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A Theoretical and Experimental Analysis of Double Diffusion Precipitin Reactions in Gels, and its Application to Characterization of Antigens

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Summary. The distribution of antigen and antibody in radial double diffusion systems was studied by means of materials labelled with ^{131}I or ^{14}C. These studies showed that, once a precipitate begins to form, the assumption that antigen and antibody obey the laws of free diffusion is invalid. They also showed that, in the systems used, no antigen and very little antibody diffused past the zone of visible precipitation.

It was found that accurate estimates of the diffusion constants of antigens could be obtained by allowing antigen and antibody to diffuse from troughs set at right angles and by measuring the angle of the precipitin line. Examples of the use of this method, and a theoretical treatment are given.

An alternative method for estimating the size of antigens is to use the 'molecular sieve' properties of gelatin gels, which are sharply graded with the concentration of the gel.

INTRODUCTION

During the past 10 years analysis of the precipitation lines formed by diffusion of antigens and antibodies in agar has been widely used for qualitative studies of antigens. Several theoretical treatments have been presented, designed to enable quantitative information to be obtained. In the latest, and most satisfactory, theoretical analysis of the Oudin linear single diffusion system, Augustin, Hayward and Spiers (1958) and Spiers and Augustin (1958) give a formula for calculating the approximate diffusion coefficients of antigens from accurate measurements of the positions of the precipitation lines at various time intervals. Great accuracy is not claimed for the method, however, and it is difficult to apply when several antigens are present. The linear double diffusion system developed by Oakley and Fulthorpe (1953) has been analysed theoretically by Preer (1956), Polson (1958) and Engelberg (1959). Radial double diffusion in flat plates has been analysed by Ouchterlony (1949, 1958) and by Korngold and van Leeuwen (1957). Most of these analyses are based on the assumption that the effect on diffusion of the antigen-antibody interaction can be neglected, and that precipitation occurs where the reactants meet in equivalent concentrations. However, Engelberg (1959) developed equations which take account of antigen-antibody interaction in the particular case of a stationary boundary.

In the first part of this paper we present a theoretical and experimental analysis of radial double diffusion precipitation systems, in which radioactively labelled antigen and antibody were used to determine the concentrations in the gel at different distances from the wells after various diffusion times. In the second part, this information has been used

to develop techniques for determining the size and chemical composition of antigens. An outline of the methods for estimating size of antigens has already been reported (Allison and Humphrey, 1959).

MATERIALS AND METHODS

LABELLED ANTIGENS

(*a*) ^{131}I-labelled human serum albumin (HSA) was prepared by iodination of crystallized HSA (Behringwerke, Marburg) with 1 atom iodine/mol. by the iodine monochloride technique of McFarlane (1958). At the time of use its specific radioactivity was 95 μC/mg. More than 99·8 per cent of the radioactivity was precipitated at equivalence by the rabbit anti-HSA serum which was used. The radioactivity soluble at antibody equivalence or excess was equivalent to 0·26 μg. HSA/ml., and represented the solubility of the antigen-antibody complex. By electrophoresis on paper at pH 8·2 in the presence of whole human serum it was shown that the ^{131}I activity was confined to the albumin.

(*b*) ^{14}C-labelled ovalbumin was prepared by administering 3 mC. ^{14}C *Chlorella* protein (Radiochemical Centre, Amersham) orally to a laying hen. The ovalbumin was prepared from the egg laid 26 hours after administration of ^{14}C, and recrystallized three times by the method of Kekwick and Cannan (1936). Its specific radioactivity was 0·026 μC/mg.

OTHER ANTIGENS

Human, rat and mouse γ globulins were prepared by chromatography on a DEAE cellulose column (Peterson and Sober, 1956); haemocyanin (*Maia squinado*) was prepared by the method of Dhéré (1908); mouse urinary protein was prepared by pressure dialysis of freshly voided urine of C3/He mice; human serum transferrin and human serum β-lipoprotein, together with rabbit antisera, were a gift from Dr. P. G. H. Gell; rat liver catalase and its antiserum were given by Dr. T. S. Work; sheep thyroglobulin and its antiserum were given by Dr. B. A. Askonas.

ANTIBODIES

Anti-HSA was prepared by repeated intravenous injection of alum-precipitated HSA into rabbits. The antibody content of the serum was 13 mg./ml. Most antisera against other proteins were prepared by immunizing rabbits or goats with purified proteins in Freund's adjuvant mixture (Freund and McDermott, 1942), followed after 1 month or more by a course of intravenous injections of alum-precipitated protein.

Labelled Anti-ovalbumin (Ea)

Rabbit antibody against crystalline hen's ovalbumin was prepared by Dr. Tozer (Microbiological Research Establishment, Porton), and was dissociated from an antigen-antibody complex prepared with ovalbumin by the method of Tozer, Cammack and Smith (1958). We are grateful to Dr. Tozer for this material. It was iodinated with ^{131}I as above, and had a specific activity 3·7 μC/mg. when used. When the labelled material was mixed with a large excess of unlabelled anti-ovalbumin serum to act as carrier, and tested carefully by the quantitative precipitation technique (Heidelberger and McPherson, 1943), with two separate preparations of crystalline ovalbumin, it was found that only 62 per cent of the radioactivity was precipitated by antigen at equivalence, although all

the radioactivity was bound to γ globulin. Corrections were therefore made in the experiment with labelled antibody for radioactivity not specifically precipitable by antigen, on the assumption that this material diffused freely as γ globulin. Such a correction could be made with considerable accuracy, since the distribution of freely diffusing labelled antibody was measured simultaneously in the same experiment.

TECHNIQUE OF DIFFUSION EXPERIMENTS

1. *Double Diffusion from Wells in Agar*

Difco agar 1·5 per cent w/v was used in M/15 Na phosphate buffer, pH 7·0, containing M/200 Na ethylene diamine tetraacetate (to increase the clarity of the agar gel) and M/100 Na azide (to prevent growth of micro-organisms). A layer of molten agar 4 mm. deep was poured on flat level glass plates 10 × 10 cm. When the agar had set at room temperature cylindrical holes were cut with a cork borer, and the bottoms sealed with molten agar. The diameter of the holes was usually 7·5 mm., and the distance between their centres 22·5 mm. The wells were filled with antigen or antibody solution and the plates were left in a moist chamber at room temperature (18–20°). When desired, small standard samples of the agar were removed by pushing a sharp-edged stainless-steel tube 1 mm. diameter down to the glass plate. The cores, which were very uniform, were expelled with a stilette on small discs of filter paper, which were placed at the bottom of standard tubes for counting ^{131}I in a well-type scintillation counter, or on to clean metal 1 $cm.^2$ planchettes containing a trace of detergent at 110° (at which temperature the agar melted and spread as a thin film) for estimation of ^{14}C in an end-window counter. The quantity of agar was so small that the sample could be considered as infinitely thin. When ^{131}I and ^{14}C were present together the counts due to ^{131}I β-emission were allowed for by recounting after 6 weeks, and using the known decay rate of ^{131}I to correct for its contribution to the total counts.

2. *Double Diffusion from Troughs at Right Angles*

An agar layer 4 mm. deep on a flat glass plate 10 × 10 cm. was prepared with two thin rectangular troughs at right angles to one another, but separated by 1 mm. This was conveniently done by placing a stainless-steel template having bars of appropriate size at 90° face downwards on the glass at the time of pouring the agar, and then carefully removing the template without disturbing the agar after it had set. The arrangement resembled that described by Elek (1949). Equal volumes of antigen and antibody solutions were placed in the troughs, containing amounts of reactants calculated to be approximately equivalent. The angle between the precipitin line and the troughs was measured with a protractor to within ½° on an image, enlarged ten times, projected on to a ground glass screen.

3. *Diffusion through Agar or Gelatin Cylinders*

As described below, the molecular sieve properties of gelatin gels were used to estimate the size of antigen molecules. For this purpose flat sheets of 1·5 per cent agar gel were prepared in the usual way, and a row of holes 7–8 mm. diameter, and about 5 mm. apart, cut out with a cork borer. These holes were filled with solutions of molten agar at 100° or of molten gelatin at 60°, made up at various concentrations in the same buffer solution as the agar sheet. After setting at room temperature, cores 3–4 mm. in diameter were cut

from the centre of each agar or gelatin plug, leaving cylinders with walls about 2 mm. thick. A narrow trough for antiserum was next cut in the agar sheet, about 7·5 mm. from the row of cylinders, and the plate was set aside for 24 hours in a moist chamber in the cold for the gelatin gels to 'mature' and attain stable and reproducible properties. Antigen solution was then placed in the cylinders, and antiserum in the trough. Precipitin lines appeared after 1–2 days in the agar between the trough and those cylinders through which antigen had been able to diffuse. According to the purposes of the experiment the plugs were filled with agar 2–7 per cent or gelatin 10–40 per cent w/v. At higher concentrations than these the molten gels are difficult to handle.

RESULTS

FREE DIFFUSION OF ANTIGENS AND ANTIBODIES IN AGAR

An experiment was performed to ascertain whether diffusion through 1 per cent agar gel occurs at the same rate as through solvent alone. HSA was trace labelled with ^{131}I (which does not detectably affect its physical properties) and 15 per cent solution prepared by dialysis under pressure against the fluid medium used for dissolving the agar. The solution was placed in small wells (1 mm. diameter) or large wells (7·5 mm. diameter) in flat agar sheets, and allowed to diffuse for varying periods of time. The concentration at various distances from the wells was measured by counting 1 mm. diameter plugs of agar, as described. Agreement between replicate samples taken at the same distance from the origin was good.

The concentration c at a point P of a substance diffusing radially from a point source through a plane XY (or from a pencil source through a thick sheet of medium) is given by expression (1) (Crank, 1956).

$$c = \frac{k}{t} \cdot e^{-(x^2 + y^2)/4\,Dt} \qquad (1)$$

where D is the diffusion constant, t = time, x, y are the rectangular coordinates of P, and k is a constant proportional to the quantity of solute placed at the origin at zero time.

The diffusion constant of HSA was calculated by this method from experimental measurements of c, t, x and y. When diffusion took place from 1 mm. wells the value was within 5 per cent of the accepted value of $6{\cdot}1 \times 10^{-7}$ cm^2. sec^{-1}. When diffusion occurred from larger wells the calculated diffusion coefficient was about 15 per cent too high. This was presumably due to the zero time error involved, owing to the fact that the material did not start from a thin pencil source. By taking measurements at two different times the error could be eliminated, and good agreement was obtained with the accepted values. It may therefore be taken that the proteins studied diffuse through a dilute agar gel as through plain solvent. This may not hold at higher concentrations of agar, or for basic proteins which can interact with the acidic groups of agar.

DISTRIBUTION OF ANTIGEN AND ANTIBODY IN AGAR WHEN PRECIPITATION OCCURS

Double diffusion experiments were performed using ^{131}I HSA and unlabelled antiserum, under the conditions described in the section on 'Methods'. Concentrations of HSA and of antibody were chosen so as to give the following ratios of antigen/antibody — 1·5:1 (antigen excess); 1:6·5 (approximate equivalence); 1:65 (antibody excess). The

distribution of antigen around the antigen wells, both in the direction of, and away from, the antibody wells, was measured at times from 44–170 hours after setting up the experiment. Some results are given in Fig. 1, in which antigen concentration is plotted on a logarithmic scale against distance from the well on a linear scale. The following points emerge:

1. The concentration of antigen immediately beyond the precipitation line is very low (0·03 μg./ml.), and is accountable in terms of the solubility of the antigen-antibody complex.

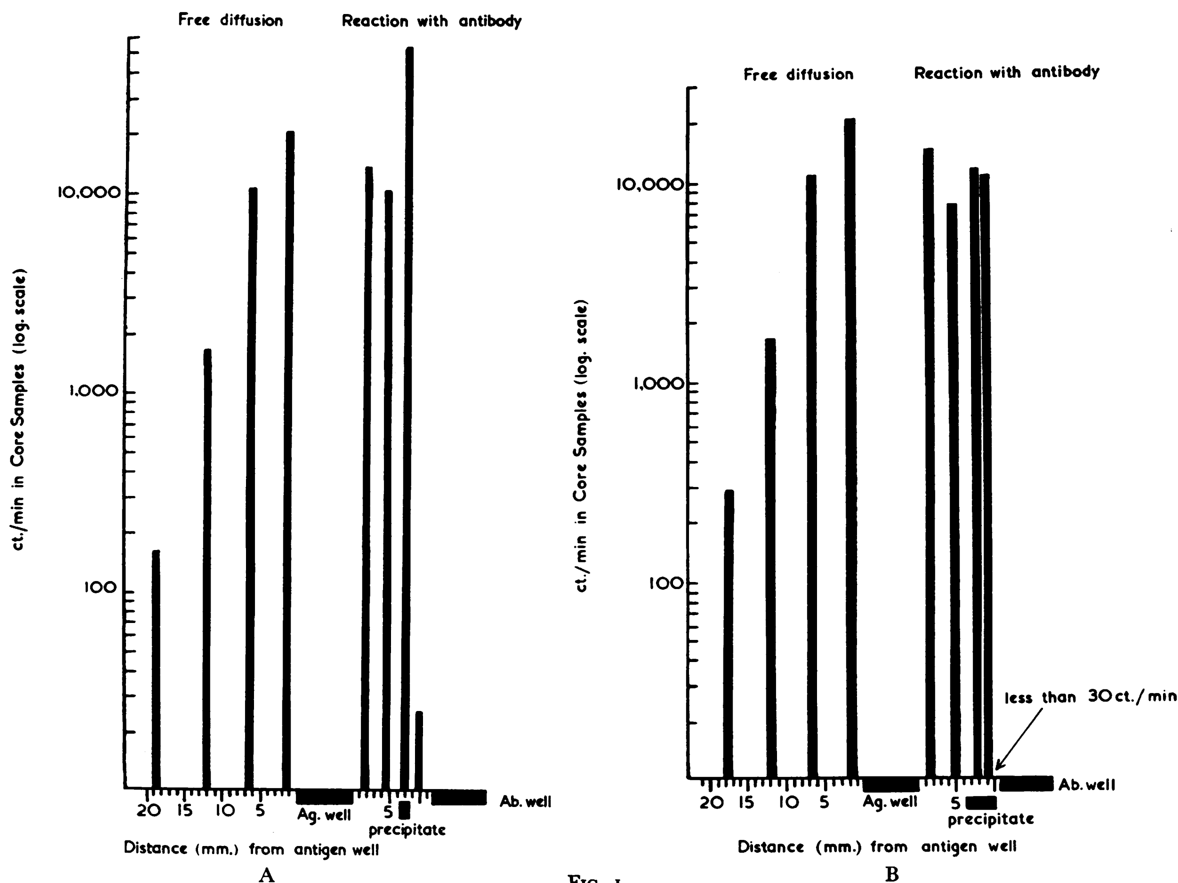

FIG. 1.

A. Distribution of antigen in agar around the well 115 hours after start of the experiment, under conditions of free diffusion and of reaction with antibody at approximate equivalence.

B. Distribution of antigen 115 hours after the start of the experiment, under conditions of free diffusion and of reaction with antibody in antigen excess.

The ordinate scale is logarithmic. The conditions of the experiment were as follows: antigen ^{131}I HSA, 2 mg./ml. in A and in B; antibody (rabbit) 13 mg./ml. in A and 2·6 mg./ml. in B; temperature 18–20° C.; depth of agar 4 mm.; diameter of core samples 1 mm.

2. Antigen accumulates markedly in the precipitate.

3. The concentration of antigen between the origin and the precipitate is less than the concentration at an equal distance on the other side (where diffusion is free).

4. The concentrations of antigen in directions away from the antibody are close to those predicted from diffusion theory, as described above, except that after long time intervals

the concentrations are slightly lower than expected. This is probably a consequence of asymmetric diffusion, due to accumulation of antigen in the precipitate.

5. With the antigen-antibody system used, the initial line of precipitation remains fixed, although when relative excess of one reagent is present the line broadens in the direction of the weaker reagent.

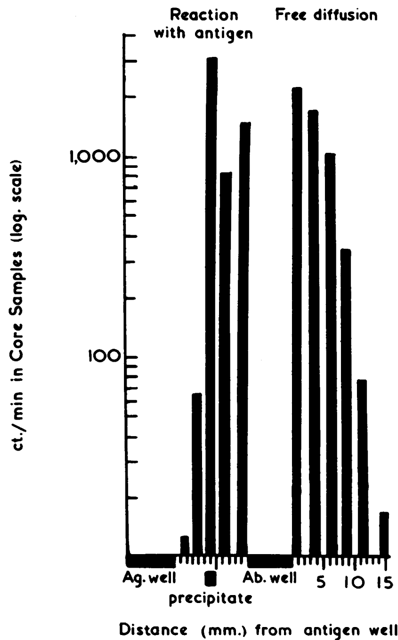

FIG. 2. Distribution of antibody in agar around the well 72 hours after the start of the experiment, under conditions of free diffusion and of reaction with antigen at approximate equivalence.

The ordinate scale is logarithmic. The conditions of the experiment were as follows: antigen ^{14}C-labelled Ea, 0·4 mg./ml.; rabbit antibody, purified by dissociation from antigen-antibody complex and labelled with ^{131}I, 6·5 mg./ml. Temperature 18–20° C.; depth of agar 4 mm.; diameter of core samples 1 mm. Only ^{131}I radioactivity is shown and the radioactivity in the samples has been corrected for the presence of 38 per cent of non-specific γ globulin.

It is evident that in the region of the precipitate a low concentration of free antigen (or antibody) develops, and that diffusion towards this region is accelerated. The assumption that the antigen will diffuse according to the laws of free diffusion is therefore, as expected, invalid.

Similar experiments were done with ^{14}C ovalbumin and a purified ^{131}I rabbit anti-ovalbumin γ globulin concentrate. As described above, allowance was made for the fact that 38 per cent of the γ globulin was not specifically precipitable by ovalbumin. The measurements of ovalbumin concentration by counting ^{14}C were relatively inaccurate,

owing to the simultaneous presence of ^{131}I and the difficulty of obtaining even spread of the agar on the planchettes, and had an error of $\pm$ 30 per cent. Nevertheless, the results clearly showed a distribution of ^{14}C ovalbumin very like that of ^{131}I HSA in Fig. 1. The distribution of labelled antibody around the precipitation line reproduces the picture obtained when only antigen was labelled (Fig. 2). However, the amount of antibody beyond the precipitate is somewhat greater than that of antigen, presumably because the antigen-antibody complex is more soluble in excess antigen than in excess antibody.

A direct visual demonstration of the phenomena described above is given by the autoradiographs shown in Fig. 3. These were obtained by conducting similar experiments in thinner layers of agar, containing M/75 phosphate buffer pH 7·0, and drying the gels after a suitable time, without washing, as thin flat sheets. The distribution of antigen and

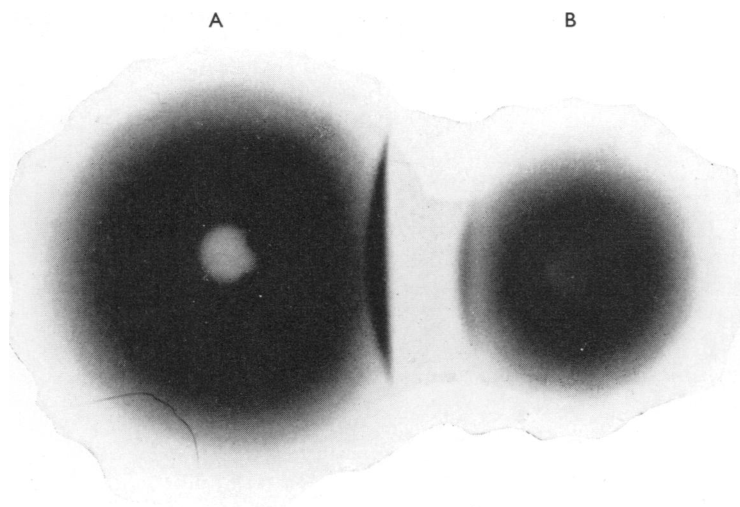

FIG. 3. Autoradiographs showing the distribution of A. ^{131}I HSA, B. ^{131}I anti-Ea in agar around the wells in double diffusion experiments against approximately equivalent amounts of the corresponding antibody and antigen.
The conditions of the experiment were as follows:
A. — as in Fig. 1A. B. — as in Fig. 2.

antibody distorted towards the precipitin lines, and their accumulation in the lines, are well seen. The lines appear rather diffuse because of the relatively wide zone of exposure of the photographic emulsion due to the high-energy β-rays emitted by the ^{131}I label.

ESTIMATION OF DIFFUSION COEFFICIENTS OF ANTIGENS

By Double Diffusion Technique

Since diffusion is no longer free when precipitation takes place, diffusion coefficients can only be calculated from the position of the precipitate in conditions where this is stationary. In such conditions the ratio of the diffusion coefficients of antigen and antibody can be measured with considerable accuracy. Korngold and van Leeuwen (1957) pointed out that when diffusion occurs from wells in a flat sheet, the line of precipitate is convex towards the well containing the reacting material with the greater diffusion coefficient. When the reactants are placed in troughs at 90°, it has been shown by Elek (1949) and by Ouchterlony (1949, 1958) that precipitation occurs at optimal proportions

along a plane which, viewed from above, appears as a line. The use of this arrangement for measuring diffusion coefficients has been briefly described by Allison and Humphrey (1959). The practical details have been given above, and the ratio of the diffusion constants of antigen (D_g) and of antibody (D_b) is given by

$$\tan\theta = (D_g/D_b)^{\frac{1}{2}} \tag{2}$$

where θ is the angle between the precipitation line and the antigen trough.

The theoretical treatment is as follows:

The diffusion of molecules from a narrow trough through a shallow motionless medium of indefinite area is represented (Crank, 1956) by a particular solution of the diffusion equation of the form

$$m = \frac{n}{(4\pi\, Dt)^{\frac{1}{2}}} \cdot e^{-x^2/4\, Dt} \tag{3}$$

where m is the concentration (number per unit area) of any given type of molecule at a distance x from the trough at any time t after the trough is filled, n is the number of molecules (per unit length) initially in the trough and D is their diffusion coefficient.

Initially one trough contains b antibody molecules per unit length, with a diffusion coefficient D_b. The perpendicular trough contains g antigen molecules per unit length with diffusion coefficient D_g. If at any later time a precipitate begins to form at a point a distance x_b from the antibody trough and x_g from the antigen trough, then at this point the two types of molecule are present in optimal proportions. Suppose the equivalence ratio of antigen molecules: antibody molecules is k, then $m_g = km_b$, i.e.

$$\frac{g}{(4\pi\, D_g t)^{\frac{1}{2}}} e^{-x_g^2/4\, D_g t} = \frac{kb}{(4\pi\, D_b t)^{\frac{1}{2}}} e^{-x_b^2/4\, D_b t}, \tag{4}$$

$$\frac{g}{b} e^{-x_g^2/4\, D_g t} = k \left(\frac{D_g}{D_b}\right)^{\frac{1}{2}} \cdot e^{-x_b^2/4\, D_b t}, \tag{5}$$

or, taking logarithms,

$$\frac{x_b^2}{4\, D_b t} + \log_e \left(\frac{g}{b}\right) = \frac{x_g^2}{4\, D_g t} + \log_e k \left(\frac{D_g}{D_b}\right)^{\frac{1}{2}}, \tag{6}$$

$$\frac{D_g}{D_b} = \left(\frac{x_g}{x_b}\right)^2 + \frac{4\, D_g t}{x_b^2} \log_e \left[k \frac{b}{g} \left(\frac{D_g}{D_b}\right)^{\frac{1}{2}}\right] \tag{7}$$

Thus, in general, the relation between x_b and x_y will be hyperbolic, but it will be seen that the second term in equation (7) vanishes when $g/b = k\ (D_g/D_b)^{\frac{1}{2}}$.

In practice the proportions of antigen and antibody are adjusted so that a narrow straight line is obtained, and for this line

$$\frac{x_b}{x_g} = \tan\theta = (D_g/D_b)^{\frac{1}{2}} \tag{8}$$

Using a number of test systems it was found that it was usually sufficient to set up plates in which the amounts of antigen and antibody per unit length of trough were such as had

been found to be equivalent by the method of optimum proportions, together with plates in which there was a 50 per cent relative excess of antigen and a 50 per cent relative excess of antibody. At least one of these plates conditions were sufficiently close to ideal to give thin straight precipitation lines, and usually they all gave lines sufficiently straight to determine θ to within ½°; the values of θ lay within 1° for all the plates. When the ratio of antigen to antibody departed grossly from the ideal (a twofold difference or greater) diffuse curved precipitation bands were formed which spread towards the trough containing the weaker reagent. With completely unknown systems approximately correct proportions had to be discovered by trial and error, and the characterisitc curvature and spread of the bands could be used for this purpose.

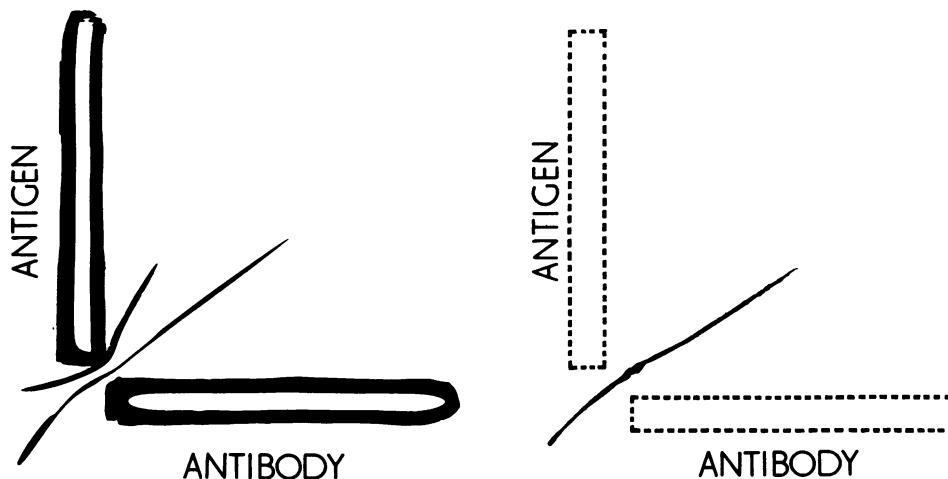

FIG. 4. Estimation of diffusion coefficients of antigens by technique using antigen and antibody troughs at right angles.

The antigen trough contains a mixture of ^{14}C ovalbumin and human serum β-lipoprotein, and the antibody trough contains a mixture of the corresponding rabbit antisera. The agar was well washed to remove unprecipitated material before being photographed.

Left. Direct photograph, showing independent behaviour of the precipitin lines.

Right. Autoradiograph, showing accumulation of ^{14}C ovalbumin in its own precipitin line only.

The reason why workable conditions are so easily achieved is that unless D_g and D_b are very different, or g/b is very different from k, the second term in equation (7) will be small compared with $(x_g/x_b)^2$, and so equation (2) is approximately valid even when the ratios of antigen to antibody concentration are not ideal. This can be shown by a numerical example. Suppose that a simple antigen-antibody system is used in which the equivalence ratio (k) has been measured. Ideally the amounts of antigen and antibody per unit length of trough should be in the ratio $k\ (D_g/D_b)^{\frac{1}{2}}$. If they were in fact applied in the ratio k, and the angle of the precipitation line, θ, were measured after 40 hours when the line extended to a point distant 1 cm. from the antigen trough (as found, for example, with the system HSA - anti-HSA, when the concentration of antibody solution was 2 mg./ml.), the error in D_g which resulted from assuming equation (2) to be true would be as follows:

nil when the diffusion coefficients were the same

4 per cent when the diffusion coefficients differed by a factor of 1·5

7 per cent when the diffusion coefficients differed by a factor of 2·0

12 per cent when the diffusion coefficients differed by a factor of 3·0

As already stated, once precipitation has begun, the assumption of free diffusion can no longer be made. Nevertheless, under the conditions prescribed, molecules of both antigen and antibody will continue to diffuse into the zone of low concentration in the region of the precipitate at rates proportional to their diffusion coefficients, so that the precipitate will continue to form in its original position. Different antigen-antibody systems were shown to precipitate independently of one another, or of other inert protein present, and

TABLE 1

DIFFUSION COEFFICIENTS ($\times 10^{-7}$ CM.^{2} SEC.$^{-1}$) OF ANTIGENS CALCULATED FROM AGAR PRECIPITATION LINES AND AS MEASURED BY OTHER TECHNIQUES

Protein	θ	*Calculated* D_{20}	*Reported* D_{20}	*Molecular weight*
Mouse urinary protein	58°	9·6	9·7	17,000
Hen's ovalbumin	54°	7·1	7·7	43,800
Human serum albumin	52°	6·1	6·1	70,000
Human serum transferrin	51°	5·7	5·8	88,000
Human γ globulin	45°	3·8	3·8	177,000
Mouse γ globulin	45°	3·8	3·9	160,000
Rat liver catalase	45°	3·8	4·1	249,000
Sheep thyroglobulin	38°	2·3	2·6	660,000
Human serum β-lipoprotein	28°	1·1	0·98	2,770,000

The references to the reported values for D_{20} and molecular weights are given in Allison and Humphrey (1959).

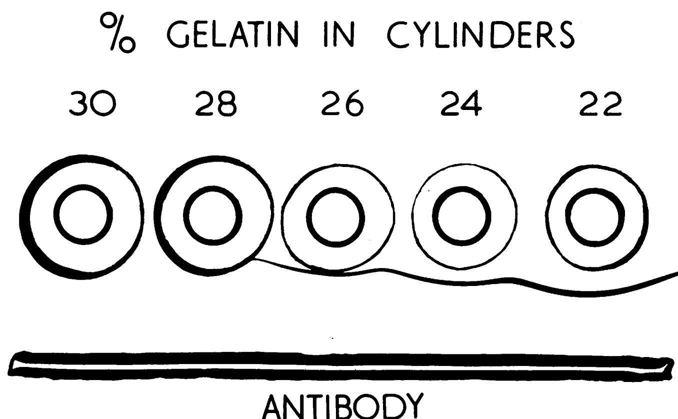

FIG. 5. Photograph of experiment for estimating size of antigen based on 'molecular sieve' properties of gelatin gels.

Each cylinder is composed of gelatin gel at a different concentration. Antigen (human serum albumin 1 mg./ml.) was placed in the cylinders and rabbit antiserum in the trough. Antigen diffused through 26 per cent gelatin but not through 28 per cent.

the diffusion coefficients of each component in a mixed system could be measured provided that conditions were chosen to be suitable for each (Fig. 4). In Table 1 are summarized the values for the diffusion coefficients calculated by this method for a range of test antigens with reference to rabbit antibodies (diffusion coefficient $= 3{\cdot}8 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, Kabat and Mayer, 1948). The agreement with values obtained by conventional techniques is good.

It is interesting that the equations derived by Engelberg (1959) for the linear double diffusion system and by Spiers and Augustin (1958) for linear single diffusion, taking into account the effects of precipitation, show that a stationary boundary is obtained when the initial molar ratios of antigen and antibody are proportional to the product of the equivalence ratio and the square root of the ratio of the diffusion coefficients.

Alternative Method for Estimating Size of Antigen

A different approach is to make use of the 'molecular sieve' properties of agar and gelatin gels, in which the 'pores' or spaces within the solid phase become smaller as the concentration of solid increases. Polson (1956) has presented evidence that penetration of proteins

TABLE 2

PERCENTAGES (W/V) OF AGAR LIMITING PENETRATION OF ANTIGENS

Protein	*Estimated molecular wt.*	*Agar concentration*
Caminella haemocyanin	6,600,000	3
Human serum β-lipoprotein	2,770,000	5
Sheep thyroglobulin	660,000	7
Jasus haemocyanin	450,000	7

The values for haemocyanins are taken from Polson (1956).

TABLE 3

PERCENTAGES (W/V) OF GELATIN LIMITING PENETRATION OF ANTIGENS

Protein	*Estimated molecular wt.*	*Gelatin concentration*
Human serum β-lipoprotein	2,770,000	5
Sheep thyroglobulin	660,000	7
Human and rat serum γ globulin	177,000	18
Human serum transferrin	88,000	26
Human serum albumin	70,000	28
Hen's ovalbumin	44,000	32
Mouse urinary protein	17,000	40

into agar gels is limited by their size. We used agar gels at concentration 1–7 per cent and gelatin gels at 4–40 per cent w/v, employing the technique described in the section on 'Methods', and illustrated in Fig. 5. From the illustration it can be seen that antigen diffuses almost unimpeded through the cylinders of gel of increasing concentration and precipitates with antibody until, with quite a small increment of gel concentration, passage of the antigen is prevented and no line is formed. The concentration of gel at which the line from the neighbouring cylinder appears to pass into the cylinder in question is taken as the end point. This is reproducible over quite a wide range of antigen and antibody concentrations, and each system in a mixture reaches its end point independently. Some results with reference antigens, using agar gels, are given in Table 2 and with gelatin gels in Table 3.

The technique permits estimation of the order of effective size of antigens to within ± 30 per cent, and enables ovalbumin (M.W. 44,000) consistently to be distinguished from human serum albumin (M.W. 70,000).

The molecular sieve effect of gelatin gels seems to be independent of electrostatic interaction between the solid phase and the solute molecules. The end points were similar with acid-processed gelatin (isoelectric point pH 9·0) or lime-processed gelatin (isoelectric point pH 4·0), although the solid phases at pH 7·0 would have opposite net charges. Passage of large molecules through gels is a complex process, and some of the problems have been discussed by Ogston (1958). An important limiting factor is the length of the greatest axis or radius of gyration. Determination of the effective size of antigens by migration through gels therefore gives information other than the simple diffusion coefficient. If molecules with similar diffusion coefficients have similar end points by this method, it is likely that they have similar sizes and shapes.

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