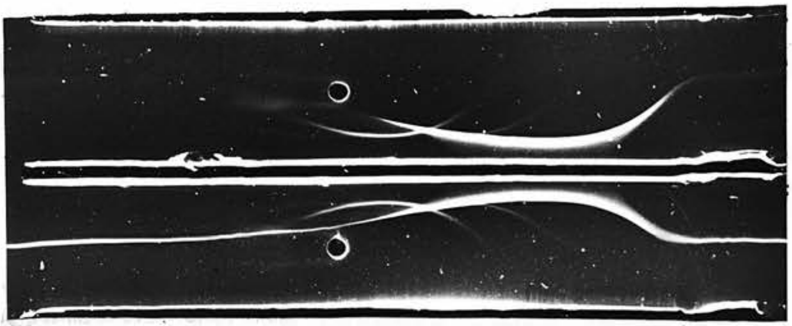
Fig. 58 MIE(65)1

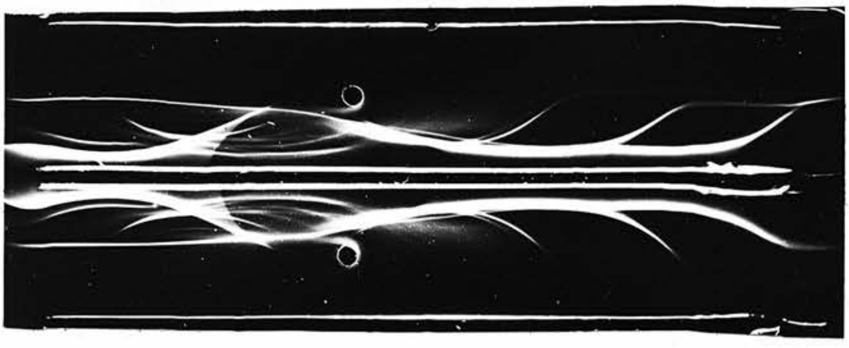
Trough	GP γ_1-globulin from fast total γ-globulin (91)
Well	GP anti-OA serum (27 pooled)
Trough	Specific rabbit anti-GP γ_2-globulin (R101 Abs)
Well	GP anti-OA serum (27 pooled)
Trough	GP γ_2-globulin (91)

Fig. 59 MIE(65)3

Trough	GP γ_1-globulin from fast total γ-globulin (91)
Well	GP anti-OA serum (27 pooled)
Trough	Rabbit anti-GPS (R94+99)
Well	GP anti-OA serum (27 pooled)
Trough	GP γ_2-globulin (91)







(c) The purity of isolated γ_1- and γ_2-globulin fractions

It has been shown in the preceding section that γ_1-globulin from normal serum is contaminated with γ_2-globulin but that γ_2-globulin was not detectably contaminated with γ_1-globulin. Similar results have been obtained with fractions isolated from immune sera.

(1) Purity of γ_2-globulin. When γ_2-globulin was prepared in a single stage, by DEAE cellulose column chromatography, the product contained traces of β-globulins. Although these could not be unambiguously detected by direct immunoelectrophoretic examination, their presence was clearly shown when this γ_2-globulin preparation (batch 88) was used for the preparation of rabbit anti-GPγ_2-globulin serum. The resulting serum (R101) contained clearly visible antibodies against at least two β-globulins. The results obtained with the specific (in the sense that it does not react with γ_1-globulin) antiserum obtained from this (R101 Abs) are shown in Fig. 58 which also shows the identity of isolated γ_2-globulin with the γ_2 -globulin arc revealed in serum.

In contrast, γ_2-globulin purified by the two stage process routinely used did not contain detectable β-globulin or γ_1-globulin contaminants. The absence of β-globulins is shown in Fig. 52 and also in Fig. 59, and, perhaps the most sensitive test, by the absence of antibodies against β-globulins in rabbit antiserum (R106) made against γ_2-globulin purified by a two stage process as shown already in Figs. 45, 56 and 57.

No γ_1-globulin impurity in isolated γ_2-globulin is visible in Figs. 47, 51, 56 or 59 and this was the case with all batches of γ-globulins with the possible exception of a very slight trace in batch 92. Fig. 60 shows the only sign ever seen of γ_1-globulin in isolated γ_2-globulin; the γ_1-globulin arc is seen to bend over slightly at its end. Further investigation with a specific anti GP γ_1-globulin serum (R102 Abs.) is shown in Fig. 61. This shows firstly that the antiserum is specific (only one arc is seen in whole immune serum) and secondly that the isolated γ_1-globulin fuses with the γ_1-globulin arc in whole serum but no trace of γ_1-globulin is visible in the γ_2-globulin fraction. The result in Fig. 61 is typical of those obtained with all batches of purified γ-globulins including the batch used in Fig. 60. The gel double diffusion method should be an even more sensitive test for impurities. The result shown in Fig. 62, in which specific anti γ_1-globulin serum was used in the central well, shows that even though the concentrations of the various batches of γ_2-globulin in the peripheral wells were between 10 and 20 times the concentration of the γ_1-globulin, no trace of γ_1-globulin contamination is visible.

(2) <u>The purity of γ_1-globulin</u>. Purified γ_1-globulin usually contained some β-globulins if it was made from an electrophoretically fast fraction of total γ-globulin. For example a β-globulin is visible in Fig. 59 and 77, though not in Fig. 60. A trace of a β-globulin is probably visible in Fig. 53, but direct immunoelectrophoresis is not the most sensitive method for detecting small amounts of impurities. The absence of β-globulins from γ_1-globulin prepared from electrophoretically slow

fractions of total γ-globulin is strongly suggested by the absence of antibodies against β-globulins in rabbit antisera prepared against such γ_1-globulin, as shown in Figs. 43, 44, 54, 55, 56, 57 and 61.

The presence of γ_2-globulin has been demonstrated in all preparations of γ_1-globulin. This is suggested in Fig. 58 in which a faint γ_2-globulin line parallel to the γ_1-globulin trough can be seen.

The impurity can be seen rather faintly in a γ_1-globulin fraction from immune serum in Fig. 57 and more clearly in the more heavily contaminated γ_1-globulin fraction from normal serum in Fig. 48. In both cases it can be seen that the γ_2-globulin impurity has a similar fast mobility to the main γ_1-globulin part of the fraction. The terms γ_1 and γ_2 are, of course, not now being used simply in their original sense, to denote mobility, but as names for antigenically characterized proteins regardless of mobility. It is not surprising that the γ_1- and γ_2-globulins coming off the DEAE cellulose column together are of similar mobility since affinity for the column and mobility both depend at least partly on net charge.

Fig. 60 MIE(65)7

Trough	GP γ_1-globulin from fast total γ-globulin (92)
Well	GP anti-OA serum (26 pooled)
Trough	Rabbit anti-GPS (R94+99)
Well	GP anti-OA serum (26 pooled)
Trough	GP γ_2-globulin (92)

Fig. 61 MIE (65)10

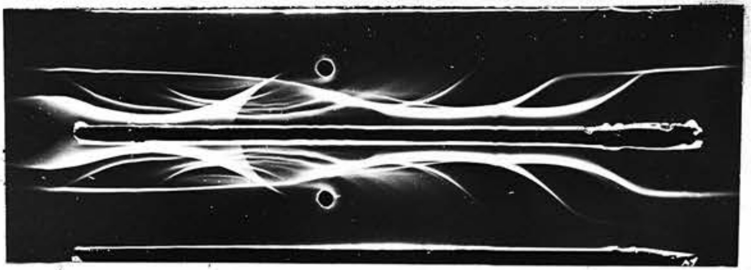
Trough	GP γ_1-globulin from fast total γ-globulin (93)
Well	GP anti-OA serum (25 pooled)
Trough	Specific rabbit anti-GP γ_1-globulin (R102 Abs)
Well	GP anti-OA serum (25 pooled)
Trough	GP γ_2-globulin (93)

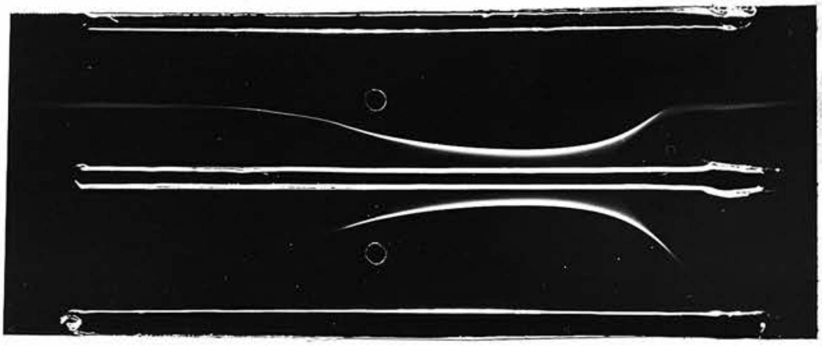
Fig. 62

Centre well	Specific rabbit anti-GP γ_1-globulin (R102 Abs)

Outer wells clockwise, starting at top.

(1)	GP γ_2-globulin.(91)	8.5 mg/ml
(2)	GP γ_1-globulin.(92)	0.46 mg/ml
(3)	GP γ_2-globulin.(92)	4.5 mg/ml
(4)	GP γ_1-globulin.(92)	0.46 mg/ml
(5)	GP γ_2-globulin.(93)	6.6 mg/ml
(6)	GP γ_1-globulin.(92)	0.46 mg/ml





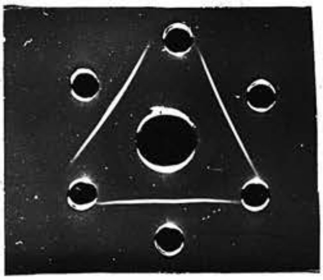


Fig. 63 O(7)2(L)

Centre well Rabbit anti-GP γ_1-globulin (R99)

Outer well. Clockwise, starting at top

(1) GP γ_2-globulin (87)
(2) GP γ_1-globulin (87)
(3) GP γ_2-globulin (87)
(4) GP γ_1-globulin (87)
(5) GP γ_2-globulin (87)
(6) GP γ_1-globulin (87)

Fig. 64 O(9)2(L)

Centre well Rabbit anti-GP γ_1-globulin (R102)

Outer well. Clockwise, starting at top

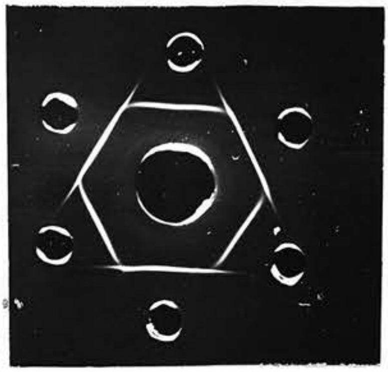
(1) GP γ_2-globulin (91)
(2) GP γ_1-globulin (91)
(3) GP γ_2-globulin (91)
(4) GP γ_1-globulin (91)
(5) GP γ_2-globulin (91)
(6) GP γ_1-globulin (91)

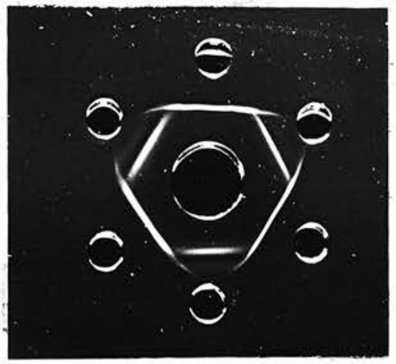
Fig. 65 O(10)2 (R)

Centre well Rabbit anti-GP γ_2-globulin (R101)

Outer well. Clockwise starting at top

(1) GP γ_2-globulin (92)
(2) GP γ_1-globulin (92)
(3) GP γ_2-globulin (92)
(4) GP γ_1-globulin (92)
(5) GP γ_2-globulin (92)
(6) GP γ_1-globulin (92)





The impurity is more clearly shown by experiments of the Ouchterlony type and the use of specific antisera. It should be noted that although the batch of γ_1-globulin used in Fig. 58, had been shown (see Fig. 59) to contain some β-globulin, this is not revealed by the anti-γ_2 serum (R101) used in Fig. 58, which, together with similar results on other batches of purified proteins, implies that the β-globulins which sometimes contaminate γ_1-globulin preparations are different from those β-globulins to which R101 contains antibodies. This means that the specific anti- γ_2 globulin serum (R101 Abs) can be used for quantitative estimates of γ_2-globulin contaminant in purified γ_1-globulin preparations. The presence of γ_2-globulin in the γ_1-globulin fractions from normal serum has already been shown in Fig. 50. Fig. 63 and 64 show the results obtained with two batches of rabbit anti-GP γ_1-globulin serum (R102 and R99). Both show that the antisera combine with both globulins but that γ_1-globulin contains antigenic determinants not present in γ_2-globulin as indicated by spur formation. A similar experiment using anti-GP γ_2-globulin serum (R101) is shown in Fig. 65. Again spur formation occurs, indicating that γ_2-globulin contains antigenic determinants not present on γ_1-globulin, but this time the spurs tend to fuse suggesting the γ_1-globulin preparations used contain γ_2-globulin. This is confirmed by the results shown in Fig. 66 in which various purified fractions are tested with a different anti-γ_2-globulin (R100). All the γ_1-globulin fractions are seen to produce, to a greater or lesser extent, a line which fuses with the spur produced by the genuine sample of γ_2-globulin, and which is therefore produced by antibodies against antigenic determinants specific for γ_2-globulin. Spur formation is seen more clearly in Fig. 67 in which another anti-γ_2-globulin serum (R108) is tested against γ_2-globulin and an electrophoretically fast batch of γ_1-globulin which contains little γ_2-globulin. The result of an experiment similar to that shown in Fig. 64, except that a later batch of the anti-γ_1-globulin serum (R102) (obtained by reboosting rabbit 102) was used, is shown in Fig. 68. In this case, in

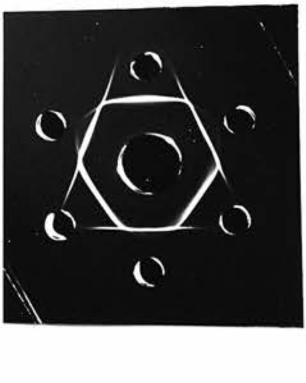


Fig. 66 O(10)13 L

Centre well. Rabbit anti-GP γ_2-globulin (R100)

Outer well. Clockwise starting at top

(1) GP γ_2-globulin (92) 1 mg/ml
(2) GP γ_1-globulin (91) 1 mg/ml
(3) GP γ_1-globulin (90) 1 mg/ml
(4) GP γ_1-globulin (92) 1 mg/ml (from slow total γ-globulin)
(5) GP γ_1-globulin (92) 1 mg/ml (from fast total γ-globulin)
(6) GP γ_1-globulin (92) 1 mg/ml (from very fast total γ-globulin)

Fig. 67. O(16)1.L.

Centre well. Rabbit anti-GP γ_2-globulin (R108)

Outer well. Clockwise starting at top

(1) GP γ_2-globulin (88)
(2) GP γ_1-globulin (86)
(3) GP γ_2-globulin (88)
(4) GP γ_1-globulin (86)
(5) GP γ_2-globulin (88)
(6) GP γ_1-globulin (86)

Fig. 68 O(16)2 L

Centre well Rabbit anti-GP γ_1-globulin (R102)

Outer well. Clockwise starting at top

(1) GP γ_2-globulin (88)
(2) GP γ_1-globulin (93)
(3) GP γ_2-globulin (88)
(4) GP γ_1-globulin (93)
(5) GP γ_2-globulin (88)
(6) GP γ_1-globulin (93).

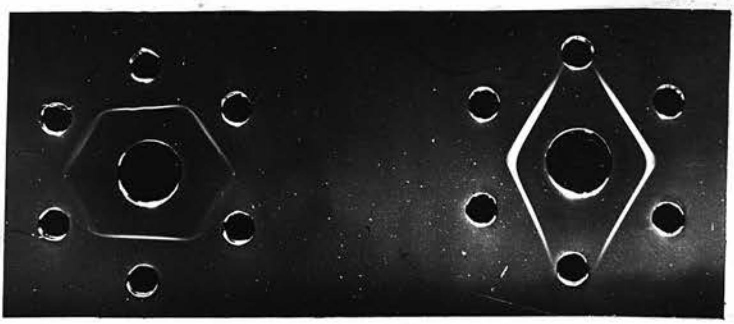


Fig. 69 O(12)1

<u>Left hand side</u>

Centre well. Specific rabbit anti-GP γ_2-globulin (R101 Abs)

Outer well. Clockwise starting at top

(1) GP γ_2-globulin (92) 0.6 mg/ml
(2) GP γ_1-globulin (92) 4.1 mg/ml
(3) GP γ_1-globulin (92) 2.3 mg/ml (from very fast total γ-globulin)
(4) GP γ_2-globulin (92) 0.6 mg/ml
(5) same as well 3
(6) same as well 2

<u>Right hand side</u>

Centre well. Specific rabbit anti-GP γ_1-globulin (R102 Abs)

Outer wells as on left hand side.

Fig. 70 O(19)1 L.

Centre well. Specific rabbit anti-GP γ_2-globulin (R106 Abs)

Outer well. Clockwise starting at top

(1) GP γ_2-globulin from normal GPS. 0.5 mg/ml (95)
(2) GP γ_1-globulin (93)
(3) same as well 1
(4) same as well 2
(5) same as well 1
(6) same as well 2

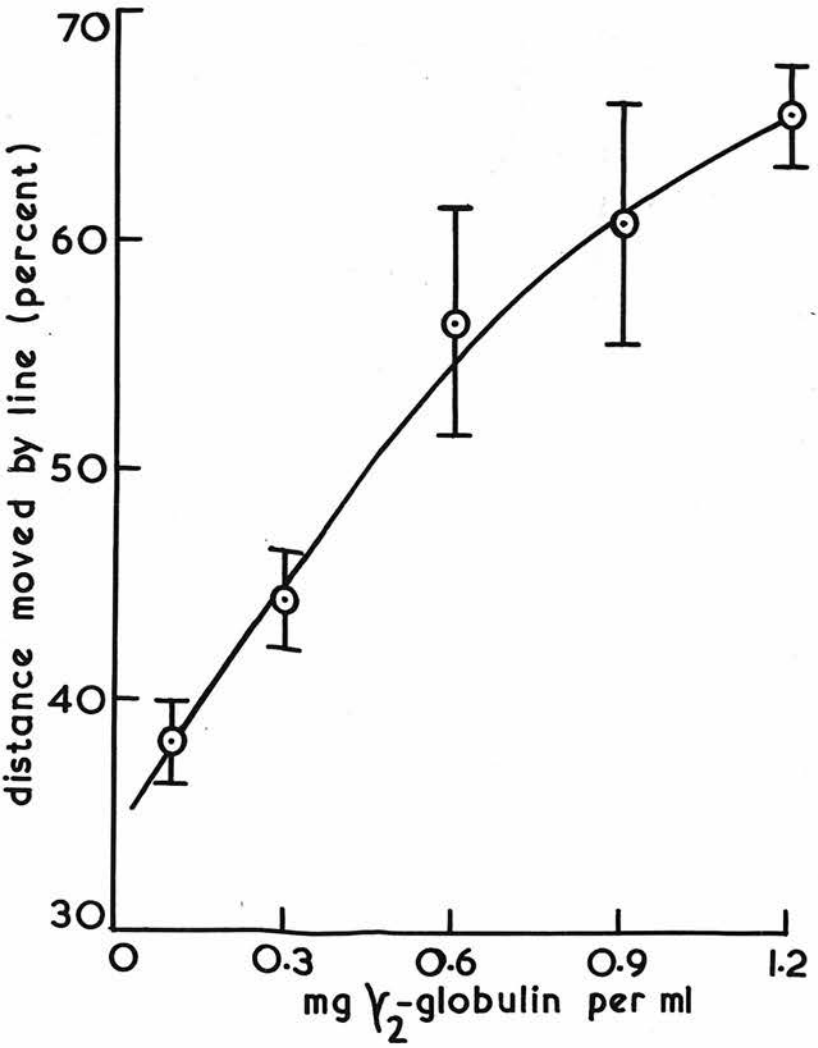
Fig. 71 O(19)1R.

Large well. Specific rabbit anti-GP γ_1-globulin (R102 Abs)

Small wells (reading from top downwards)

(1) GP γ_2-globulin (95) 0.5 mg/ml
(2) Specific rabbit anti-GP γ_2-globulin (R106 Abs)
(3) GP γ_1-globulin (93) 0.5 mg/ml.

Fig. 72 Standard curve for estimation of GP γ_2-globulin using specific rabbit anti-GP γ_2-globulin serum (R101 Abs). Standards made from GP γ_2-globulin (batch 92). Vertical bars indicate 95% confidence limits for means.


70
60
50
40
30
distance moved by line (percent)
0
0.3
0.6
0.9
1.2
mg γ_2-globulin per ml

addition to the spurs seen in Fig. 64, loops facing the γ_1-globulin wells are also seen. These are clearly due to the fact that the γ_1-globulin used for producing anti-γ_1-globulin serum was contaminated with γ_2-globulin so R102 also contains some anti-γ_2-globulin which precipitates with the γ_2-globulin contaminant producing the loops seen in Fig. 68.

The results of experiments with specific antisera are shown in Fig. 69. It can be seen quite unambiguously that all the γ_1-globulin preparations tested contain material which gives a reaction of identity with γ_2-globulin using specific anti-γ_2-globulin serum R101 Abs. Fig. 70 shows a different specific anti-γ_2-globulin (R106 Abs) with a batch of γ_1-globulin containing relatively little γ_2-globulin. Again no γ_1-globulin is detectable in γ_2-globulin. Fig. 71 shows the result of a test carried out during preparation of the specific antisera. It shows that R102 Abs contains an excess of γ_2-globulin whereas R106 Abs is at equivalence.

Preliminary quantitative estimates of the extent of impurity have been obtained using a small scale modification of the method of Darcy (1960). The results of one of these experiments is shown in Fig. 72. The γ_1-globulin obtained from an electrophoretic fraction of total γ-globulins of medium mobility was estimated to contain 8.5% of γ_2-globulin, and the γ_1-globulin obtained in the same experiment but from electrophoretically fast total γ-globulins contained only about 3.5%. A similar estimation on γ_1-globulin preparations obtained in a different experiment indicated that the γ_2-globulin contents of preparations obtained from fast, medium and slow total γ-globulins were about 6%, 8% and 22% respectively.

The antibody content of guinea pig antiovalbumin sera and purified globulins

To characterize as far as possible the antisera used it is desirable to know (a) whether all the antibody is directed against ovalbumin and (b) which immunoglobulins contain antibody.

When antiserum and isolated γ_1- or γ_2-globulins are tested by immunoelectrophoresis with ovalbumin in one of the troughs the mobility of precipitating anti-ovalbumin can be seen. In Figs. 73 and 74 the result of such

Fig. 73 MIE (68)14

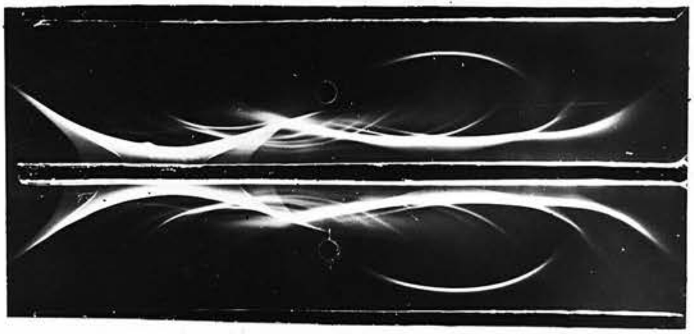
Trough	Ovalbumin (Mann, 5 x cryst.) 0.1 mg/ml
Well	GP anti-OA serum (62/A)
Trough	Rabbit anti-GPS (R90+102)
Well	GP anti-OA serum (16-18)
Trough	Ovalbumin (Mann, 5 x cryst.) 0.1 mg/ml

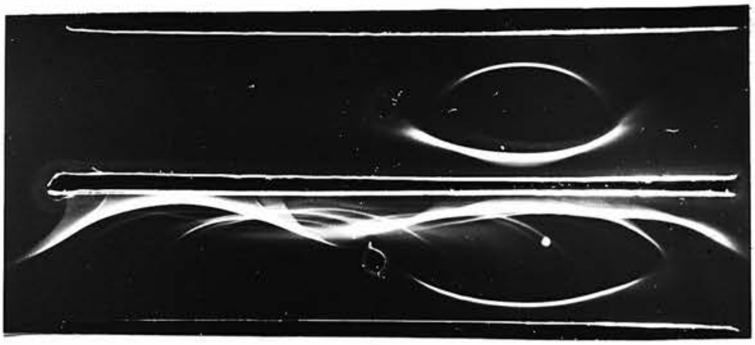
Fig. 74 MIE (68)15

Trough	Ovalbumin (Mann, 5 x cryst) 0.1 mg/ml
Well	GP γ_1-globulin (from serum GP 19-21) (94)
Trough	Rabbit anti-GPS (R90+102)
Well	GP anti-OA serum (19-21)
Trough	Ovalbumin (Mann, 5 x cryst) 0.1 mg/ml

Fig. 75 MIE(53)4 (stained slide)

Trough	Ovalbumin (Mann, 5 x cryst.) 0.1 mg/ml
Well	GP γ_1-globulin (82) from serum GP 16-18
Trough	Rabbit anti-GPS (R90)
Well	GP γ_2-globulin (82) from serum GP16-18
Trough	Ovalbumin (Mann, 5 x cryst) 0.1 mg/ml.





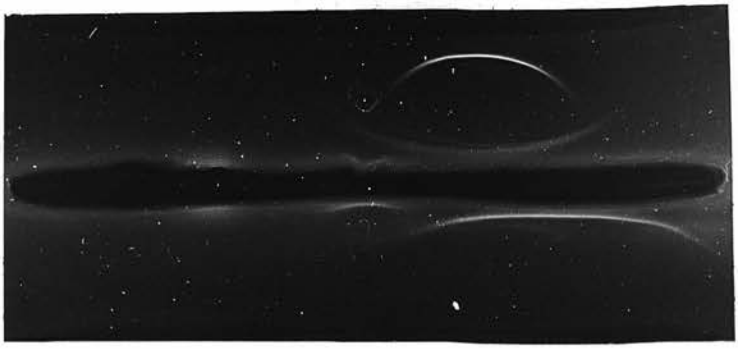


Fig. 76 MIE(62)5

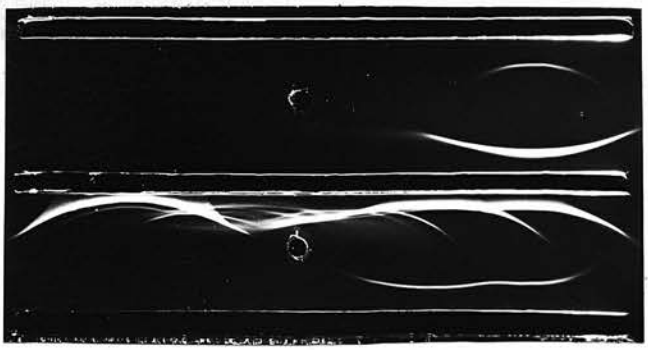
Trough	Ovalbumin (Mann, 5 x cryst) 0.05 mg/ml
Well	GP γ_2-globulin (87). From serum GP 22 (pooled)
Trough	Rabbit anti-GPS (R94+99)
Well	GP anti-OA serum (22 pooled).
Trough	Ovalbumin (Mann, 5 x cryst) 0.05 mg/ml

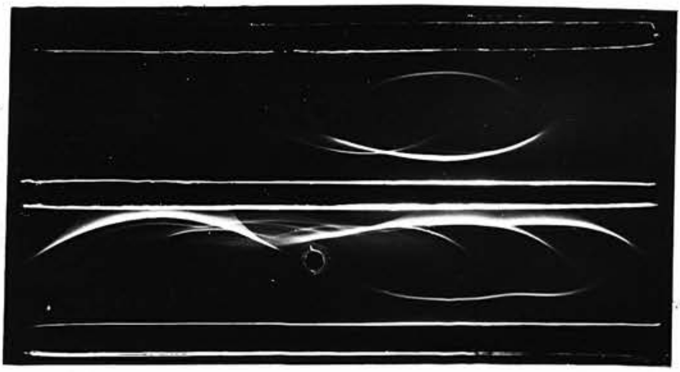
Fig. 77 MIE (62)8

Trough	Ovalbumin (Mann, 5 x cryst) 0.05 mg/ml.
Well	GP γ_1-globulin (87). From serum GP 22 (pooled).
Trough	Rabbit anti-GPS (R94+99)
Well	GP anti-OA serum (22 pooled)
Trough	Ovalbumin (Mann, 5 x cryst) 0.05 mg/ml

Fig. 78 MIE(64)6

Trough	GP γ_2-globulin (92)
Well	Ovalbumin (Mann, 5 x cryst) 10 mg/ml
Trough	GP anti-OA serum (26 pooled)
Well	Ovalbumin (Mann, 5 x cryst.) 10 mg/ml
Trough	GP γ_1-globulin (92)





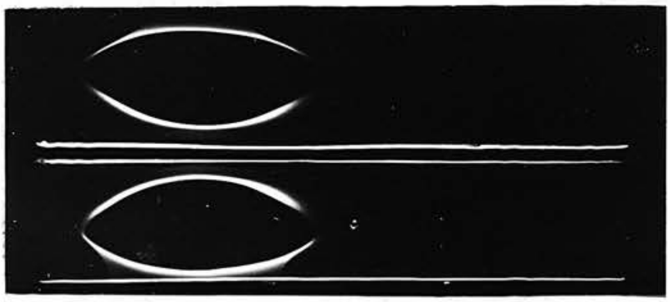


Fig. 79 MIE (68)16

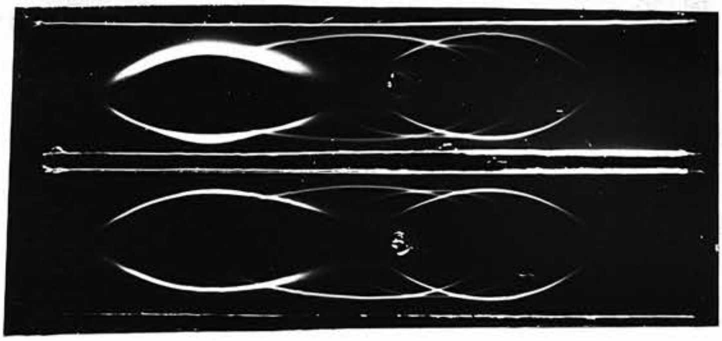
Trough	GP anti-OA serum (19-21, pooled)
Well	Egg white (10%)
Trough	GP anti-OA serum (62/A, pooled)
Well	Egg white (10%)
Trough	GP anti-OA serum (16-18, pooled)

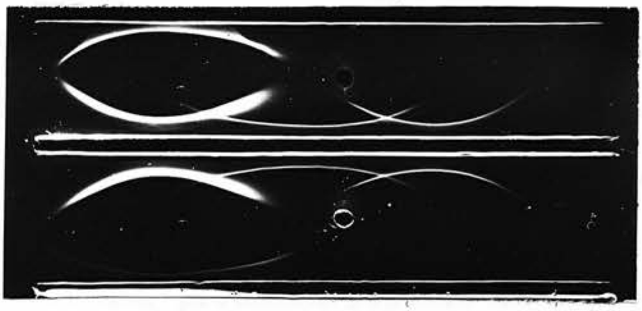
Fig. 80 MIE (67)7

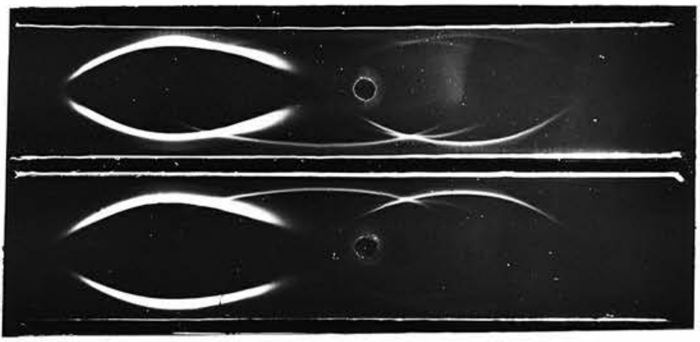
Trough	GP γ_1-globulin (91)
Well	Egg white (10%)
Trough	GP anti-OA serum (25, pooled)
Well	Egg white (10%)
Trough	GP γ_2-globulin (91). From fast total γ-globulin.

Fig. 81 MIE(68)10

Trough	GP γ_1-globulin (93)
Well	Egg white (10%)
Trough	GP anti-OA serum (25, pooled)
Well	Egg white (10%)
Trough	GP γ_2-globulin (93).







experiments with the earlier batches of GP anti-ovalbumin serum are shown. Only a single band of anti-ovalbumin with γ_1 mobility is visible. Results of this sort were obtained with batches GP 16-18, GP 19-21 and GP62/A. Isolation of γ_1- and γ_2-globulins from these sera showed that precipitating anti-ovalbumin was detectable only in the γ_1-globulin fraction as shown in Figs. 74 and 75. It is shown later that some antibody was detectable in the γ_2-globulin by haemagglutination. These batches, especially GP 62/A, were all prepared by a relatively prolonged course of immunization using Weybridge type Freund's adjuvant. All other batches produced results of the sort shown in Figs. 76 and 77. The whole antiserum shows a double arc of precipitating antibody and the isolated γ_1- and γ_2-globulins are both found to contain precipitating anti-ovalbumin in comparable amounts. These antisera (batches 22 to 27) were all prepared by relatively short immunization procedures using adjuvant containing Weybridge type mycobacteria, M. butyricum and M. tuberculosis H37Rv.

When the ovalbumin (5 x crystallized) used for immunization is separated electrophoretically and developed with purified anti-ovalbumin γ_1- and γ_2-globulins it appears (Fig. 78) that all the antibody in both globulins is directed against ovalbumin. However when whole egg white was used for immunoelectrophoresis, as illustrated in Figs. 79 and 80, antibodies against 3 or 4 of its constituents can be seen. Comparison with the results of Weigle and McConahey (1962) suggests that most of the antibody is against ovalbumin, some against conalbumin and ovomucoid and sometimes small amounts against a 4th component. These substances must therefore be present in 5 x crystallized ovalbumin in sufficient amounts to provoke antibody production, though their concentration is not high enough for them to be detected by immunoelectrophoresis. No gross difference was noticeable between the ranges of antigens to which γ_1- and γ_2-globulin contained antibodies. This is illustrated in Figs. 80 and 81.

The sensitization of skin and lung by guinea pig γ_1- and γ_2-globulins

Skin sensitization was measured using passive cutaneous anaphylaxis (PCA). When the arithmetic mean of square roots of the products of longest and shortest lesion diameters was used as a measure of response it was found in virtually every experiment that no deviations from linearity were detected, and in only a few experiments did the log-dose response lines for γ_1- and γ_2-globulins deviate significantly from parallelism when the results were inspected by the conventional analysis of variance for parallel line assays.* The slope of the log dose response line was usually such that a threefold increase in dose increased the mean lesion diameter by between 3 and 4 mm. In almost all experiments application of Bartlett's test gave no evidence for heteroscedasticity when the above transformation was used. The error standard deviation of a single response was between 2.5 and 4 mm in almost every experiment. In some experiments the differences in response level between guinea pigs could be eliminated in the analysis of variance, though

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* A typical analysis (for the assay of fast against slow γ_1-globulins shown in Fig. 86) is given in Table 8a.

Table 8a

Analysis of variance of (3 + 3) dose assay of fast against slow γ_1-globulins by passive cutaneous anaphylaxis (see Fig. 86)

Source	d.f.	SS	MS	VR	P
Linear reg.	1	442.9	442.9	80.2	0.001
Between preps.	1	10.41	10.41	1.89	0.1 - 0.2
Deviations from parallelism	1	0.051	0.051	1	N.S.
Quadratic reg.	1	1.143	1.143	1	N.S.
Difference of quadratics	1	0.1341	0.1341	1	N.S.
Between treatments	5	454.5			
Between guinea pigs	11	548.9	49.9	9.04	0.001
Residual	55	303.4	5.52		
Total	71				

The calculated regression lines (constrained to be parallel) are:

For slow γ_1-globulin

$$Y = 8.41 + 3.04\,x$$

and for fast γ_1-globulin

$$Y = 7.65 + 3.04x$$

where Y is the expected response and x is the logarithm to the base 3 of the dose (μg protein/0.05 ml).

they did not always reach statistical significance so the gain was usually rather small.

It can be seen from the graphs in this section that the amounts of protein needed to produce sensitization were small. Incubation of lung tissue for one or two hours with 0.1 μg/ml (total protein) γ_1-globulin produced definite though submaximal sensitization with most batches. A dose of 0.1 to 0.3 μg (total protein) γ_1-globulin in 0.05 ml usually produced clear submaximal sensitization in PCA tests. The amount of antibody in these fractions is unknown. Testing isolated fractions against a guinea pig antiserum (GP 19-21) containing a known amount of precipitating antibody (about 10mg/ml) by haemagglutination would indicate an antibody content of about 10% if haemagglutination measured precipitating antibody, but this cannot be assumed.

It was found, in agreement with the recent results of other workers, that most of the skin sensitizing activity lay in the γ_1-globulin antibody fraction. In all experiments except one it was found easily possible to give a large enough dose of γ_2-globulin to produce a skin response which looked very similar to the responses produced by γ_1-globulin antibodies. It was felt, in some experiments at least, that it was possible to distinguish between the lesions produced by γ_1- and γ_2-globulin antibodies, the latter tending perhaps to produce rather paler lesions for a given size. However, even though readings of size and intensity of lesions were made blind it is doubtful whether they are completely free from subjective bias and no difference in the lesions can be established on the basis of the present results.

Control experiments showed that even the highest doses of γ_2-globulin produced no blue lesion when dye without antigen was injected I.V. 4 hours after the intradermal antibody. Sometimes, however, there was pronounced petechial haemorrhage at the site of the injection but it was never blue.

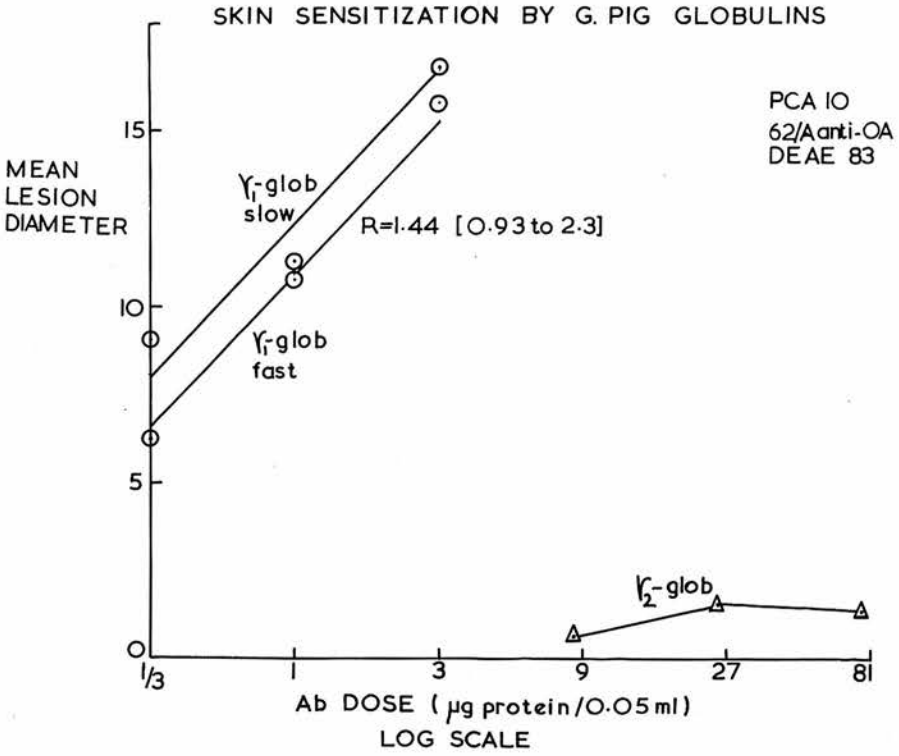
It has also been found that the ability to sensitize chopped guinea pig lung tissue <u>in vitro</u> is almost exclusively a property of γ_1-globulin antibody. Again by using a fairly high concentration of γ_2-globulin it was usually possible to obtain some sensitization and an estimate of the potency ratio between the two globulins.

For comparison of potency ratios on skin and lung knowledge of the relative antibody content of the γ_1- and γ_2-globulin fractions is not needed. However to compare the potencies of the antibodies themselves this knowledge is needed and an approximate estimate has been made by means of haemagglutination titration. In most preparations containing both γ_1- and γ_2-globulin precipitating antibodies the latter had between 50% and 100% of the agglutinating power of the former. Where figures are available potency ratios based on relative antibody contents are given. In one haemagglutination experiment it was found that γ_2-globulin from serum batch GP 19-21 (which did not contain detectable precipitating antibody in the γ_2-globulin fraction) actually had 12% (or rather more) of the antibody content of the γ_1-globulin fraction so appeared to be far from free of antibody. However this point has not been further investigated.

The obvious explanation for sensitization by γ_2-globulin is that it is contaminated with γ_1-globulin. The fact that log dose-response lines hardly ever deviate significantly from

Fig. 82 Passive cutaneous anaphylaxis (PCA 10). Fractions (DEAE 83) from GP62/A serum. Calculated least squares parallel lines.

Fig. 83 Lung sensitization with same proteins as used in Fig. 82.


SKIN SENSITIZATION BY G. PIG GLOBULINS
PCA 10
62/A anti-OA
DEAE 83
MEAN LESION DIAMETER
15
10
5
0
γ_1-glob slow
γ_1-glob fast
R=1.44 [0.93 to 2.3]
γ_2-glob
1/3
1
3
9
27
81
Ab DOSE (µg protein/0.05 ml)
LOG SCALE

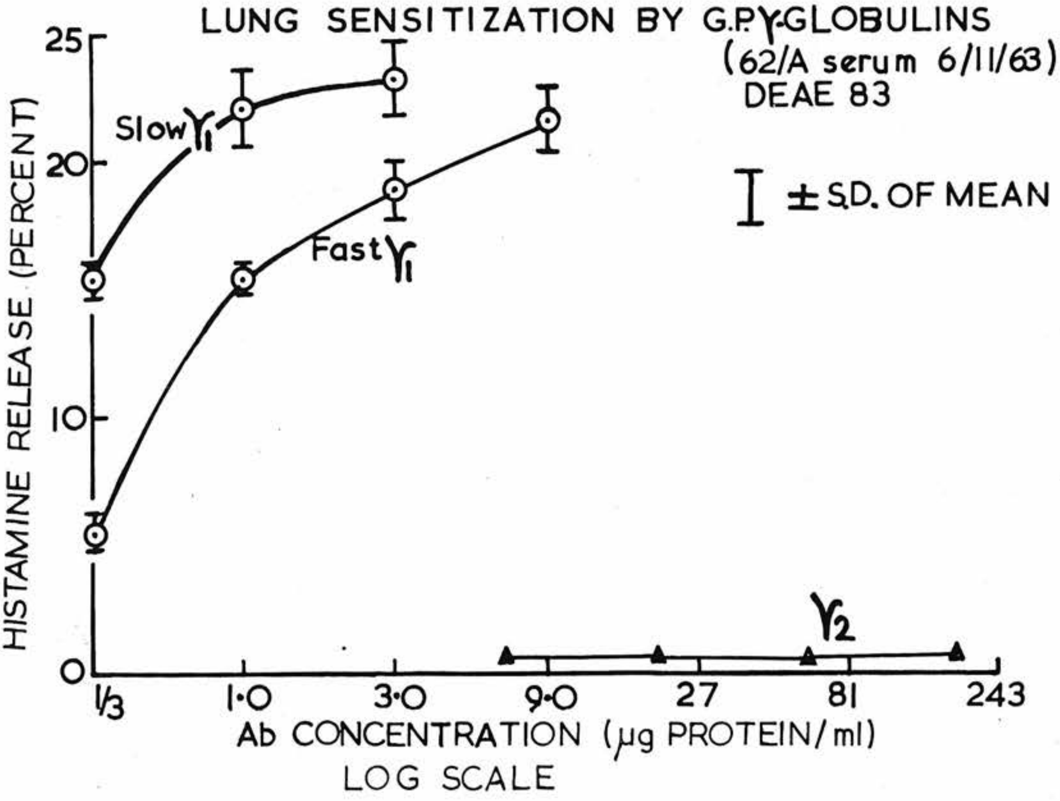
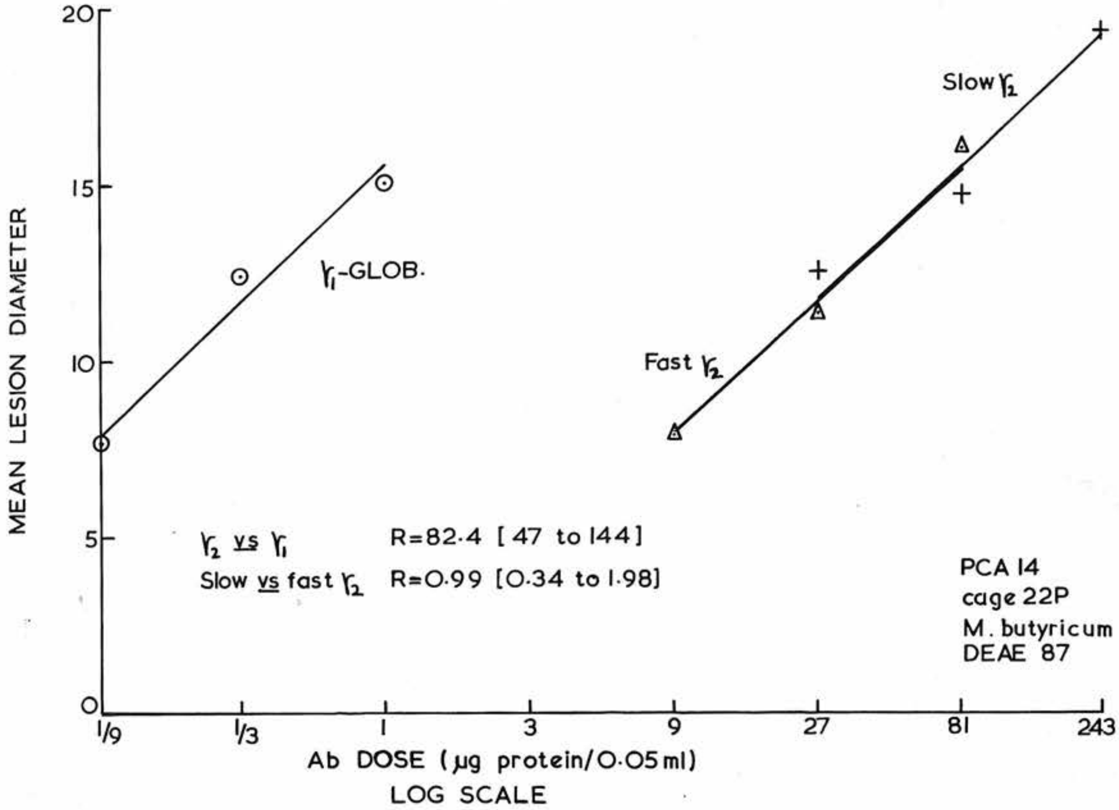
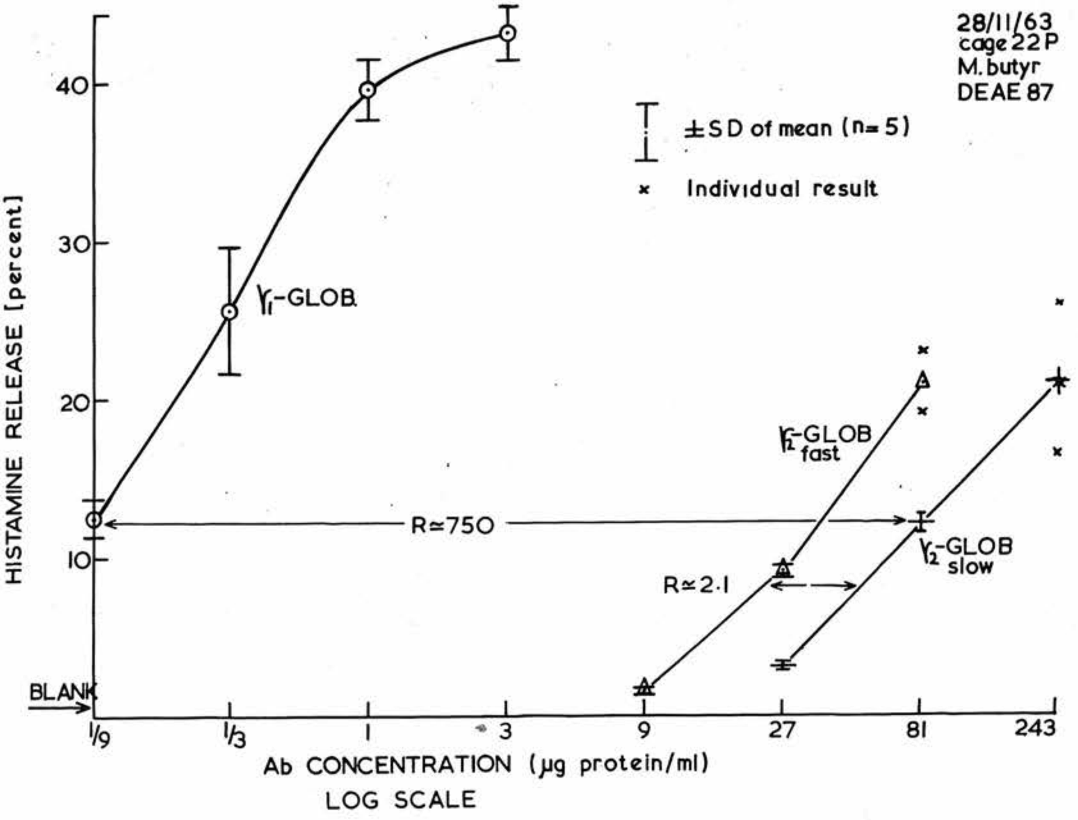

LUNG SENSITIZATION BY G.P. γ-GLOBULINS
(62/A serum 6/11/63)
DEAE 83
± S.D. OF MEAN
HISTAMINE RELEASE (PERCENT)
25
20
10
0
Slow γ_1
Fast γ_1
γ_2
1/3
1·0
3·0
9·0
27
81
243
Ab CONCENTRATION (µg PROTEIN/ml)
LOG SCALE

Fig. 84 PCA(14). Fractions (DEAE 87) from serum GP22 (pooled). R= least squares estimate of potency ratio with 95% fiducial limits.

Fig. 85 Lung sensitization by same proteins as used in Fig. 84. Potency ratio estimated graphically.



MEAN LESION DIAMETER
0
5
10
15
20
γ1-GLOB.
Fast γ2
Slow γ2
γ2 vs γ1 R=82.4 [47 to 144]
Slow vs fast γ2 R=0.99 [0.34 to 1.98]
PCA 14
cage 22P
M. butyricum
DEAE 87
1/9
1/3
1
3
9
27
81
243
Ab DOSE (μg protein/0.05 ml)
LOG SCALE



28/11/63
cage 22P
M. butyr
DEAE 87
HISTAMINE RELEASE [percent]
10
20
30
40
± SD of mean (n = 5)
× Individual result
γ1-GLOB.
γ2-GLOB fast
γ2-GLOB slow
R≃750
R≃2.1
BLANK
1/9
1/3
1
3
9
27
81
243
Ab CONCENTRATION (μg protein/ml)
LOG SCALE

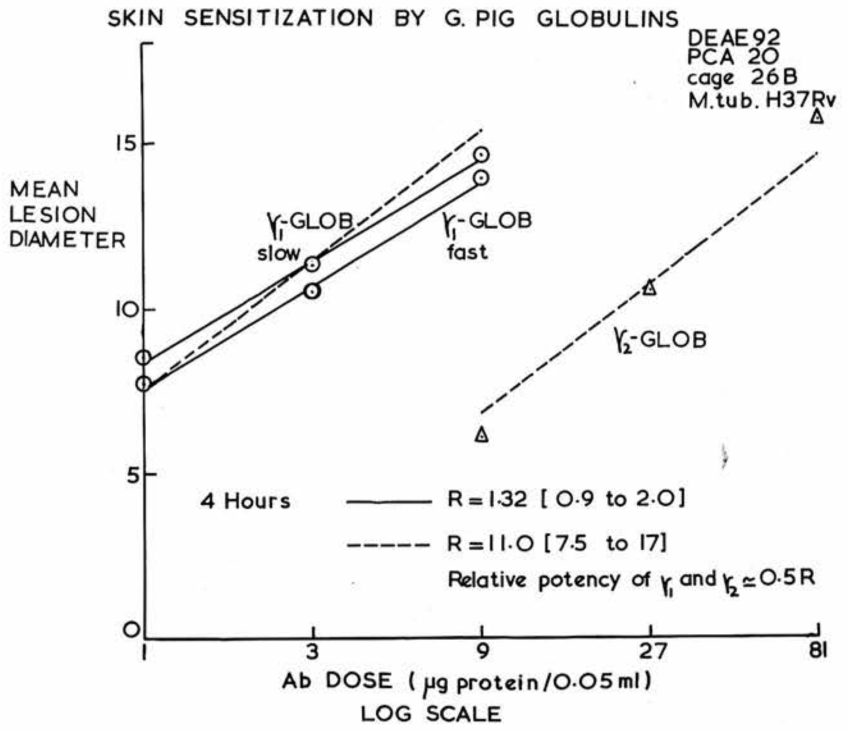
parallelism would be consistent with this idea if γ_2-globulin did not interfere with sensitization. However it will be shown that there is good reason to believe that γ_2-globulin antibody itself has some power to sensitize skin.

The highest skin potency ratios (on a total protein basis) between γ_1- and γ_2-globulins were obtained with those batches which did not contain detectable precipitating antibody in the γ_2-globulin fraction, though the potency ratios of the globulins on lung tissue was similar to those found with other batches of globulins (see Table 9). The results with globulins from serum 62/A (which was produced by prolonged immunization) are shown in Fig. 82 (skin sensitization) and Fig. 83 (lung sensitization). In both cases the potency ratios were unmeasurably large with the concentrations used. Evidently, if γ_1-globulin is responsible for the sensitization produced by the γ_2-globulin fraction, there is very little such contamination in these experiments, especially since it might be expected that γ_1-globulin impurity would be more effective in these experiments in which the bulk of the γ_2-globulin fraction contains little antibody and therefore does not compete for antigen.

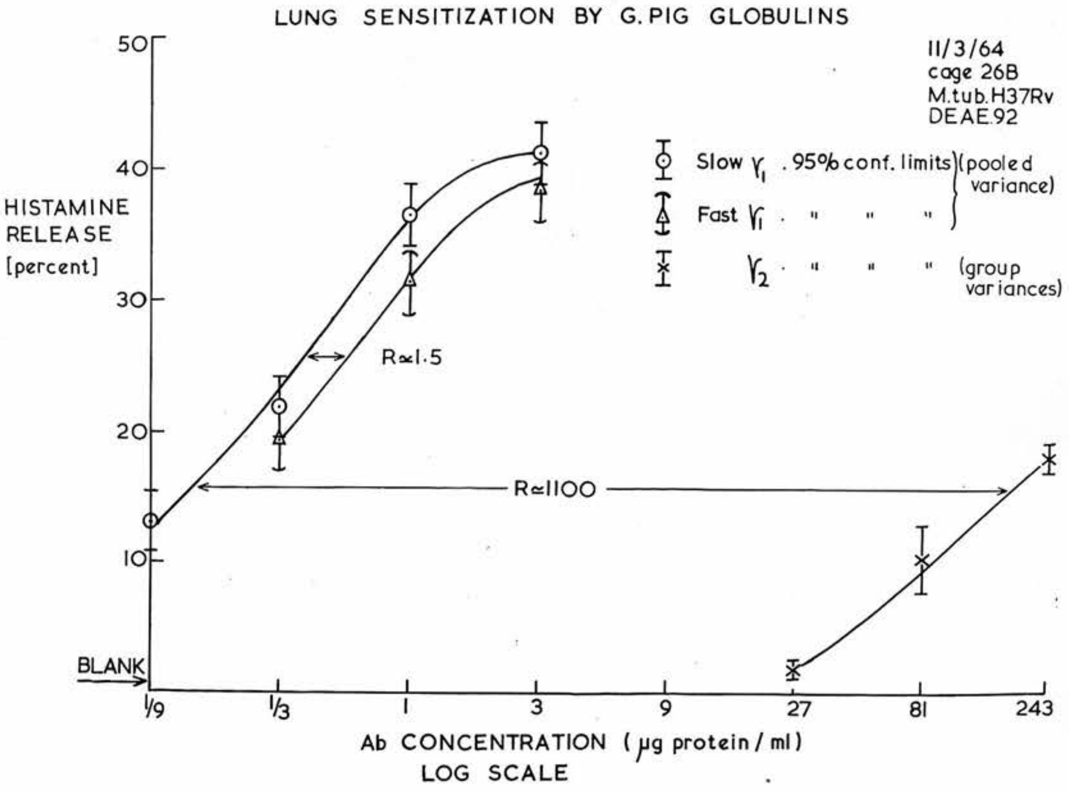
In the PCA test it was found that the potency ratios (total protein basis) between γ_1- and γ_2-globulins for batches in which both globulins contained comparable amounts of precipitating antibody were always lower than for batches in which most of the antibody was in the γ_1-globulin fraction. Furthermore the potency ratio between the globulins (for any given batch in which both globulins contain precipitating antibody) was always greater, usually considerably greater, when it was measured on lung than when it was measured by PCA (see Table 9). The results of a set of experiments in which the potency ratios on skin and lung were most similar, though even in this experiment they differ by a factor of about 9, are shown in Fig. 84 (skin) and Fig. 85 (lung). It is interesting that fast and slow preparations of γ_2-globulin appear equipotent on skin, but on lung the fast preparation appears to be about twice as potent as the slow. Although there is no further evidence on this point, and the difference might be within experimental error, this observation is consistent with the view that there is a very low level of contamination of γ_2-globulin with

Fig. 86 PCA (2). Fractions from GP26 anti-OA serum. The lines are calculated least squares estimates constrained to be parallel (so the estimated slope of the slow γ_1-globulin curve is different when it is compared with each of the other two preparations, as shown). R= least squares estimate of potency ratio on protein basis. As the γ_2-globulin only contained about half as much antibody as the γ_1-globulin when tested by haemagglutination, the potency ratio based on antibody content is about 0.5R

Fig. 87. Lung sensitization by same proteins as used in Fig. 86. R(=potency ratio on protein basis) estimated graphically.



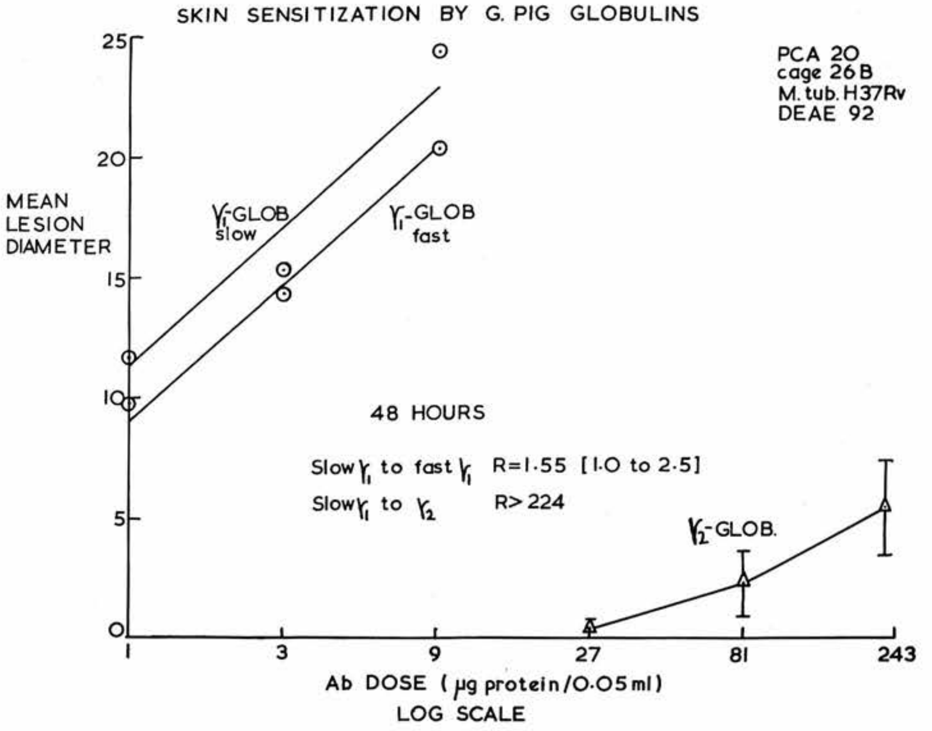
SKIN SENSITIZATION BY G. PIG GLOBULINS



LUNG SENSITIZATION BY G. PIG GLOBULINS

Fig. 88 PCA(20). As for Fig. 86 but 48 hour interval between antibody and antigen, instead of 4 hour.

Fig. 89 PCA(22). Fractions (DEAE 93) from GP 25 serum. Least squares estimates of R (with 95% fiducial limits) at 4.7 hours and 51 hours.


SKIN SENSITIZATION BY G. PIG GLOBULINS
PCA 20
cage 26 B
M. tub. H37Rv
DEAE 92
MEAN
LESION
DIAMETER
25
20
15
10
5
0
γ1-GLOB slow
γ1-GLOB fast
48 HOURS
Slow γ1 to fast γ1 R=1.55 [1.0 to 2.5]
Slow γ1 to γ2 R> 224
γ2-GLOB.
1
3
9
27
81
243
Ab DOSE (μg protein/0.05 ml)
LOG SCALE

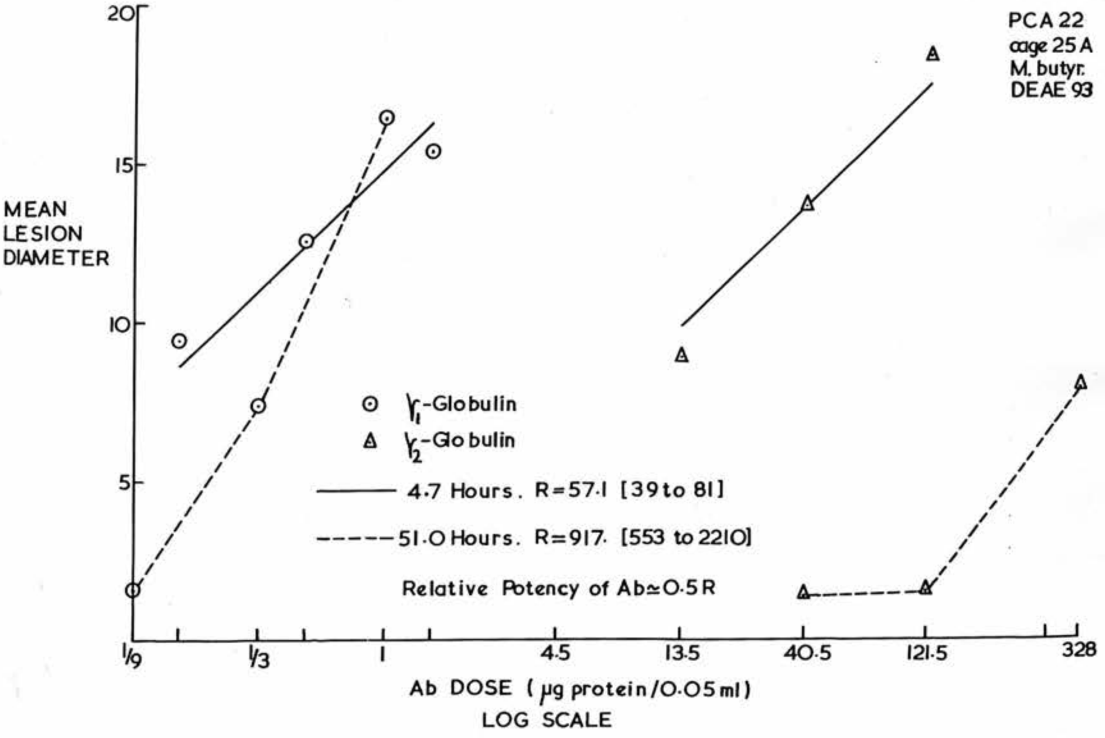
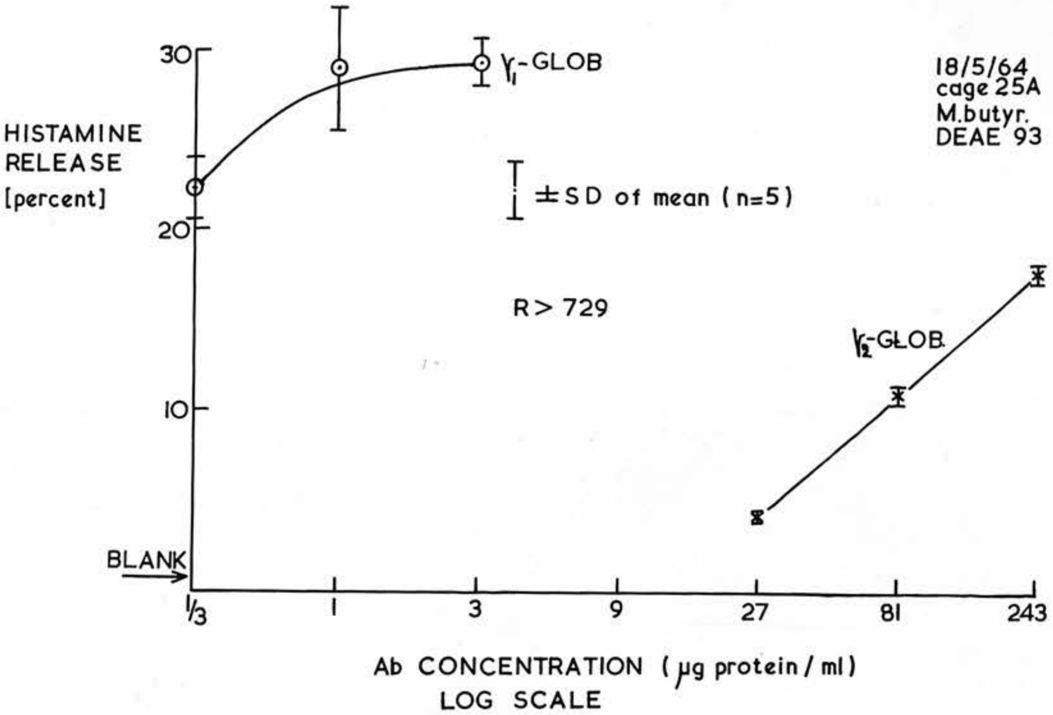

PCA 22
cage 25 A
M. butyr.
DEAE 93
20
15
10
5
MEAN
LESION
DIAMETER
⊙ γ1-Globulin
Δ γ2-Globulin
4.7 Hours. R=57.1 [39 to 81]
51.0 Hours. R=917. [553 to 2210]
Relative Potency of Ab≃0.5 R
1/9
1/3
1
4.5
13.5
40.5
121.5
328
Ab DOSE (μg protein/0.05 ml)
LOG SCALE

Fig. 90 Lung sensitization by proteins used in Fig. 89.


30
20
10
HISTAMINE RELEASE [percent]
BLANK
γ_1-GLOB
γ_2-GLOB
± SD of mean (n=5)
R > 729
18/5/64
cage 25A
M.butyr.
DEAE 93
1/3
1
3
9
27
81
243
Ab CONCENTRATION (µg protein/ml)
LOG SCALE

γ_1-globulin and this would be expected to be greater in fast batches of γ_2-globulin (which were made from fast total γ-globulin which was predominantly γ_1-globulin). This slight contamination would not be noticeable in PCA experiments if γ_2-globulin itself had some skin sensitizing ability, as is suggested by its greater potency with respect to γ_1-globulin when measured by PCA.

The results of experiments on the most extreme batch of globulins, in which the potency ratio between γ_1- and γ_2-globulins (on total protein basis) was about 100 times larger on lung than on skin, are shown in Figs. 86 and 87. These potency ratios were confirmed in independent experiments (see Table 9). In an experiment on lung tissue using the same batch of γ_1- and γ_2-globulins labelled with ^{131}I, a potency ratio of about 1094 was found after one hour incubation with antibody agreeing remarkably with the result shown in Fig. 87. In this experiment the potency ratio on lung was also measured at 4 hours in case it was lower (nearer the value on skin) after more prolonged incubation with antibody because, for example,

γ_2-globulin had the effect of slowing down sensitization due to a γ_1-globulin impurity in it. In fact the potency ratio was, if anything, larger after 4 hours incubation with antibody than after 1 hour, being about 1660, though this apparent increase may well be within experimental error. Again the simplest explanation of the discrepancy of potency ratios on lung and skin is that γ_2-globulin sensitizes the latter to some extent.

It seemed possible that γ_2-globulin might show some sensitizing power on lung tissue in vitro if complement were present. However when complement (2% fresh normal guinea pig serum) was added at the same time as antigen there was no increase in the amount of histamine released by γ_2-globulin antibody.

Change of potency ratio on skin with time. The results shown in Fig. 86 were obtained with the standard interval of 4 hours between the intradermal injection of antibody and intravenous injection of antigen and dye. If, instead of 4 hours, an interval of 48 hours is allowed to elapse, the potency ratio between γ_1- and γ_2-globulins is seen (Fig. 88) to have risen from about 11 to something greater than 200 (approximate extrapolation suggests a ratio of about 700). Another experiment with a different batch of globulins, the results of which are shown in Fig. 89, again showed an increase in potency ratio with time from 57 at 4.7 hours to 917 at 51 hours. The latter figure is comparable with the potency ratio of $>$ 729 observed with the same batch of globulins on lung tissue (see Fig. 90). In spite of the wide fiducial limits for the potency ratios (see Figures and Table) the change with time is obvious and strongly suggests that different substances must be responsible for producing skin sensitization in the γ_1- and γ_2-globulin antibody preparations.

When two different preparations of γ_1-globulin antibody (fast and medium mobility, both from the same batch of serum viz. GP 26) were assayed against each other by PCA, the potency ratio between them at 4 hours (1.32, 95% fiducial limits 0.9 - 2.0) was not significantly different from the potency ratio at 48 hours (1.55, limits 1.0 - 2.5) as shown in Figs. 86 and 88. The potency ratio between these preparations was also similar when measured by sensitization of lung tissue, viz. about 1.5 as shown in Fig. 87.

Perhaps the most powerful reason for believing that γ_2-globulin has some skin sensitizing ability is that, if the γ_2-globulin fraction produced sensitization only because it was contaminated with γ_1-globulin, the amount of γ_1-globulin needed should, in most cases, be easily detectable by immunochemical methods, whereas in fact none is detectable (with one exception, though even this is rather uncertain and the amount must be very small). For example the potency ratio between γ_1- and γ_2-globulins (column 92) of about 11 shown in Fig. 86 (or 9.3 in a duplicate experiment), if it arose by contamination, would necessitate the presence of about 10% of γ_1-globulin in the γ_2-globulin preparation if the γ_2-globulin did not interfere with sensitization, or even more if the γ_2-globulin did interfere. If this were present then the γ_2-globulin preparation (at a concentration of 4.5 mg protein/ml) should produce a line of about equal intensity to γ_1-globulin (at a concentration of 0.46 mg protein/ml) with specific anti-γ_1-globulin serum, whereas in fact, as has already been shown in Fig. 62, no γ_1-globulin at all is detectable in these circumstances.

Table 9

Results of sensitization experiments

All sera except GP 62/A and GP 16-18 contained precipitating antibody in the γ_2-globulin as well as the γ_1-globulin fraction.

Serum batch	Globulin batch	Potency ratio (R) of γ_1- and γ_2-globulins based on total protein content			Potency ratio based on antibody	
		Skin		Lung	Skin	Lung
		Interval (hours)	R, (95% fid. limits for R)			
GP 62/A	83(1) I and (2) II	4	$\gg$ 225	$\gg$ 540		
GP 16-18	82 I and II	4	202 (58 - 3300)	~ 367*		
GP 23	86(1) I and (1) II	4	97.8 (60.6-146)	$\geqslant$ 270**		
GP 22	87(1) I and (2) II 87(1) I and (2) II	4 4	82.4 (47.1-144) 106 (51.8-235)	750	about 59 about 77	about 540
GP 27	91(1) I and (2) II	3½	13.2 (6.5- 21.6)	260-470**	13.2	260-470
GP 26	92(1) I and (2) II 92(1) I and (2) II 92(1) I and (2) II	4 3 48	11.0 (7.5 -16.6) 9.34 (5.8 -13.3) > 224 (about 700 *)	1100 1094 †	about 5	about 500
GP 25	93(1) I and (2) II	4.7 51	57.1 (39.2-81.1) 917 (553 - 2208)	> 729	about 28	> 360

* obtained by extrapolation

** exact figure not obtainable as dose-response lines not parallel

† labelled with ^{131}I.

The adsorption of guinea pig γ-globulins by lung tissue.

It was uniformly found in six experiments that no difference could be detected in the amounts of labelled γ_1- and γ_2-globulins adsorbed at (or at least near) equilibrium.

The mean (5 replicates) values of the adsorption constant K' observed in an experiment with labelled γ_1- and γ_2- globulins (serum GP26, batch 92) are given in Table 10 . The amount adsorbed is seen to rise with time, as equilibrium is not reached in one hour. The values of K' are in the same range as those found with rabbit γ_2-globulin (see Figs. 29 and 30). In this experiment a high concentration of γ_2-globulin was used (labelled at 13.9 nc/mg, 1.03 atoms iodine/mole protein), and a low concentration of γ_1-globulin (labelled at 57.1 µc/mg, 0.7 atoms iodine/mole protein). The sensitization potency ratio between γ_1- and γ_2-globulins was found to be very similar to that found with unlabelled proteins of the same batch (see Fig. 87 and Table 9).

Table 10

Adsorption constants (K') for labelled GP γ_1- and γ_2-globulins on lung tissue

Time	Protein	Conc. (μg/ml)	K'	95% confidence limits for K'	Histamine release (percent)
1 hour	γ_2-globulin	250	47.5	± 2.8	7.8
		77.7	38.2	± 9.4	2.4
	γ_1-globulin	0.433	27.1	± 4.6	12.0
		0.134	37.1	± 12.4	3.0
4 hours	γ_2-globulin	250	70.4	± 9.6	10.3
		77.7	70.6	± 29.4	5.3
	γ_1-globulin	0.433	71.8	± 14.1	21.6
		0.134	96.1	± 8.3	9.8
					Control 0.8%

It can be seen that there is no consistent difference between the adsorption constants of γ_1- and γ_2-globulins. Unless γ_2-globulin actually has a higher adsorption constant than γ_1-globulin (shown not to be the case in experiments using low concentrations of both), this implies that the adsorption isotherm must be linear (within experimental error) up to a concentration of 250 µg γ-globulin/ml. Analysis of these results as a factorial experiment (2 proteins x 2 concentrations x 2 times), shown in Table 12 confirms the impression that there is no evidence for any difference in K' between proteins or concentrations, and that K' increases with time. The significant 1st order interactions must be random fluctuations or small systematic errors in the experiment. They did not occur in other experiments.

The results of another experiment, in which labelled γ_1- and γ_2-globulins were tested at equal (low) concentrations, are given in Table 11 . The duration of incubation with antibody was 2 hours and two concentrations of each protein were tested. In addition samples of the proteins were each separately labelled by the two procedures mentioned in the methods section. It can again be seen that there is no evidence for any difference between the adsorption constants of γ_1- and γ_2-globulins and that the method of iodination appears to have no influence on this result. These inferences are confirmed by the analysis of the results as a factorial experiment (2 proteins x 2 concentrations x 2 methods of iodination) shown in Table 13 . Owing to a fault in the inulin estimations in this experiment, a mean value from previous experiments was used. The mean of 106 determinations was 52.2 µl/100mg. Almost all the values in previous experiments were between 48 and 55 µl/100mg and, in any case, the value has no influence on the comparisons between proteins or between methods since a single figure for the the inulin space is subtracted from all of the results (values of n'/n - see "Measurement of adsorption at equilibrium" section).

The sensitization results in this experiment were a bit erratic but indicated that if there is any difference in sensitizing power of

γ_1-globulin labelled by the two methods it must be small. The γ_2-globulin in this experiment was from normal, not immune, serum. It behaved in the same way as immune γ_2-globulin in other experiments.

It is also of interest that the percentage of the label not precipitable with trichloracetic acid, and the specific activity seemed to depend on the protein, not the method of iodination. The specific activities of γ_2-globulin labelled by the two methods were 37.5 and 40.3 μc/mg and the percent non-precipitable values were 0.56% and 0.57%. For γ_1-globulin labelled by the two methods the specific activities were 23.5 and 27.6 μc/mg and the percent non-precipitable values were 2.3% and 2.2%. Although the proportion of the label not precipitable with TCA is excessively large in the case of γ_1-globulin, the fact that in this experiment both γ_1- and γ_2-globulin give similar adsorption results to those seen in other experiments in which the percent non-precipitable values were quite different suggests that this is not a crucial factor.

The lack of any significant difference between the adsorption constants of γ_1- and γ_2-globulins was confirmed in four other experiments.

Table 11

Adsorption constants (K') for γ_1- and γ_2-globulins labelled by different methods

Method	protein	conc. (μg/ml)	K'	95% confidence limits for K'
Protein injected into ^{131}ICl	γ_2-globulin	0.75	23.9	± 4.4
		3.0	28.2	± 4.8
	γ_1-globulin	0.75	29.0	± 4.8
		3.0	21.5	± 3.0
^{131}ICl injected into protein	γ_2-globulin	0.75	25.8	± 3.2
		3.0	22.8	± 1.9
	γ_1-globulin	0.75	24.9	± 6.5
		3.0	25.8	± 3.9

Table 12

Analysis of variance for adsorption constants shown in Table 10

Source	d.f.	SS	MS	VR	P
Between proteins	1	18.63	18.63	<1	N.S.
Between concentrations	1	398.8	398.8	3.27	0.10–0.05
Between times	1	15805	15805	130	$\ll 0.001$
times x conc.	1	353.4	353.4	2.90	0.10
times x proteins	1	1472.6	1472.6	12.1	0.01–0.001
conc. x proteins	1	1182.7	1182.7	9.69	0.01–0.001
times x conc. x proteins	1	14.0	14.0	<1	N.S.
Between treatments	7	19244.7			
Within treatments	32	3904.0	122.0		
Total	39	23148.8			

Table 13

Analysis of variance for adsorption constants shown in Table 11

Source	d.f.	SS	MS	VR	P
Between proteins	1	0.915	0.915	<1	N.S.
Between concentrations	1	10.29	10.29	<1	N.S.
Between methods	1	3.65	3.65	<1	N.S.
proteins x conc.	1	32.13	32.13	2.5	0.2-0.1
proteins x methods	1	9.87	9.87	<1	N.S.
conc. x methods	1	2.53	2.53	<1	N.S.
proteins x conc.xmethods	1	155.1	155.1	12.0	0.01-0.001
Between treatments	7	214.505	30.6	2.4	0.05
Within treatments	32	411.905	12.87		
Total	39	626.410			

DISCUSSION

Histamine Assay Method

The results and calculations given in appendix (2) show the simple method used to be adequate in practice. The errors involved are:

(a) The error caused by using linear interpolation when logarithmic interpolation might be more appropriate. Equations have been derived which show that the maximum error of interpolation, as long as the unknown lies between the two standards, would be 2.07% in the case when the ratio between high and low standard doses was 1.5 (it was hardly ever higher than this and often lower). The average error of interpolation, if the concentrations of unknown solutions fall equally often at different points between the two standards, has been shown to have a value of only 1.375% in the above case. These figures, of course, refer to the difference between the estimates obtained by the two methods of interpolation and therefore are properly referred to as errors only if the logarithmic interpolation were the correct one, which is itself only approximately true. It is also shown in the appendix that the interpolation error increases rapidly as the ratio between high and low standard doses increases. For example when this ratio is 4.0, instead of 1.5 as in the above example, the maximum interpolation error is 26.4%, and the average error 17.1%. It is of interest that the equations in appendix (2) show that when the unknown response is a given distance from a standard response, the error of interpolation will always be less if the unknown response is near the upper rather than the lower standard, though this effect is not likely to be large enough to matter in practice.

(b) Error owing to interpolation by eye instead of measuring responses. This might be expected to be an individual matter. The example analysed in appendix (2) shows that the author quite consistently underestimates, on the average by about 2%, the value for the unknown concentration obtained by linear interpolation with measurement of responses. In fact this effect more than compensated for the interpolation error so that visual linear interpolation gave values for the unknown concentrations which were, on the average, 0.9% <u>less</u> than those obtained by logarithmic interpolation with measured responses. If the histamine releases were expressed as percentages of the total tissue histamine the difference between the two methods of interpolation was even less.

(c) Error of replicate assays on the same sample. As shown in appendix (2) the coefficient of variation of the mean of two potency estimates on the same sample was between 3% and 6%, and since this was based on a large number of degrees of freedom the 95% confidence interval would be between ± 6% ± 12%. Usually 5 replicate samples of each treatment were done.

It is evident that the accuracy of the simple assay procedure is quite adequate.

Diffusion and Adsorption of γ-globulin

The calculations and results presented show that even in the small particles of chopped lung tissue diffusion of γ-globulin is not fast enough to be ignored. It probably takes 15 or 30 minutes before the concentration of γ-globulin in the extracellular space has substantially equilibrated with that in the external solution. The calculations also indicate that it is improbable that the standard washing procedures lasting only a few minutes, which have been used by various workers to remove γ-globulin from the extracellular space, do in fact remove it all in the case of chopped lung tissue. It seems likely that diffusion may be an equally important factor in the studies of adsorption on guinea pig ileum in which the washing period was short. In accordance with these conclusions it was found that adsorption, when measured by the inulin method, was rather less than that found by washing the tissue three times with Tyrode solution. However if this washing was followed by a 15 minute or longer incubation in Tyrode's solution, the amount of γ-globulin remaining in the tissue was, as expected, less than the amount originally adsorbed.

The possibilities that the method of labelling, the extent of iodination, or self-irradiation damage might influence the measured extent of adsorption have been discussed already. No evidence was obtained that any of these factors were important.

In order to carry out calculations on the effect on diffusion rate of the rate and extent of adsorption, it has been necessary to consider the adsorption rate and equilibrium constants from a more rigorous point of view than has previously been appropriate. Details have been given in appendix (5). It has been shown that although the amount of γ-globulin adsorbed usually appears to increase slowly even after one or two hours incubation, this is not likely to cause serious error in the assumption that the extracellular space has equilibrated at this time. The main conclusion to be drawn from the calculations of diffusion rate is that it is not possible to make inferences concerning the mechanisms of adsorption and sensitization from observations of their rates over short time periods. Although adsorption itself is certainly not a fast process, and the observed rate of adsorption cannot be accounted for entirely by diffusion, nevertheless detailed inferences from observations of rates at incubation times of less than 30 minutes are probably not justified. The rate of adsorption observed by Humphrey and Mota (1959) using guinea pig mesentery, in which diffusion should be fast, also shows that adsorption itself is not particularly rapid. It is of interest that the time course of uptake of γ-globulin by various "false tissues" observed by Brocklehurst et al. (1961), and the proportion of γ-globulin remaining after washing procedures, are roughly what would be expected from the calculated diffusion rate confirming the authors' suggestion that the observed uptake and wash-off curves might be accounted for partly or largely by diffusion. As diffusion should be even slower in tissue particles than in agar gel particles of the same external dimensions it seems improbable that factor chiefly responsible for the shape of the curves is reversible adsorption, though this will undoubtedly influence their shape. No calculations have been made for guinea pig ileum but it seems probable that similar remarks apply to the results of Nielsen et al. (1959) and Feigen et al. (1962).

Is there a fast phase in the adsorption or sensitization processes?

The problem of diffusion is also relevant to the possibility of the existence of a fast initial stage in the sensitization process as suggested by Mongar and Schild (1960, 1962). In the present work no consistent evidence for such a stage has been seen (see Figs. 17, 19, 22, 23, 24), even though measurements were made after 5 minutes incubation in some of the experiments, and the lung tissue was perfused before chopping which would be expected to favour the appearance of a fast phase. The possibility that such a phase exists cannot be totally ruled out on the basis of the present work but no real evidence for it has been obtained. It may be noted that the calculated curves for diffusion of γ-globulin into the extracellular space rise very steeply at first but flatten out considerably later. It is, perhaps, not impossible that purely diffusion controlled phenomena might give rise to the appearance of a fast initial phase.

The adsorption isotherm.

In the present work no consistent evidence has been obtained against a linear adsorption isotherm. This might be expected in the low concentration range used, the concentration of γ-globulin usually being less than 3 µg/ml. 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 (see Table 10, p.142). Although adsorption isotherms of various forms have been reported the present results are probably not inconsistent with those of earlier workers as mentioned in the review section.

Since adsorption is a monotonically increasing function of sensitization, and sensitization (at least of a whole tissue) is not an all-or-nothing phenomenon, it is,a priori, inevitable that there will exist a range of antibody concentrations over which the degree of sensitization is correlated with the amount of antibody adsorbed, and equally a priori inevitable that no conclusions could be drawn from this fact alone unless the isotherm had an unusual form in the region in which sensitization was submaximal.

The extent of adsorption observed in the present experiments was of the same order as, or rather less than that reported by other workers. K' was in the region of 40 µl/100mg for all the γ-globulins examined. With most guinea pig γ_1-globulin anti-ovalbumin preparations definite sensitization was produced by a concentration of 0.1 µg total protein /ml, i.e. about 40 ng of adsorbed protein per gram of wet tissue. Since only part of this protein is antibody, and roughly half of the adsorbed material can be removed without reducing the sensitization it appears that less than 10 ng antibody/g tissue is necessary to produce observable histamine release. A solution of γ-globulin of molecular weight 160,000 and 0.1 μg/ml is about 6×10^{-10} molar, which compares well with the most potent drugs. For example a concentration of at least 1 ng/ml of histamine is usually needed to produce a contraction of the guinea pig ileum. This is about 9×10^{-9} M, i.e. about 15 times more concentrated than the total protein concentration needed to produce detectable histamine release from lung tissue. For near-maximum sensitization about 1 μg/ml γ_1-globulin was usually needed, corresponding to 0.4 μg protein /g tissue which is comparable with the values of less than 1 μg/g observed by Brocklehurst et al. (1961) using rabbit γ-globulin. Nielsen et al. (1959) found that the smallest amount of antibody that must be taken up by guinea pig ileum to give a detectable Schultz-Dale response was about 0.8 μg/g. This may be compared with 0.6 μg/g found by Ishizaka et al. (1957) and 0.02 μg/g found by Brocklehurst et al. (1961). The present figure of 0.04 μg total γ_1-globulin /g (probably implying not more than 0.01 μg antibody/g required) is at the bottom end of the range of published values.

What proportion of receptors need be occupied to produce sensitization?

The present work allows no estimate of the proportion of the putative "specific receptors" for γ-globulin which need be occupied by antibody to produce sensitization. Mongar and Schild (1962) suggest that it must be less than 1% since rapid maximal passive sensitization can be produced by a quantity of guinea pig antibody equivalent to about 1% of the total γ-globulin in the circulation. However, although the directly measured affinities of guinea pig γ_1- and γ_2-globulins for lung tissue are the same, the results of passive cutaneous anaphylaxis experiments obtained by Ovary et al. (1963) suggest that the affinity of γ_1-globulin may be much larger than that of γ_2-globulin for the receptors concerned in sensitization. Since the present experiments show that the proportion of γ_1-globulin in the total γ-globulin is much greater in immune than in normal serum it seems likely that the amount of γ_1-globulin injected was a good deal more than 1% of the γ_1-globulin in the circulation. Nevertheless it seems probable that experiments of this sort would lead to the conclusion than only a small proportion of receptors need be occupied by sensitizing antibody.

Mongar and Schild (1962) calculated, from the results of Nielsen et al. (1959) that more than 30% of the available surface of guinea pig ileum had to be covered with antibody to produce a maximal Schultz-Dale response. However, as has been discussed in the review section, the claim that adsorption is Langmuirian has not been confirmed by other workers or by the subsequent work of Feigen et al. (1962), so there is no evidence for monolayer formation.

The effect of ionic strength on adsorption and sensitization.

In the present experiments it has been shown that the amount of antibody adsorbed increases rapidly as ionic strength is increased, but no increase in sensitization results (possibly owing to an adverse effect of low ionic strength media on the tissue). The lack of increase in sensitization is in agreement with Mongar and Schild (1960) who mention that "Appreciable sensitization even occurred in sucrose solution". The lack of effect of calcium ion concentration on sensitization confirms the results of Mongar and Schild (1960) who found that sodium edetate had no effect on the sensitization process.

Binaghi, Liacopoulos, Halpern, Liacopoulos-Briot and Bloch (1961) found that the rate of sensitization of guinea pig ileum by rabbit anti-ovalbumin sera was very greatly increased in solutions of low ionic strength. The effect appeared to depend roughly on the ionic strength of the medium (i.e. $\Gamma/2 = \frac{1}{2} \sum_i c_i z_i^2$ where c_i is the molar concentration and z_i the charge of the i th ion) and not on the nature of the ions present. These results, and the fact that increased adsorption at low ionic strength would be expected on physicochemical grounds, were the reasons for performing the present experiments. The explanation of the increased adsorption can be looked at from two viewpoints.

Firstly, since γ-globulin is a charged molecule, it would be expected from the Debye-Hückel theory that its activity coefficient would decrease with ionic strength. This, of course, is an explanation of the salting-in effect seen with most charged molecules (at higher ionic strengths often masked by a larger salting-out effect) (see, for example, Edsall, 1947). The solubility of γ-globulin increases with ionic strength (i.e. it behaves like a euglobulin) when it is on the alkaline side of its isoelectric point (as in Tyrode's solution) though not when it is on the acid side (Edsall, 1947). This implies that the activity coefficient of γ-globulin should decrease with increasing ionic strength, so decreased adsorption would be expected with increasing ionic strength, as observed. (This argument assumes that the activity coefficients of γ-globulin in the solid and in the adsorbed phases do not depend on the ionic strength of the solution).

Secondly, the situation can be viewed from a molecular rather than a thermodynamic point of view. According to the Gouy-Chapman theory, and more recent improvements on the simple theory, the surface potential and thickness of the ionic double-layer near to charged surfaces should decrease with increasing ionic strength. Since it is very probable that the cells, like the γ-globulin molecules, are charged, electrostatic interaction between cell and protein should increase as their surface potentials increase, i.e. as ionic strength decreases. As this increased interaction leads to increased adsorption, it might be inferred that the interaction was attractive, i.e. that the combining sites on cell and protein had opposite charges. However it is possible that increased adsorption might result solely from an increase in activity coefficient of the dissolved protein even if the cellular receptor had no charge or a charge the same as that of the protein. If both receptor and protein were charged, the activity coefficients of <u>both</u> should increase as ionic strength decreases, whatever the sign of the charges. This should tend to cause an increase in adsorption and, at first sight appears to contradict the non-thermodynamic argument which would predict an increase in adsorption with decreasing ionic strength only if the protein and receptor had charges of opposite sign. The resolution of this apparent paradox lies, probably, in the assumption (mentioned above) that the chemical potential of the γ-globulin in the adsorbed phase was independent of the ionic strength of the solution. If the cellular receptor was charged and exposed to the external solution this assumption might well not be fulfilled.

Even if it were fulfilled, and the adsorption equilibrium constant went up in spite of receptor and protein having like charges, the expected increase in adsorption on lowering the ionic strength might never be observed because of the large potential barrier (i.e. increased activation energy of adsorption) which would result from the increased surface potentials of like charge.

Although glycine is not a non-electrolyte, but a dipolar molecule with the properties, to a diminished extent, of an electrolyte, the increase in adsorption of γ-globulin observed in glycine solutions was almost as large as that observed in non-electrolyte solutions, and Binaghi et al. (1961) found that an increase in rate of sensitization in glycine, alanine and γ-aminobutyrate solutions similar to that in non-electrolyte solutions. These findings are consistent with the fact that, although glycine increases the solubility of many proteins, it does not (for reasons unknown) increase the solubility of "serum globulin" (Edsall, 1947).

It is of interest that γ-globulin (and to a greater extent β_{1C}- and β_{1E}-globulins) have been shown to be adsorbed onto red cells at low ionic strengths (Mollison and Polley, 1964) and that the association of antibody with various antigens is increased at low ionic strength (see Hughes-Jones, 1963).

Adsorption from the dynamic point of view

The fundamental relationship governing adsorption, when it is viewed from a molecular point of view, is

$$s = n\tau$$

where s is the number of molecules adsorbed on unit area of surface at equilibrium (cm^{-2}), n is the number of molecules which strike unit area of the surface in unit time and become adsorbed, i.e. the effective collision rate ($cm^{-2} sec^{-1}$) and τ is the average length of time for which a molecule stays on the surface before becoming desorbed (sec) (see, for example, de Boer, 1953 and Davies and Rideal, 1961).

It had been hoped, when the work on adsorption was started, that interesting results might be obtained using this relationship. The results could only be approximate since the surface area available for adsorption could only be roughly estimated, but useful information might derive from "order of magnitude" calculations. The situation turned out to be even more difficulat than expected so it is probably not worth including the tentative numerical results obtained here, but the method used will be outlined as it should be applicable in the future when better experimental results are obtainable, perhaps from isolated mast cell preparations.

Given an estimate of surface area, the adsorption, s, can be obtained directly from the experimental observations. The time τ can also be determined experimentally since it is easily shown that the assumption of an average life on the surface implies that desorption must be exponential in time, and the time constant for desorption (i.e. the time needed for $100(1-{}^{1}/e)= 63.2\%$ of adsorbed molecules to leave the surface) must be τ. This could, in principle, be simply determined by measuring the time constant for desorption of labelled protein into a well stirred solution containing an excess of unlabelled protein (to prevent readsorption of desorbed molecules). In practice this could not be done, firstly because of the complication introduced by diffusion through the tissue, and secondly because of the great heterogeneity of the surface (implying that a single average life is insufficient to describe the situation). If the results given by the inulin method represent real adsorption (and, as discussed earlier, most sources of error would tend to make the figures too high rather than too low), then it is clear from Fig. 32, and from the results obtained by other workers, that part of the adsorbed material desorbs quite rapidly, and part very slowly. A rough estimate of τ for the putative receptors involved in sensitization might be obtained from the persistence of sensitization under some circumstances (e.g. PCA in vivo), although it cannot reasonably be assumed that the response is proportional to the number of such receptors occupied.

If estimates of s and τ were available then n could be calculated, and compared with the theoretical value for the actual collision rate with the surface, and so an estimate of the proportion of collisions which lead to adsorption calculated. Various interpretations of this figure are possible. If the activation energy for adsorption was low (not necessarily, or even probably, true in view of the above discussion of the electrostatic forces operating between the protein and the surface), or if an estimate of it could be made (e.g. by calculating the electrostatic barrier to adsorption which might be possible since the charge densities on both the cell and the protein could be estimated), then the proportion of effective collisions i.e. the proportion of those molecules striking the surface which became adsorbed, might be interpreted as the proportion which approached the surface with the correct orientation, and from the known (or calculated) rotational diffusion coefficient of γ-globulin, an estimate of the size of the part of the molecule involved in adsorption might be made.

If the theoretical figure for the actual number of collisions with the surface is calculated from the gas kinetic formula, as advocated by Moelwyn-Hughes (1947), then even the most extreme assumptions concerning the values of s and τ lead to the conclusion that the proportion of effective collisions is very small. In fact the gas kinetic formula almost certainly overestimates the actual number of collisions, and an estimate based on Einstein's treatment of Brownian movement and diffusion would be more appropriate.

Rate control by diffusion at the interface

A possible rate controlling factor is diffusion at the interface. Adsorption will result in a lowering of the γ-globulin concentration near to the cell surface and, especially in solutions of low concentration, diffusion into this depleted zone may be a rate controlling factor. The correct equations describing this situation have been given by Ward and Tordai, (1946), but their application in the present case has not been possible owing to the complication of diffusion through the tissue, lack of knowledge of the surface area for adsorption and the heterogeneity of the surface.

The purification and properties of guinea pig immunoglobulins

The results herein reported were started on the basis of the preliminary report by Ovary and Benacerraf (1962). The method adopted for the preparation of the two immunoglobulins was based on that used by Fahey and Horbett (1959) for the preparation of human immunoglobulins. It has been shown in the present work that this produced γ_2-globulin without any detectable impurity, but γ_1-globulin which always contained some γ_2-globulin; the contamination probably being greatest in the preparation of γ_1-globulin from normal serum in which the ratio of γ_2- to γ_1-globulin in the total γ-globulins is high. γ_1-globulin was also contaminated with β-globulins if a fast electrophoretic fraction of the total γ-globulin was used in its preparation. It seems likely, from the methods of preparation used, that these impurities were present in the preparations of the other workers discussed in the review, but no results have been presented concerning the question of purity. Yagi and Maier (1962a) rechromatographed the fractions they obtained by single stage chromatography from whole serum and stated that the results "showed that there was practically no cross-contamination between them." However it has been shown that the γ_2-globulin which contaminates γ_1-globulin preparations is of similar (fast) mobility to the γ_1-globulin and it is therefore to be expected that if the preparation is rechromatographed both γ_1- and γ_2-globulins will, being of the same mobility, emerge as a single peak. Rechromatography is therefore no criterion for purity in the case of proteins like γ-globulins, which have a distribution of isoelectric points, and which can therefore be separated into fractions which differ in mobility but are antigenically identical. It is possible that the extent of contamination could be altered by starting with a different buffer as this has been shown by Fahey and Horbett (1959) to alter the proportion of the total γ-globulin emerging in different peaks from DEAE cellulose columns, but in view of the nature of the contamination it seems unlikely that it could be eliminated altogether using the present method of fractionation. The present results are very similar to those obtained with human immunoglobulins (by, for example, Fahey, 1962). Total human γ-globulins, when fractionated on DEAE cellulose, are separated into a first peak containing pure 6.6S γ-globulin and a second peak containing γ_{1A}-globulin plus some 6.6S γ-globulin, traces of the latter being eluted right up into the region where albumin comes off the column. There appears to be no work concerning the possible presence of macroglobulins in guinea pig serum and they have not been looked for in the present study.

It would appear that the chromatographic, immunoelectrophoretic, ultracentrifugal and sensitizing properties of guinea pig γ_1-globulin are sufficient to establish it as the analogue of human γ_{1A}-globulin. This is supported strongly by the finding of Thorbecke et al. (1963) that the antigenic difference between guinea pig γ_1- and γ_2-globulins lies in papain fragment F, and therefore presumably in the heavy polypeptide chains. No results concerning carbohydrate content have been reported though preliminary experiments in the present study suggested that there may be little difference between the carbohydrate contents of γ_1- and γ_2-globulins. If true this would be different from the situation found in the human analogues.

Although no criteria have been agreed for deciding when immunoglobulins of other species can be considered analogues of the human proteins, it seems safe on the above mentioned grounds to consider the analogy established. Consequently it is proposed that the guinea pig proteins should no longer be called γ_1- and γ_2-globulins, but renamed in accordance with the very recent decision of the World Health Organization ("Nomenclature for Human Immunoglobulins", 1964) as γA and γG (or IgA and IgG) respectively, although, for the sake of consistency, these terms will not be used in the rest of this report.

The results concerning the antigenic differences between γ_1- and γ_2-globulins are consistent with those of other authors. They confirm the care that must be taken to be sure that the antisera used for gel-diffusion analysis contain antibodies against all relevant antigens. For example, in the first report of Ovary and Benacerraf (1962) it was reported that the two globulins gave a reaction of identity. This must have been because the antiserum used contained antibodies against only the antigenic determinants common to both globulins. The commercial antisera, and some rabbit anti-guinea pig sera, used in the present study were of this sort. The antisera used by Benacerraf et al. (1963) contained no antibodies to determinants unique to γ_1-globulin, whereas most of the antisera used for routine immunoelectrophoresis (except of course R100, R101, R106 and R108) in the present study were deficient in antibodies against determinants unique to γ_2-globulin. This accounts for the difference in direction of the spur seen in the immunoelectrophoretic study of whole serum in the present work and that of Benacerraf et al. (1963).

The γ-globulin fractions used in the present study were incomplete in the sense that all mobilities present in the original serum were not also present in the purified protein. This must have been true in other studies also, especially when, as in the work of Ovary et al. (1963) and of Bloch et al. (1963), separation was accomplished solely by electrophoresis and adequate purity obtained by taking only the slowest γ_2-globulin and fastest γ_1-globulin.

The additional lines observed in the γ mobility region on immunoelectrophoresis by Ovary et al. (1963) and Thorbecke et al. (1963) have not been observed in the present study (except possibly in degraded samples). The reason for this is obscure at the moment.

The results of sensitization experiments are largely consistent with those of other authors. Results on sensitization of lung tissue in vitro have not been previously reported. The finding that lung sensitization is a property of the γ_1-globulin antibody is what might be expected on the basis of the report by Ovary et al. (1963) that this antibody was responsible for passive sensitization leading to systemic anaphylaxis. Austen (private communication) has obtained similar results.

The present results concerning sensitization of skin are not inconsistent with those presented by White et al. (1963). The potency ratio between γ_1- and γ_2-globulin antibodies appeared to be rather higher in the experiments of Ovary et al. (1963) than in those of White et al. (1963), though systematic investigations of the potency ratios have not been published. The potency ratio of greater than 100 inferred from one set of results given by Ovary et al. (1963) is somewhat larger than was observed in any of the present experiments in which both γ_1- and γ_2-globulins contained precipitating antibody. The following evidence suggests that, in the present experiments, γ_2-globulin antibody itself was producing some skin sensitization (though certainly less than the γ_1-globulin antibody):

(1) The potency ratio between γ_1- and γ_2-globulin antibodies was always greater when measured on lung than when measured on skin when both globulins contained precipitating antibodies. When no detectable precipitating antibodies were present in the γ_2-globulin the potency ratio measured by PCA was higher than when such antibody was present, and seemed to approach that measured on lung. These facts suggest that different principles were responsible for sensitization in the γ_1-globulin and γ_2-globulin fractions. It might be argued that in both cases γ_1-globulin was responsible, but that the γ_2-globulin with which it was mixed in the γ_2 fraction was more effective

in inhibiting (by competition for antigen) the response to γ_1-globulin in lung than in skin. If this were so then batches in which the γ_2-globulin fraction contained little antibody should have a potency ratio as low on lung as that found on skin. In fact both the quantitative experiments carried out with such batches suggested that the potency ratio in this case was larger on skin rather than lower on lung. As it seems implausible that the γ_2-globulin could actually enhance the response on skin to a γ_1-globulin contaminant in it, and as the results of Ovary et al. (1963) and of Kourilsky et al. (1963) suggest that the only likely interference by γ_2-globulin is through competition for antigen and that the proportion of γ_2-globulin in γ_1-globulin preparations is too small to have any effect, the conclusion that the skin response is not due to a γ_1-globulin contaminant, and is therefore presumably due to γ_2-globulin itself, seems inescapable.

(2) The rate of disappearance of skin sensitization by γ_1- and γ_2-globulin fractions has been shown, with two different

batches, to differ considerably. Although γ_2-globulin might conceivably alter the rate at which a hypothetical γ_1-globulin contaminant mixed with it produced sensitization, it does not seem plausible to imagine that the rate of loss of an established sensitization could be altered. Again the implication is that different substances are responsible for sensitization in the two fractions.

(3) The amount of γ_1-globulin contamination required to produce the sensitization observed with the most active batches of γ_2-globulin should be easily detectable by immunochemical methods using specific anti γ_1-globulin sera even if the γ_2-globulin did not interfere at all with sensitization (if it did interfere, even more γ_1-globulin would be needed to produce the observed response). In fact it has been shown that, with a single rather uncertain exception, no γ_1-globulin was detectable in any of the γ_2-globulin preparations and in no case was there any sign of the amount necessary to produce the observed responses.

The conclusion that γ_2-globulin can itself produce some sensitization is in disagreement with the conclusion of Ovary et al. (1963), and the one potency ratio which can be inferred from their results seems somewhat higher than those observed in the present experiments. The reasons for this disagreement are uncertain but it is possible that the methods of preparing the fractions might influence the results. The fractions used by Ovary et al. (1963) were prepared by specific precipitation of the antisera, dissociation of the precipitate and electrophoretic separation, compared with electrophoretic and chromatographic separation of antiprotein sera in the present experiments. This means that the proportion of precipitating antibody in the preparations of Ovary et al. (1963) would be higher than the proportion in the work here reported. They would, however, contain some residual bound hapten, and would not contain much non-precipitating antibody. There is no good evidence on the denaturant effects of different methods of preparation although Austen (private communication) points out that lung sensitization was produced in the present experiments by a lower concentration of protein than he had observed using the preparative methods of Benacerraf et al. (1963). Also no mention is made by Benacerraf et al. (1963) of the methods used for storing proteins. In the present experiments no isolated γ-globulin solutions were frozen. A difference in experimental method which might be important is the dose of antigen used in PCA experiments. Ovary et al. (1963) used 120 µg of haptenic antigen or 60 µg of ovalbumin per guinea pig compared with a dose of between 20 and 30 mg of ovalbumin used in the present experiments. However White et al. (1963) used a dose of only about 12.5 µg ovalbumin per guinea pig and the results they obtained in the two published experiments were probably not inconsistent with the present results, although potency ratios were not systematically investigated.

Since the results of Bloch et al. (1963) suggest that γ_2-globulin antibody is rather more active than γ_1-globulin antibody in producing the reversed passive Arthus reaction (ovalbumin 2mg I.V. followed immediately by antibody intradermally) it might be suggested that what was observed with γ_2-globulin was in fact a modified or mild Arthus reaction, but since the lesion had the typical appearance of a PCA lesion and was produced by the method normally used for PCA experiments this approach does not seem to be useful. In spite of the similarity in appearance of the lesions it seems likely that the two globulins were acting by different mechanisms but no evidence is available on this point.

Does fixation mean adsorption?

The term fixation is usually used in the immunological literature as though it meant adsorption, but in fact what is measured is sensitization. The use of the term is, in the words of Austen and Humphrey (1963), "essentially vague".

The fact that there is no apparent difference between the extent to which γ_1- and γ_2-globulins are adsorbed (using the inulin method) is consistent with the finding of Humphrey and Mota (1959) that antibodies from "non-sensitizing species" (horse, rat and goat) were adsorbed onto guinea pig mesentery to a similar extent to sensitizing antibodies (rabbit). (In a single experiment in the present series it was found that antibodies from non-sensitizing species were adsorbed onto lung tissue far less than rabbit antibody, but as no more experiments have been done it does not seem wise to rely on this observation at the moment). Both findings imply that the adsorption measured is not the same as fixation. This is, perhaps, not particularly surprising since it is quite possible that those sites on the tissue which can adsorb γ-globulin and produce histamine release on addition of antigen ("specific sites") are only a small proportion of the total number of sites; and further, that the proportion of "specific sites" which need to be occupied in order to produce maximum histamine release may also be small. If the affinity of fixing and non-fixing γ-globulins differed only for the specific sites but not (because of a different sort of attachment e.g. attachment through a different part of the γ-globulin molecule) for the non-specific sites, it would be surprising if any difference in total adsorption could be observed. As discussed in the review, the results of Feigen et al. (1962) can be plausibly interpreted as being due to a different ΔH for the reaction of sensitizing

γ-globulin with specific and non-specific sites. If this interpretation were true it would imply the reaction with specific sites was more endothermic than that with non-specific sites, i.e. ΔH was more positive for specific sites. According to the results of Feigen et al. (1962) the amount adsorbed, assumed to be mainly on non-specific sites, alters little with temperature implying that ΔH is near to zero for non-specific sites (as it is for many protein-protein interactions). If the affinity (i.e. $-\Delta F$) of γ-globulin for specific sites is no less than for non-specific sites, then the implication is that the entropy increase, ΔS, is greater for reaction with specific sites than that with non-specific sites which could mean that the former reaction involves more extensive dehydration or less restriction of the conformation of the antibody molecule than the latter. This interpretation, although largely speculative, seems to be less in conflict with thermodynamic theory than that offer~~r~~ed by Feigen et al. (1962).

The question remains of whether fixation means adsorption on specific sites which cannot be detected by the currently available methods for adsorption measurement. There is the additional possibility that non-fixing antibody has just as great an affinity for specific sites, but that, even though adsorbed, it has no ability to release histamine when antigen is added. In pharmacological terms (Stephenson, 1956) such an antibody molecule might be referred to as having affinity, but no efficacy. It would be expected that such a molecule would antagonize the effect of antibodies with non-zero efficacy to an extent dependent on their relative affinities and concentrations. It was found by Ovary et al. (1963) that guinea pig γ_2-globulin did not inhibit skin sensitization produced by γ_1-globulin (except by competition for antigen) but the experiments reported were not really quantitative. Binaghi, Liacopoulos, Halpern and Liacopoulos-Briot (1962) reported that the sensitization of guinea pig ileum by rabbit antibody was inhibited by normal (non-specific) γ-globulins from various species in the following order of effectiveness: rabbit > man > dog > guinea pig > rat > horse > cattle > pig > chicken > goat. This order resembles the order of effectiveness of antibodies from the same species for passive sensitization of guinea pig tissues, but even globulins that themselves consistently fail to sensitize produced some degree of inhibition, horse γ-globulin producing considerable inhibition at a concentration of about 6 mg/ml in this system. Biozzi, Halpern and Binaghi (1959) found that horse γ-globulin had no effect on PCA responses at 10 mg/ml and Mongar and Schild (1960) found that bovine γ-globulin did not inhibit sensitization of guinea pig lung by rabbit antibody when the concentration of γ-globulin was 4 mg/ml. A slight inhibition, probably within experimental error was seen at 10 mg/ml. These results suggest that γ-globulins from non-sensitizing species do not act as effective antagonists and therefore it would be inferred, if the pharmacological model is realistic, that the γ-globulins from "non-fixing" species have a low affinity for the specific sites, i.e. are not adsorbed onto them to any great extent. This being so the question of their efficacy is hypothetical although the results of Binaghi et al. (1962) mentioned above suggest that they do have some affinity, but no efficacy as even the highest concentrations will not produce sensitization. The bulk of the evidence at the moment, however, does seem to suggest that "non-fixing" γ-globulins are in fact not well adsorbed on specific sites.

Binaghi et al. (1962) suggest that since tissue could still be sensitized in the presence of normal γ-globulin, although at a slower rate, the model used in the above argument, of competition for receptors, is invalid, and propose that antibody and non-specific γ-globulins interact in some way in solution. There appears to be no evidence for this proposition and it seems far from obvious, at the moment, that the results of Binaghi et al. (1962) are in fact incompatible with a competitive theory. The sensitization did not reach an equilibrium level in any of their experiments and prediction of the expected time course of the approach to equilibrium on the competitive hypothesis, for comparison with the observed rates, would require calculations based on at least the adsorption and desorption rate constants of both antibody and non-specific γ-globulins and also some hypothesis concerning the relation between the proportion of specific receptors occupied and the observed anaphylactic response. Until such model calculations are attempted it does not seem wise to reject the competitive model.

APPENDIX (1)

Derivation of equation giving amount of thiosulphate present in iodine -131 solutions allowing for the radiative oxidation of thiosulphate

It has been found (Burgess and Partington 1960) that the rate of decomposition of thiosulphate at any time is proportional to the activity of iodine -131 (mc/ml) present at that time i.e.

$$-\frac{d(\text{thio})}{dt} = ka_t \qquad -(1)$$

where (thio) = concentration of thiosulphate (mMole/ml)

a_t = activity of ^{131}I at time t (mc/ml)

k = 4.3×10^{-5} mMole mc^{-1} day^{-1} (Burgess and Partington 1960)

and since $ka_t = ka_o \exp(-k't)$ -(2)

where a_o = initial activity of ^{131}I (mc/ml)

k' = decay constant for ^{131}I = 0.0862 days^{-1}

We have

$$-\int_{(\text{thio})_o}^{(\text{thio})_{t=t}} d(\text{thio}) = ka_o \int_{t=o}^{t=t} \exp(-k't)\ dt$$

$$\therefore (\text{thio})_o - (\text{thio})_t = (\text{thio})_{\text{oxidized}}$$

$$= \frac{k}{k'} (a_o - a_t) \qquad -(3)$$

Thus the total amount of thiosulphate destroyed as $t \rightarrow \infty$ i.e. as $a_t \rightarrow o$, from equation (3),

$$\frac{(\text{thio})_{\text{oxid}}}{a_o} = \frac{k}{k'} = \frac{4.3 \times 10^{-5}}{0.0862}$$

$$= 5 \times 10^{-4} \text{ mMole/mc}$$

If the amount of thiosulphate added at t=o is

$$(\text{thio})_o = xa_o \quad \text{mMole/ml}$$

$$\text{i.e.} \quad \frac{(\text{thio})_o}{a_o} = x \quad \text{mMole/mc}$$

then, from (3),

$$(\text{thio})_t = xa_o - \frac{k}{k'} (a_o - a_t)$$

$$= \left(x - \frac{k}{k'}\right) a_o + \frac{k}{k'} a_t \quad \text{mMole/ml}$$

It is stated that $x = 6 \times 10^{-4}$ mMole/mc of thiosulphate are added at $t = o$ so when $t \rightarrow \infty$ i.e. when $a_t \rightarrow o$ there should be $(6-5)a_o \times 10^{-4}$ mMole/ml of thiosulphate remaining in the solution. In other words

$$\frac{(\text{thio})_{t\to\infty}}{a_o} = \left(x - \frac{k}{k'}\right) = 1\text{x}10^{-4}\,\text{mMole/mc.}$$

It can also be seen from (3) that

$$\frac{(\text{thio})_t}{a_t} = \left(x-\frac{k}{k'}\right)\frac{a_o}{a_t} + \frac{k}{k'}$$

$$= \left(x - \frac{k}{k'}\right)e^{k't} + \frac{k}{k'}$$

from which it is clear that if $x = k/k'$ the amount of thiosulphate per mc of radioiodine will not change with time (and will be k/k'), and if, as in fact, excess thiosulphate is added so $x > k/k'$, this ratio will rise to infinity with time. If $x < k/k'$ the amount of thiosulphate per mc of radioiodine will fall to zero in a finite time. (That the thiosulphate can disappear in a finite time was shown by Burgess and Partington 1960)

The time for total disappearance of thiosulphate (t_o say) is found from equation 4 to be

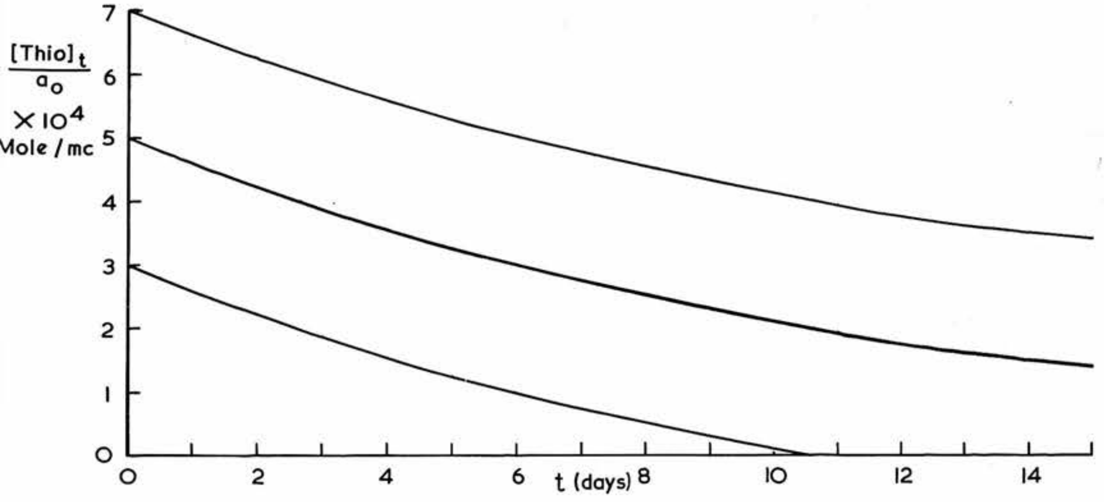
$$t_o = -\frac{1}{k'} \ln \left(1 - \frac{k'x}{k}\right) \text{ days}$$

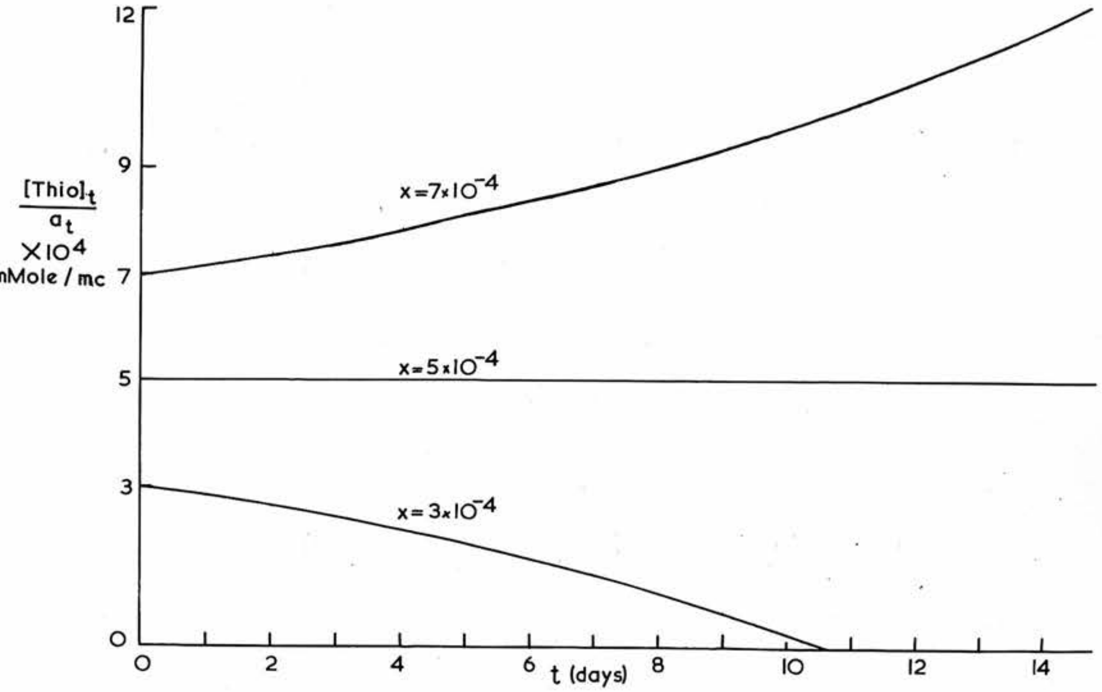
which is finite when $x < k/k'$. $^1/k = 11.6$ days.

For example if $x = 3 \times 10^{-4}$ m Mole/mc, as shown in Fig. 1.2, $t_o = 10.6$ days. If x were 1×10^{-4} mMole/mc then it is predicted that all thiosulphate would disappear in 2.6 days. Values calculated from this equation agree, within experimental error, with the time for disappearance of thiosulphate observed by Burgess and Partington in their solutions A, B and C.

Fig. 1.1 Plot of ratio of thiosulphate concentration present at time t to radioactivity originally present.

Fig. 1.2 Plot of ratio of thiosulphate concentration present at time t, to radioactivity at the same time.


[Thio]$_t$ / a$_o$ × 10^4 Mole / mc
0
1
2
3
4
5
6
7
0
2
4
6
t (days)
8
10
12
14


[Thio]$_t$ / a$_t$ × 10^4 nMole / mc
12
9
7
5
3
0
x = 7 × 10^{-4}
x = 5 × 10^{-4}
x = 3 × 10^{-4}
0
2
4
6
t (days)
8
10
12
14

APPENDIX 2

An analysis of the errors involved in a rapid method of assaying large numbers of histamine solutions using visual linear interpolation between two standards

A schematic bracket assay is shown in Fig. (2.1). The following definitions will be used:

Z_{HS}	High concentration of standard
Z_{LS}	low concentration of standard
Z_T	estimated concentration of unknown
y	response
$D = \frac{Z_{HS}}{Z_{LS}}$	ratio of standard concentrations
$\alpha = \frac{\Delta' y}{\Delta y}$	response to unknown as proportion of distance between standard responses.

Interpolation The formulas for interpolation are

(a) assuming response linearly related to concentration (referred to as linear interpolation)

$$Z_T = Z_{LS} + \alpha(Z_{HS} - Z_{LS})$$

(b) assuming, more reasonably, that response is linearly related to the logarithm of the concentration (referred to as logarithmic interpolation)

$$Z_T = Z_{LS} \ D^{\alpha}$$

Difference between methods of interpolation

Obviously linear interpolation will give a larger estimate of the unknown concentration than logarithmic interpolation. Taking the latter method as the "best" estimate of the unknown, it can be shown that the percentage by which linear interpolation overestimates it is given by:

$$e=\left[\frac{Z_T(\text{linear})-Z_T(\text{log})}{Z_T(\text{log})}\right]100 \ = \left[\frac{1 + \alpha(D-1)}{D^{\alpha}}-1\right]100 \qquad (2.1)$$

This is referred to as "interpolation error".

Fig. 2.2 shows the interpolation error plotted against α for various values of D, and Fig. 2.3 shows a similar plot for D=1.5 over a wider range of α. This shows that the error involved in extrapolation (i.e. α outside the range 0 to 1) rapidly becomes very large, although of course outside this range it cannot be expected that either the linear or the logarithmic dose-response curves will be straight for long.

It can be seen that e = o when $\alpha = o$ and when $\alpha = 1$ as expected, because when the unknown response is the same as either of the standards there is obviously no interpolation error.

The point at which the interpolation error is a maximum can be found by equating the first derivative of e to zero and turns out to be

$$\alpha_{\text{max}} = \frac{1}{\ln D} - \frac{1}{D-1} \qquad (2.2)$$

Application of L'Hospital's rule shows that this tends to 0.5 as $D \rightarrow 1$ and to 0 as $D \rightarrow \infty$ i.e. the point of maximum interpolation error is always at $\alpha \leqslant 0.5$, and interpolation errors are always rather smaller when the response to the unknown is near the upper standard than when it is near the lower standard.

The maximum error is found by substituting (2.2) into (2.1). It can also be shown, by integration of (2.1), that the mean expected percentage error over the range $0 \leqslant \alpha \leqslant 1$

(if the unknowns fall equally often at each point between the standards) is given by

$$\bar{e} = 100\left[\frac{(D-1)^2}{D(\ln D)^2} - 1\right] \qquad (2.3)$$

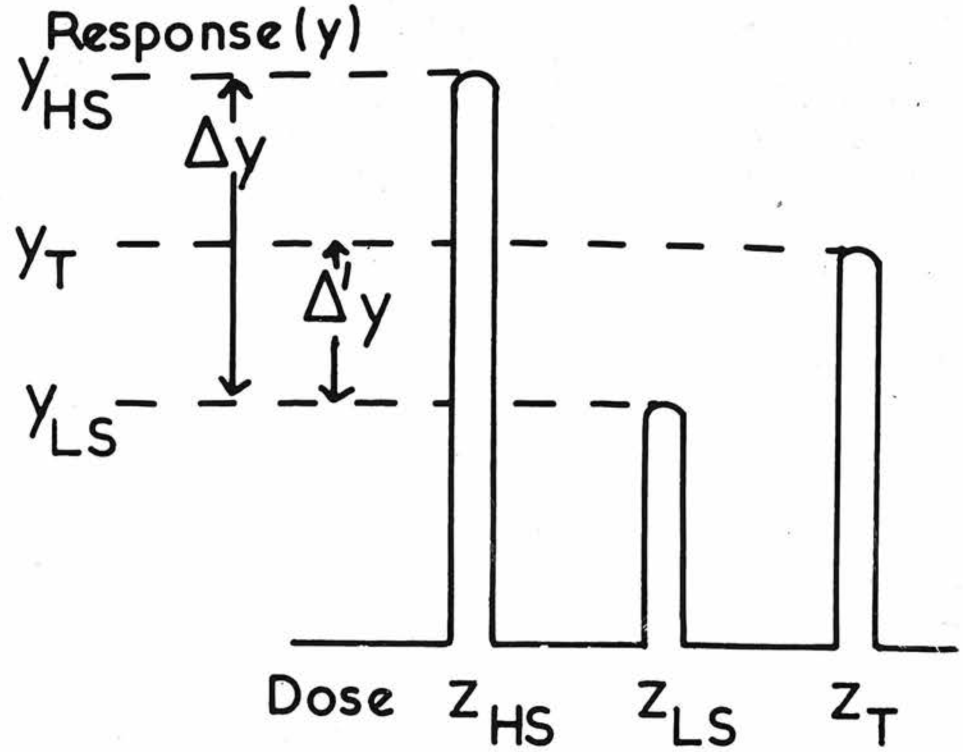
and it can be shown that $\bar{e} \rightarrow 0$ as $D \rightarrow 1$, and $\bar{e} \rightarrow \infty$ as $D \rightarrow \infty$. Fig. (2.4) shows the mean and maximum interpolation errors plotted against D.

If D is not larger than 1.5, as was usually the case, and if all values are interpolated not extrapolated, then the error of interpolation is negligible. It can be seen, however, that e_{max} and $\bar{e}$ rise rapidly with increasing D and if D is larger than 1.5 they become quite important.

Table (2.1) shows the analysis of an actual set of histamine assays by various methods. The average percentage by which linear interpolation exceeds logarithmic interpolation is seen to be 1.16% compared with 1.375% calculated from equation (2.3). The fact that this figure was consistently slightly less than theoretical probably means that there was a tendency for the assays to be conducted in such a way that the unknown responses were near to the standard responses. The average value of α in this set of assays (24/10/63) was in fact 0.58, rather than 0.50.

Fig. 2.1 Schematic bracket assay.

Fig. 2.2 Interpolation error (e) as a function of α for various values of D.


Response (y)
y_HS
Δy
y_T
Δ'y
y_LS
Dose
Z_HS
Z_LS
Z_T

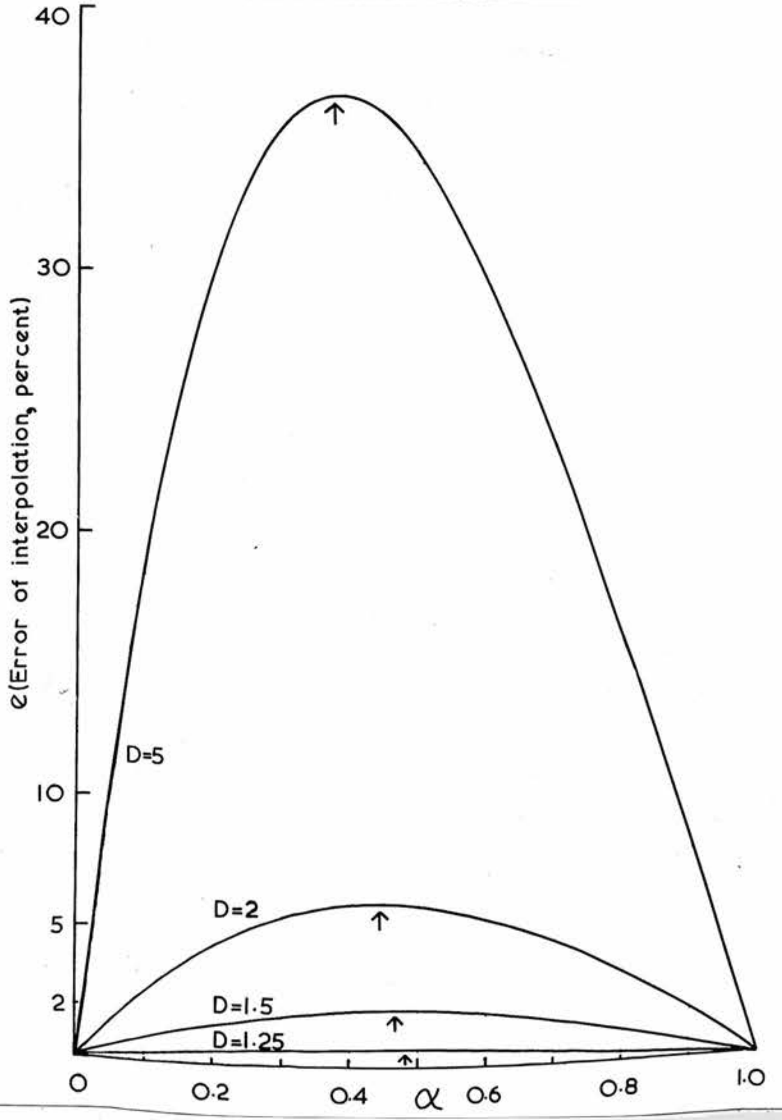
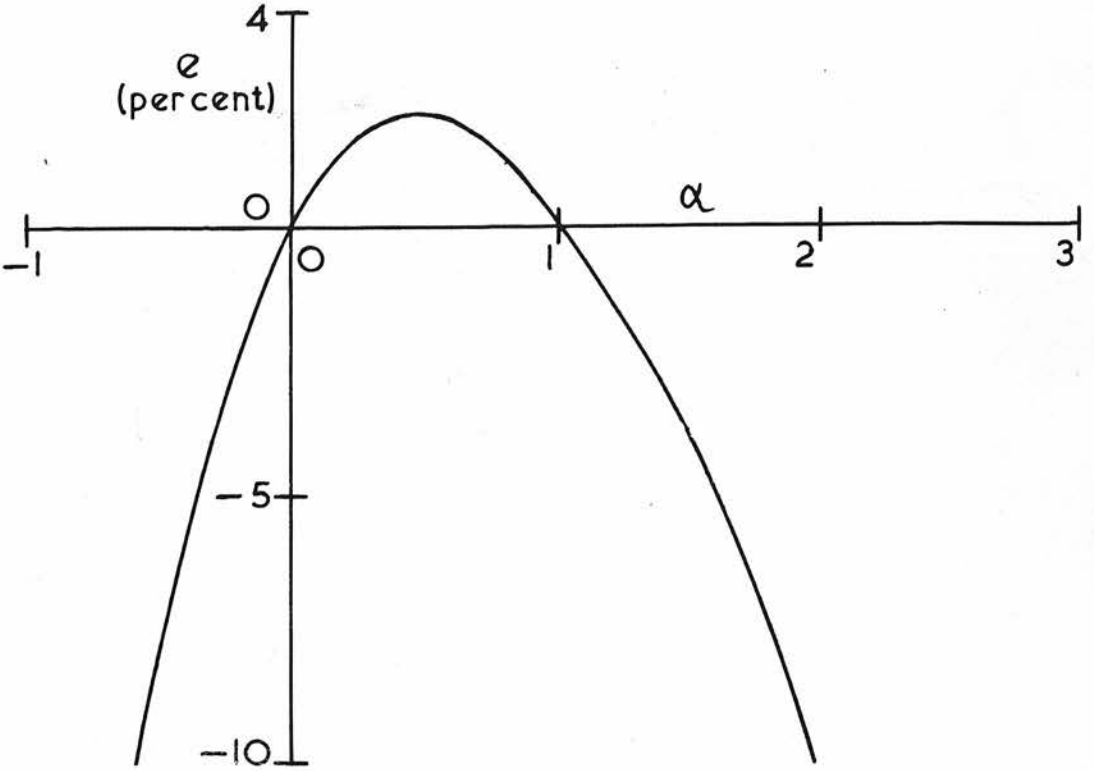
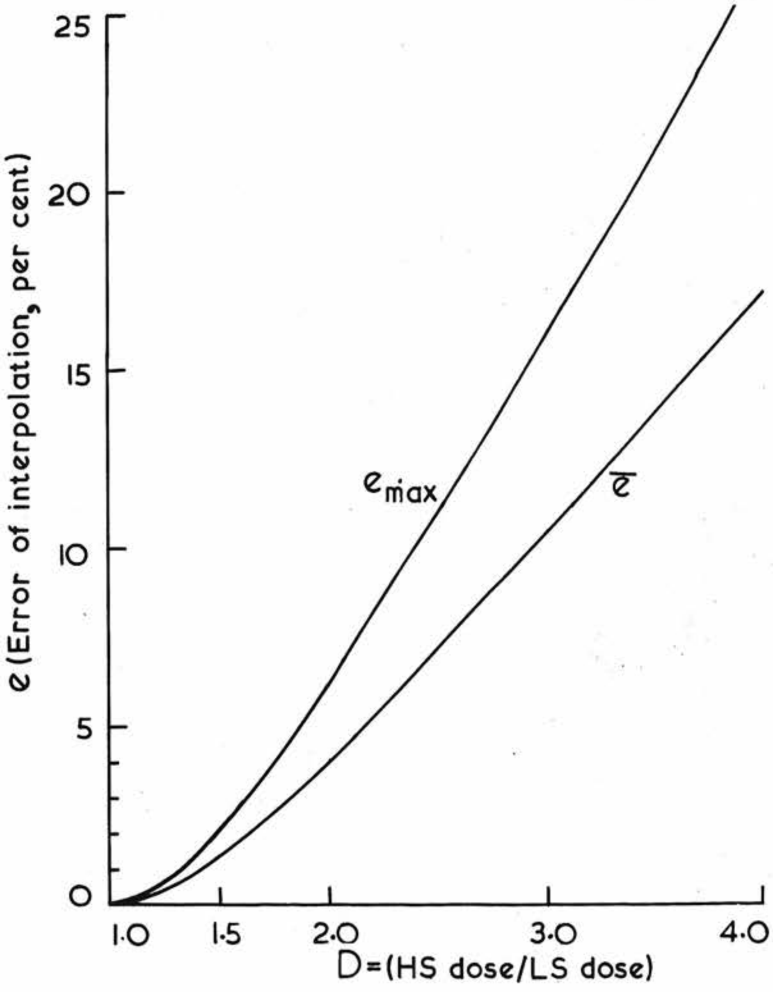

e (Error of interpolation, percent)
40
30
20
10
5
2
0
0.2
0.4
α
0.6
0.8
1.0
D=5
D=2
D=1.5
D=1.25

Fig. 2.3 Interpolation error (e) for D=1.5 over a larger range of α than shown in Fig. 2.2.

Fig. 2.4 Maximum (e_{max}) and mean ($\bar{e}$) interpolation error as function of D assuming all unknowns are in the range $0 < \alpha < 1$.


4
e
(percent)
0
α
−1
0
1
2
3
−5
−10


25
20
15
10
5
0
e (Error of interpolation, per cent)
e max
ē
1.0
1.5
2.0
3.0
4.0
D=(HS dose/LS dose)

Error of visual interpolation

It can be seen from table (2.1) (Column 6a) that there was a small but remarkably consistent bias in visual estimation, the visual linear interpolation being on the average 2.02% lower than the estimate obtained by linear interpolation with measurement of responses. This more than cancels out the error of interpolation so that the average difference between linear (visual) and logarithmic (measured) estimates was only -0.89% (Column 5a). When the results are expressed as percent histamine release even these small errors mostly cancel out (Columns 4b, 5b and 6b), so the final average difference between the rapid method of interpolation used and the "best" method is only +0.37% (Column 5b), varying between -0.323% and +1.81% for different treatments tested in the experiment.

Error of replicate assays on the same sample

In assessing the difference between two treatments the variability of the histamine assay figures for the five or so replicate samples each given the same treatment is usually considered. However from the point of view of the histamine assay method the relevant variable is the value of potency obtained in replicate assays of the same sample. This is subsumed in the variability of replicate samples of the same treatment. The potencies of the solutions to be assayed vary over a wide range so it is necessary to consider the variability of the potencies observed on the dilutions of the original solutions which give a response lying between the two standards. These cover only a narrow range so can reasonably be assumed to have constant variance ("within samples" variance). As dilutions were made separately for each bracket assay this subsumes the random dilution errors. Different samples will of course give dilutions lying at different points between the standards, and the "between samples" variance is, as expected statistically significantly larger than the within samples variance.

Similar analysis of two other experiments (11/3/64 and 25/5/64) gave the following results: Coefficient of variation of mean of two potency estimates, 3.7% (11/3/64) and 3.4% (25/5/64) Mean value of α, 0.68 (11/3/64) and 0.56 (25/5/64).

It is interesting that in all three assays analysed the average value of α differs from 0.5 which suggests that the interpolation error will be usually even less than $\overline{e}$ shown in Fig. 2.4 as was found in Table 2.1.

It may be noted that the above simple analysis, although giving a legitimate measure of the scatter of replicate analyses on the same sample, is not consistent with the usual assumption that the response is a normally distributed variable, since if this were true the potency estimates would not be normally distributed. The mean potency estimate for each sample and its fiducial limits could in fact be calculated by the full (2 + 1) dose bioassay procedure but in this case no simple pooled estimate of the variability of the method (such as the coefficients of variation quoted above) would be available.

Column number			(1)		(2)		(3)		(4)		(5)		(6)	
Treatment		No. of replicate samples	Mean histamine release						Deviations (per cent)					
			Log interpolation (measured)		Linear interpolation (measured)		Linear interpolation (visual)		$\frac{(2)-(1)}{(1)} \times 100$		$\frac{(3)-(1)}{(1)} \times 100$		$\frac{(2)-(3)}{(2)} \times 100$	
			(a) ng per sample	(b) percent total H	(a) ng per sample	(b) percent total H	(a) ng per sample	(b) percent total H	(a)	(b)	(a)	(b)	(a)	(b)
γ_2-glob.	4.0µg/ml	5	10.72	0.9139	10.86	0.9141	10.56	0.9111	+1.305	+0.0219	-1.49	-0.306	+2.76	+0.328
	16.0µg/ml	5	18.16	1.548	18.34	1.544	18.00	1.553	+0.991	-0.258	-0.881	+0.323	+1.854	-0.583
	64.0µg/ml	5	97.98	8.353	99.26	8.355	96.60	8.335	+1.306	+0.0275	-1.408	-0.217	+2.680	+0.244
	256.0µg/ml	2	313.0	26.68	316.5	26.64	309.0	26.66	+1.118	-0.161	-1.278	-0.086	+2.370	-0.075
γ_1-glob.	1.0µg/ml	5	393.2	33.52	400.0	33.67	388.2	33.49	+1.729	+0.447	-1.272	-0.089	+2.950	+0.535
	4.0µg/ml	4	471.2	40.17	475.5	40.02	474.0	40.90	+0.902	-0.361	+0.583	+1.810	+0.315	-2.178
	16.0µg/ml	5	470.8	40.14	472.5	39.77	469.6	40.52	+0.3611	-0.904	-0.255	+0.952	+0.614	-1.873
	64.0µg/ml	3	427.7	36.65	433.3	36.47	427.7	36.90	+1.309	-0.472	0.000	+0.698	+1.292	-1.176
Remaining tissue histamine		9	987.3		1000.0		974.5		+1.286		-1.291		+2.545	
Total no. of samples		43					Weighted mean deviations		+1.16	-0.191	-0.892	+0.367	+2.02	-0.563

Table (2.1) Analysis of histamine assay results by various methods (see text). The standard concentrations were 2 and 3 ng/ml so D = 1.5. Between 2 and 4 replicate brackets were performed on each solution.

An analysis of variance for the experiment shown in Table 2.1 is given in Table 2.2.

Table 2.2

Analysis of variance of replicate analyses on the samples assayed in Experiment 24/10/63.

Source	d.f.	SS	MS	VR	P
Between samples	42	9.272	0.2208	4.6	<0.001
Within samples	46	2.186	0.0475		
Total	88	11.458			

Thus the variance of a single potency estimate (or of the difference between two means of two estimates) is 0.0475, and the standard deviation 0.218 ng/ml. Since at least two estimates were made on each sample a more relevant figure is the standard deviation of the mean of two estimates, i.e. 0.154 ng/ml. The standards in this assay were 2.0 and 3.0 ng/ml and the average potency of all diluted samples was 2.58 ng/ml (so, as interpolation is linear, the average value of α is 0.58 as mentioned before). The average value of the coefficient of variation of the mean of two potency estimates is therefore 100 x 0.154/2.58 = 6.0% and if the error of dilution is neglected this figure will also apply to the original undiluted solution.

APPENDIX 3

Calibration of tuberculin syringes and comparison of consistency and heterogeneity of glass and disposable tuberculin syringes

Syringes were calibrated by measuring to 0.1 mg the weight of freshly boiled and cooled distilled water they delivered. The nominal volume delivered was 0.05 ml and the syringes were used as in actual PCA experiments. The syringes were filled to the 0.05 ml mark (using the lower edge of the rubber washer as reference point in disposable syringes), and ejecting the contents by pushing the plunger as far as it would go. The weights were converted to volumes using the density of water at the temperature of the balance room (with which the temperature of the water was allowed to equilibrate).

An early experiment in which three measurements were made on each of six all glass 1 ml tuberculin syringes showed that there were significant differences between the volumes delivered by individual syringes ($P < 0.01$) and the mean volume delivered was 56.35 µl with a within syringe standard deviation of 1.3 µl and a between syringe component (measuring heterogeneity of syringes) of 1.9 µl. The largest deviations of mean volumes delivered by individual syringes from the mean for all syringes were -4.2% and +5.2%.

The results of a second experiment are given in more detail below. Nine all glass tuberculin syringes which had been specially selected for the accuracy of fit of their plungers, and nine disposable plastic tuberculin syringes, (Johnson's Ethical Plastics Ltd., Slough, Bucks) with evenly fitting rubber washers, were used in the experiment. Four calibrations were done on each syringe and the sequence in which the syringes were tested was randomized using a table of random numbers.

The mean volume delivered by all syringes was 53.87 µl, again slightly more than the nominal 50 µl. If the scatter of volumes delivered in a series of trials is the same for all sorts of syringe (as is shown to be approximately so below), the results can be pooled and their analysis of variance is shown in table (3.1). It can be seen that syringes definitely differ in the volumes they deliver but there is no evidence at all (whether the analysis is considered as model I or model II) of any difference between the amounts delivered by glass and plastic syringes (53.98 µl and 53.76 µl respectively).

Analyses of the results for the two sorts of syringe separately are given in tables (3.2) and (3.3). If these are treated as model II analyses, the appropriate model is

$$x_{ij} = \xi + y_i + Z_{ij}$$

where x_{ij} = observed volume of j th delivery from i th syringe

ξ = true mean of all readings $\text{Var}[\xi] = 0$

y_i = effect due to i th syringe. $\text{Var}(y_i) = w^2$ where w^2, the "between syringes component of variance" is the variance of the true means for <u>individual</u> syringes about the true mean for <u>all</u> syringes, thus $\text{Var}(\xi + y_i) = w^2$. This is referred to as the <u>heterogeneity</u> of the syringe population.

Z_{ij} = effect due to j th delivery by the i th syringe, or "deliveries within syringes effect". Z_{ij} is assumed to be normally distributed about zero with variance $\mathrm{Var}(Z_{ij}) = \sigma^2$, which is the variance of individual observations on a single syringe about the true mean for that syringe i.e. $\mathrm{Var}\,(\xi + Z_{ij}) = \sigma^2$. This is referred to as the consistency with which a syringe delivers its liquid.

The figures in the analysis of variance table are calculated from weights rather than volumes. The estimated components of variance calculated from the tables, and converted to volume units, are:

<u>glass</u> $\hat{\sigma} = 2.113$ µl $\hat{\omega} = 3.774$ µl

<u>disposable</u> $\hat{\sigma} = 2.369$ µl $\hat{\omega} = 1.987$ µl

It is seen that the two types of syringe are very similar in consistency but the glass ones are, if anything more heterogeneous, though this difference does not reach statistical significance. When the ratio of the two estimates of w^2 is tested as a variance ratio with the χ^2 in the numerator and denominator assigned degrees of freedom according to the approximate distribution of variance components given by Satterthwaite (1946), the result is $0.1 < P < 0.2$.

95% confidence limits for the volume delivered by a given disposable syringe are given by

$\pm t(P=0.95,\ 27\text{d.f.}) \times 2.369 = \pm 4.86$ µl

i.e. about ± 9% of mean delivery for the particular syringe (which will be about 54 µl).

And it is similarly estimated that 95% of syringes in the population have true mean deliveries within the limits:

<u>glass</u> $\pm t(P=0.95,\ 7\text{d.f.}) \times 3.774 = 8.92$ µl

i.e. ± 16.5% of mean delivery

<u>disposable</u> $\pm t\ (P=0.95,\ 4\text{d.f.}) \times 1.987 = 5.52$ µl

i.e. ± 10.3% of mean delivery

where the number of degrees of freedom associated with the between syringe component of variance is calculated according to Satterthwaite (1946).

Table (3.1). Comparison of glass and plastic disposable syringes.

Source	df	SS	MS	VR	P
Between glass and plastic (materials)	1	0.889	0.889	< 1	Not sig.
Between syringes within materials	16	659.8	41.24	8.22	< 0.001
Between syringes	17	660.7	38.87		
Between deliveries (within syringes)	54	270.9	5.016		
Total	71				

Table (3.2). Glass syringes

Source	df	SS	MS	VR	P	E [MS]
Between syringes	8	489.2	61.15	13.76	< 0.001	$\sigma^2 + 4\omega^2$
Between deliveries (within syringes)	27	120.0	4.445			σ^2
Total	35	609.2				

Table (3.3) Plastic disposable syringes

Source	df	SS	MS	VR	P	E [MS]
Between syringes	8	170.6	21.33	3.817	~ 0.01	$\sigma^2 + 4\omega^2$
Between deliveries (within syringes)	27	150.9	5.587			σ^2
Total	35	321.5				

APPENDIX 4

Theoretical considerations concerning the diffusion of inulin in chopped lung tissue

(1) Diffusion coefficient of inulin. An estimate was made using the method of Schantz and Lauffer (1962). The end of the cylindrical agar gel was immersed in a large volume (about 3 litres) of well stirred 1% inulin (British Drug Houses Ltd. Batch No. 730910) in 0.154M saline. After 19 hours at 36.5°C (Max. error ±0.5°C) 1.0 mm slices of the gel were taken and analysed for inulin. The resulting plot of $\text{erf}^{-1}\ (1-c'/c_0)$ against distance, where c' is the concentration of inulin per unit volume of gel, c_0 is the concentration in the external solution, and erf^{-1} is the inverse error function, should be linear but in fact deviated from linearity at a distance from the gel surface. When the concentration of inulin fell below about 10% of its value at the gel surface the concentrations found were rather higher than predicted. There are several possible reasons for this but as the diffusion coefficient estimated, after the appropriate corrections,

from the first 8 points (which were linear), viz. $D_{3s,sal} = 2.29 \times 10^{-6}\ cm^2\ sec^{-1}$, was within the range of other values reported in the literature this value was adopted. Values of the inverse error function were obtained from National Bureau of Standard Tables (1954).

(2) Correction of diffusion coefficients for variations of temperature and solvent. These were done in the usual way from the formula

$$D(s_2, T_2) = D(s_1, T_1) . \quad \frac{T_2 . \eta(s_1, T_1)}{T_1 . \eta(s_2, T_2)}$$

(see, for example, Longsworth 1955), where D is the diffusion coefficient, s_1 and s_2 are solvents, T_1 and T_2 are absolute temperatures and η is the coefficient of viscosity. Values of the viscosity were obtained by interpolation from values for the viscosity of water, and for the relative viscosity of water and sodium chloride solutions, given in International Critical Tables.

(3) The solution of the diffusion equation. The solution for cylinders of constant radius shown in the upper half of Fig. 12 is well known. The derivation is given, for example by Bowman (1958). This solution is also given by Crank (1956) (p66) who uses a slightly different notation, It was found quite sufficiently accurate to read values of $f(t) = m(t)/m(\infty)$ from curve 0 of Fig. 5.7 in Crank (1956). It may be noted in this connection that Crank's equations 5.21 and 5.22 (p 66) both contain misprints.

Values of Bessel functions and of their roots were taken from the tables of Jahnke and Emde (1945).

For a population of cylinders with discontinuously distributed radii the equation in Fig. 12 can be derived as follows:

Assume a population containing proportion p_i of cylinders of radius a_i and length h_i so $\sum_i p_i = 1.0$. At time t the proportion of the final uptake achieved by the population will be

$$\frac{M(t)}{M(\infty)} = \frac{\sum_i p_i \; m_i(t)}{\sum_i p_i \; m_i(\infty)}$$

where $m_i(t)$ is the mass of inulin taken up at time t by a cylinder of radius a_i.

This will not be the same thing as the weighted mean of the proportional uptakes of the individual cylindrical fragments unless all the $m_i(\infty)$ are the same, i.e. unless the cylinders all have the same volume.

If the concentration of inulin (mass per unit volume of tissue) at equilibrium is $c(\infty)$ then

$$m_i(\infty) = \pi a_i^2 \, h_i \; c(\infty) \qquad \text{(mass)}$$

and if we define the proportional uptake in cylinders of radius a_i as $f_i(t) = m_i(t)/m_i(\infty)$, given by the expression in Fig. 12, then

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

thus

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

and therefore:

(a) if all the cylinders have the same length, $h_i = h$, then

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

(b) if the length of cylinders is proportional to their radius, $h_i = ka_i$ where k is a proportionality constant, then

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

APPENDIX (5)

Concerning the dimensions and magnitudes of adsorption equilibrium and rate constants and the effect of the continuous slow uptake of γ-globulin on validity of the assumption that the concentration of γ-globulin in the extracellular space is the same as that in the external solution.

(1) The equilibrium rate of adsorption

The model assumed is of diffusion in an infinite cylinder with constant external concentration. The use of the solution of the diffusion equation for a cylinder implies the assumption that the cylindrical tissue particle can be treated as a homogeneous cylinder in which diffusion takes place as though the solute had the diffusion coefficient D'. The size of internal obstructions in the tissue is therefore assumed to be small compared with the size of the cylinder so that once the obstruction effect has been allowed for by using the effective diffusion coefficient D' instead of D, the cylinder can be treated as internally homogeneous.

The linear adsorption isotherm, which is obeyed by γ-globulin over the range of concentrations used, may be written

$$s = Kc \tag{5.1}$$

where s is the adsorbed concentration (ng/µl), c is the free concentration (ng/µl) and K is the equilibrium adsorption constant. If adsorption equilibrium is reached rapidly compared with the rate of diffusion so that there is local equilibrium between free and adsorbed material at every point in the cylinder, then the equation for diffusion with concurrent adsorption will be:

$$\frac{\partial c}{\partial t} = D' \operatorname{div} \operatorname{grad} c - \frac{\partial s}{\partial t}$$

$$= \frac{D'}{K+1} \operatorname{div} \operatorname{grad} c \tag{5.2}$$

The effect of concurrent adsorption is seen to be to further slow down adsorption in such a way that the effective diffusion coefficient is reduced by a factor of (K + 1).

It is clear from (5.2) that K must be dimensionless. The relation between K and the customary adsorption constant K′ which is the

the adsorption in ng/100 mg tissue for a bulk concentration of 1 μg/ml, and therefore has the dimensions μl/100 mg, is derived as follows.

Let

m_a = mass adsorbed per unit length of cylinder ($\mathrm{ng\ cm^{-1}}$)

m_f = mass free in solution per unit length of cylinder ($\mathrm{ng\ cm^{-1}}$)

V = volume of cylinder per unit length $= \pi a^2$ ($\mathrm{\mu l\ cm^{-1}}$)

V_e = volume of extracellular space inside cylinder per unit length ($\mathrm{\mu l\ cm^{-1}}$)

M = mass of cylinder per unit length ($\mathrm{100\ mg.\ cm^{-1}}$)

E = V_e/M, the extracellular space in μl/100 mg

then

$$s = \frac{m_a}{V} \quad (\mathrm{ng\ \mu l^{-1}})$$

and $$c = \frac{m_f}{V} \quad (\mathrm{ng\ \mu l^{-1}})$$

so $$K = \frac{s}{c} = \frac{m_a}{m_f}$$

However, the customary units for measuring adsorption and free concentration are

$$s' = \frac{m_a}{M} \quad (\text{ng}/100 \text{ mg})$$

and $$c' = \frac{m_f}{V_e} \quad (\text{ng}/\mu\text{l})$$

and $$s' = K' c'$$

where K' has the dimensions µl/100 mg.

Thus $K' = \frac{s'}{c'} = \frac{m_a}{m_f} \frac{V_e}{M} = KE \quad (\mu\text{l}/100 \text{ mg})$

$$\text{or} \quad K = \frac{K'}{E} \qquad (5.3)$$

Since E is usually about 50 µl/100 mg and K' is not usually more than about 50 µl/100 mg (i.e. $s' = 50$ ng/100 mg for $c' = 1$ µg/ml) then $K \simeq 1$ (the exact value of K' is uncertain as, even after four hours, equilibrium is usually not completely attained, but the value given above should certainly not be too low).

(2) The steady uptake of γ-globulin

Rosenfalck (1961) gives the steady state ($t \to \infty$) solution of the equation for diffusion with a concurrent first order reaction with rate constant k,

$$\frac{\partial c}{\partial t} = D' \operatorname{div} \operatorname{grad} c - kc, \qquad (5.4)$$

for an infinite cylinder of radius a, surrounded by a constant concentration c_o, which was quoted in the results section, viz.

$$\frac{c(r,\infty)}{c_o} = \frac{I_o\,(r\sqrt{k/D'})}{I_o\,(a\sqrt{k/D'})} \qquad (5.5)$$

I_o is the modified Bessel function of the first kind of zero order and r is the radial distance from the centre of the cylinder. The <u>amount</u> of free, unreacted solute present in the cylinder per unit length in the steady state, M (∞), will therefore be

$$m_f(\infty) \equiv M(\infty) = \int_v c(r,\infty).dV = 2\pi\int_o^a r.c(r,\infty).\,dr$$

(5.6)

Substituting from (5.5) gives

$$M(\infty) = \frac{2\pi\, c_o}{I_o(a\sqrt{k/D'})}\int_{r=o}^{r=a} r.\,I_o\,(r\sqrt{k/D'})\,.\,dr$$

$$= \frac{2\pi\, a^2 c_o}{(a\sqrt{k/D'})}\;\frac{I_1\,(a\sqrt{k/D'})}{I_o(a\sqrt{k/D'})}$$

Where I_1 is the modified Bessel function of the first kind of order one (modified Bessel functions of the first kind of order n are defined as

$I_n(x) = i^{-n}\,J_n\,(i\,x)$, where $i = \sqrt{-1}$).

Now the amount of free (unreacted) solute which would be present per unit length of cylinder if true equilibrium were reached (k=o) is

$$m_{fo} \equiv M_o = \pi a^2 c_o \qquad (5.8)$$

Substituting this in (5.7) gives

$$\frac{M(\infty)}{M_o} = \frac{2 \ I_1 \ (a\sqrt{k/D'})}{a\sqrt{k/D'} . \ I_o(a\sqrt{k/D'})} \qquad (5.9)$$

which is the solution quoted in the results section.

The same problem is considered by Danckwerts (1951) who gives the following expression for the rate at which material is taken up across the surface of unit length of cylinder in the steady state.

$$f(\infty) = c_o \, 2\pi a \sqrt{kD'} . \ \frac{I_1 \ (a\sqrt{k/D'})}{I_o \ (a\sqrt{k/D'})} \qquad (5.10)$$

This has the dimensions ng sec^{-1} cm^{-1}. If it is divided by πa^2 the rate of uptake through the surface of the cylinder in the steady state per unit volume of cylinder, with dimensions ng μl^{-1} sec^{-1}, is obtained

$$F \ (\infty) = \frac{2 \ c_o \sqrt{kD'} . \ }{a} \frac{I_1 (a\sqrt{k/D'})}{I_o(a\sqrt{k/D'})} \qquad (5.11)$$

Equation (5.9) can also be derived from (5.11) since, using the notation of the first section of this appendix, $M(t)$ has been defined as $m_f(t)$, and, by definition of $\overline{c}$,

$$\overline{c}(t) = \frac{m_f(t)}{V} \equiv \frac{M(t)}{V} \quad (\text{ng } \mu l^{-1}) \tag{5.12}$$

and by definition of the rate of a first order reaction, if k is independent of r,

$$F(t) = k\,\overline{c}\,(t) \tag{5.13}$$

Now $V = \pi a^2$ (μl cm^{-1}) so, from (5.12) and (5.13),

$$F(t) = \frac{k\,M(t)}{\pi a^2} \tag{5.14}$$

and, since $M_o = \pi a^2 c_o$ (ng cm^{-1})

$$\frac{M(t)}{M_o} = \frac{F(t)}{k\,c_o} \tag{5.15}$$

which, substituted in (5.11) (with $t \rightarrow \infty$), agrees with (5.9).

In order to obtain numerical values for k (which, as equations 5.4 onwards show, must have the dimensions sec^{-1}), the observed rate must be expressed in the correct units. By analogy with the previous section define

$$F = k\,c$$

Now, using the definitions of the previous section

$$F = \frac{\partial s}{\partial t} = \frac{1}{V}\frac{\partial m_a}{\partial t}$$

and since $c = m_f/V$

$$k = \frac{F}{c} = \frac{1}{m_f}\frac{\partial m_a}{\partial t} \quad (\text{sec}^{-1}) \tag{5.16}$$

However, using the customary definitions we would write,

$$F' = k'c' \tag{5.17}$$

where $c' = m_f/V_e$ $(\text{ng}\ \mu\text{l}^{-1})$. Thus if F' is expressed in $\text{ng}(100\text{mg})^{-1}\ \text{sec}^{-1}$, k' will have the dimensions $\mu\text{l}\ (100\ \text{mg})^{-1}\ \text{sec}^{-1}$.

$$F' = \frac{\partial s'}{\partial t} = \frac{1}{M}\frac{\partial m_a}{\partial t} \quad \text{ng}\ (100\ \text{mg})^{-1}\ \text{sec}^{-1}$$

$$\text{so} \quad k' = \frac{F'}{c'} = \frac{1}{M}\cdot\frac{\partial m_a}{\partial t}\cdot\frac{V_e}{m_f}$$

$$= \frac{E}{m_f}\frac{\partial m_a}{\partial t}$$

$$= Ek \quad (\mu\text{l}\ (100\text{mg})^{-1}\ \text{sec}^{-1})$$

$$\text{or} \quad k = \frac{k'}{E} \tag{5.18}$$

which is analagous with (5.3).

The present case is unlike that of equilibrium adsorption in that k' cannot be obtained directly from the observations since c is not a constant but varies throughout the tissue. The direct observation is of $F'(\infty)$.

From (5.17) and (5.18) we have

$$k' = \frac{F'}{c'} = Ek = E\frac{F}{c}$$

hence

$$F' = F\frac{Ec'}{c}$$

and $$F = \frac{F'\,V_e}{E\;\;V}$$

It can be seen from this that the relation between F' and F involves the density of the cylinder (as does the analagous relation between s' and s in the previous section). This is not required, however since we also have

$$c_0 = c'_0\ \frac{V_e}{V}$$

and substitution of these into (5.11) gives

$$\frac{F'(\infty)}{E} = \frac{2\ c'_0\sqrt{k\ D'}}{a}\ \frac{I_1\,(a\sqrt{k/D'})}{I_0\ (a\sqrt{k/D'})} \tag{5.19}$$

with the same dimensions (ng μl^{-1} sec^{-1}) as $F(\infty)$ but differing numerically by a factor of V_e/V. From this equation the curve shown in fig. 25 was calculated by substituting various values of k (sec^{-1}) in the RHS with $c'_0 = 1.0$ μg/ml (i.e. 1.0 ng/μl). The values so obtained are converted to $F'(\infty)$ in $ng(100mg)^{-1}$ $(hour)^{-1}$ by multiplying by 3600E.

For the example quoted in the results section, in which $F'(\infty) = 16.4$ ng(100mg)$^{-1}$ (hr)$^{-1}$ it was found that $k = 9.77 \times 10^{-5}$ sec^{-1} using the graph plotted from (5.19). This corresponds to $F'(\infty) = 4.55 \times 10^{-3}$ ng(100 mg)$^{-1}$ sec^{-1}. Now if the free concentration were $c'_0 = 1.0$ng/µl throughout the tissue, as it would be if true equilibrium were reached, then

$$k' = \frac{F'(\infty)}{c'_0} = 4.55 \times 10^{-3} \ \mu l(100mg)^{-1} \sec^{-1}$$

and therefore, from (5.18), when E = 48µl per 100 mg,

$$k = \frac{k'}{E} = 9.5 \times 10^{-5} \ \sec^{-1}$$

This value is slightly lower than the proper value of k calculated from (5.19), i.e. 9.77×10^{-5} sec^{-1}. This, of course, is because the average free concentration in the extracellular space is not c'_0, but slightly (about 1.8%) less, because of the concentration gradient maintained by the slow uptake.

Values of modified Bessel functions used in these calculations were taken from the tables of Jahnke and Emde (1945).

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