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THE ALTERED PATTERN OF DIFFUSION IN A DOUBLE-DIFFUSION SYSTEM*

I. BETWEEN TWO SOURCES OF ONE REACTANT

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SUMMARY

The altered diffusion pattern in a double-diffusion system results from the altered properties of the medium. Assuming, in accordance with the available evidence, that the diffusion of reactant depends on the ratio of concentration gradients of the reactant and dissolved substances in the gel which diffuse against the reactant, an increase of this ratio in the area between two diffusing sources results in an altered rate of diffusion.

The diffusion experiments with and without chemical reaction revealed that the mechanism of the alteration of the diffusion rate in the system is independent of the presence of reagent in the gel.

INTRODUCTION

Although diffusion of inorganic precipitating reactants has been studied for many years, simultaneous diffusion from two sources has not been investigated. It is well known, however, that in the OUCHTERLONY[1] double-diffusion system in which the central antibody is surrounded by a number of peripheral antigens, it is possible for adjacent antigens to influence each other's rate of diffusion. OUCHTERLONY[2] has pointed out that the diffusion of each reactant can be considered independently. It is commonly assumed that antigens influence each other's rate of diffusion only if they come into contact. From the data presented by OUCHTERLONY[3] it is difficult to form a clear concept of the physical phenomena involved in this interaction, and to explain why an asymmetrical pattern of precipitation with displacement of the precipitation line toward the well with lower antigen concentration is obtained in the presence of identical antigen of differing concentration. OUCHTERLONY's technique represents diffusion without chemical reaction because the antigens diffuse through gel which is initially free of antibody. OUDIN's technique[4,5] involves a chemical reaction, because the antigens diffuse through the gel loaded with antibody.

In this paper, systems consisting of two diffusing sources are investigated, one with crystalloids and one with specific proteins, with and without chemical reaction.

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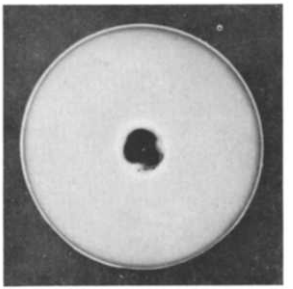

Fig. 1. Diffusion from one source.

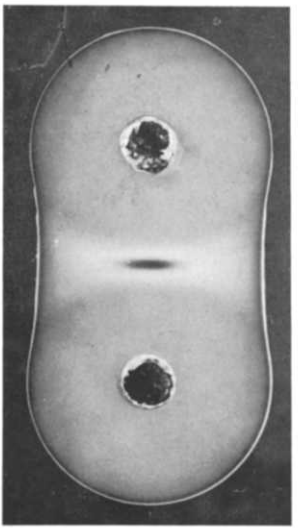

Fig. 2. Diffusion from two equal sources, with occurrence of a translucent zone.

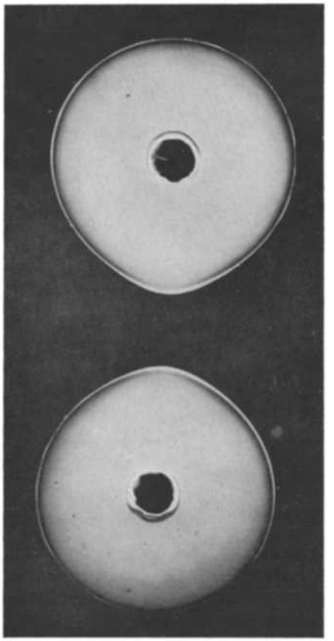

Fig. 3. Diffusion from two equal sources, with displacement of the precipitation patterns.

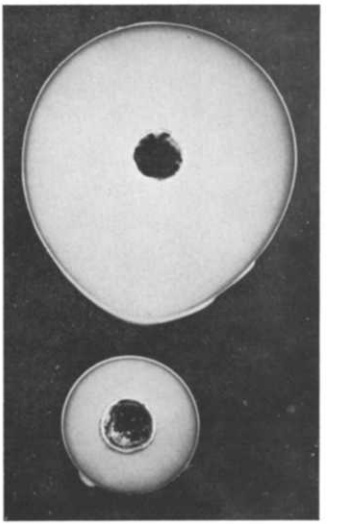

Fig. 4. Diffusion from two unequal cources with, displacement of one precipitation pattern.

EXPERIMENTAL

Crystalloids: Deionized water saturated with air was used throughout. Gel: 0.5% purified Difco-agar and containing 0.01 M $BaCl_2$ in 1 M or 0.25 M solutions of $(NH_4)_2SO_4$.

10 ml of melted agar with indicator were poured into a petri dish (9.2 cm dia.). After solidification, two wells for the double diffusion and one well for the single-diffusion experiments, were punched with a tube (0.6 cm outer dia.). A series was prepared with the wells receiving 0.02 ml $(NH_4)_2SO_4$ of equal or different concentrations. The experiments were carried out in a humid chamber at about 25°. After 2 days when the diffusion process was complete, photographs were taken in diffuse light.

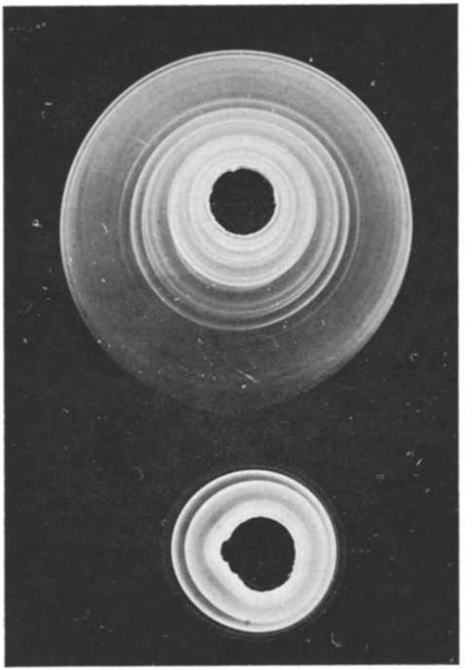

Fig. 5. The experiment shown in Fig. 4, with specific proteins.

Specific proteins: The antigen used was normal human serum. Antisera were prepared by giving rabbits six 0.5-ml injections of normal human serum, intravenously (occasionally part was given subcutaneously) over a 3-week period. Bleedings were made after the 4th week.

Purified Difco-agar (0.75%) in saline was melted, and cooled to 55°. 3 ml of the antiserum heated to 55° was put into a petri dish which had been warmed to the same temperature. Then 7 ml of the melted agar were added from a warm pipette. The fluids were mixed by shaking gently, care being taken to avoid bubbles. The serum agar was allowed to set at room temperature. Two wells for the double diffusion and one well for the single-diffusion experiments were punched with a tube. Each well received 0.02 ml of normal human serum. The experiments were carried out in a humid chamber at about 25°. Readings were made 7 days after setting up the dishes.

RESULTS

Crystalloids: Fig. 1 represents a single-diffusion experiment. The diffusion process results in a radially symmetrical precipitation pattern. Fig. 2 shows a diffusion experiment involving two sources of reactant of equal concentration. The reactant from both sources diffused at the same rate, but between the two sources diffusion was opposed and a clear zone without precipitate was produced. In Fig. 3 the distance between the centers containing equal concentrations of reactant is greater. The precipitation patterns are displaced toward the opposite source. Fig. 4 shows the results obtained with unequal concentrations of the diffusing reactant. The more dilute reactant forms a radially symmetrical precipitation pattern, and the pattern due to the more concentrated one is displaced towards the opposite source.

Specific proteins: Fig. 5 shows the result obtained with unequal concentrations of antigen which is similar to that with crystalloids.

The following generalizations can be made on the basis of these five experiments: when the concentrations of the two diffusing sources are the same, diffusion from one source is opposed by that from the other and the clear zone without precipitate appears. Increasing the distance between the sources results in increased diffusion of the reactant towards the opposite source.

With unequal concentrations of reactant the diffusion of the more dilute reactant will cease before the influence of the stronger reactant can influence its rate of transfer. Consequently, only one diffusing reactant is displaced.

The intensity of the displaced part of the precipitate decreases as a function of the displacement, but this effect is not clearly visible in the photographs.

DISCUSSION

These experiments illustrate the process of simultaneous diffusion and chemical reaction. The diffusion takes place in a gel containing reagent which forms a precipitate with the diffusing reactant. STILES[6] has shown that the rate of diffusion in a system of inorganic precipitating reagents (1) is proportional to the square root of time; (2) is dependent upon the initial concentration of the diffusing reactant; and (3) depends also on the concentration of the internal reactant; the lower the concentration of internal reactant, the higher the rate of diffusion.

OUDIN[7] showed empirically that for a given antigen–antibody system an increase of antiserum concentration in the gel affects k, the slope of the straight line obtained by plotting x, the distance the leading edge of the precipitate moves in time t, against $t_{\frac{1}{2}}$. The similarity between the two statements is evident.

On the other hand, there is an important difference between the equivalence point as the condition for precipitation of the antigen–antibody complex and the solubility product of crystalloids. Since the solubility product of $BaSO_4$ is large enough to permit appreciable concentrations of $(NH_4)_2SO_4$ ahead of the precipitation zone, one can assume that as the edges of the two diffusates come into contact there is no concentration gradient and hence no net flow, though interdiffusion still continues. Thus a more rapid increase of reactant concentration between the sources and the content plane occurs and the SO_4^{2-} concentration first reaches precipitation level along a line joining the sources, *i.e.*, before this concentration is reached along other radii. A changed precipitation pattern may result. According to this interpretation it is, however, difficult to explain the existence of the clear zone demonstrated in Fig. 2.

The hypothesis[8] put forward to explain the diffusion of antigen into the antibody-loaded gel is as follows: The precipitation line occurs at the point of equivalence. Behind the leading edge of the precipitate the excess antigen will dissolve the precipitate, whilst in the plane immediately in front of the leading edge the excess antibody will stop the diffusion of antigen until the concentration required for precipitation is reached. On the basis of this hypothesis and Oudin's observation (*loc. cit.*), the displacement of the leading edge of the precipitate demonstrated here may occur if the antibody concentration in the displacement area is decreased.

Using coloured solute BRADFORD[9] demonstrated that there was no internal reactant in the gel in the vicinity of the precipitate. We obtained the same effect if the dish was plunged into $(NH_4)_2SO_4$ solution at different stages of the diffusion process and immediately after development of the precipitation patterns described. This procedure resulted in a continuous precipitate in the surrounding medium, and in the clear "halo" just outside the advancing edge of the precipitation pattern. This effect could be due to adsorption[9] or inward diffusion[10]. The results presented suggest inward diffusion, because diffusion is the only feasible mechanism over longer distances: adsorption acts only over short distances. SPIERS AND AUGUSTIN[11] in their immunodiffusion studies also assumed inward diffusion, *i.e.*, diffusion of the internal reactant against incoming diffusing reactant.

If we consider the system described as composed of two independent sources of diffusion, we can suppose that the diffusion against incoming diffusing reactant is uniform and equal to the effect that each reactant acting alone would produce. When two zones of inward diffusion combine, the rate of inward diffusion in this area is equal to the effects of both diffusing regions. In this case, the concentration of the internal reactant is not uniform in the medium but is minimal along a line joining the centers where combination first occurs, and gradually increases along other radii. Thus, under the conditions where displacement occurs, the diffusing reactants behaved in a fashion quantitatively identical with the behavior of the reactant tested against proportionally increasing concentration of internal reactant.

In the experiment involving unequal concentrations of the diffusing sources, the diffusion of more dilute reactant ceases before the two zones of inward diffusion come into contact, thus giving a radially symmetrical precipitation pattern. As diffusion of more concentrated reactant continues, the displacement occurs. This clearly indicates continued inward diffusion after the precipitation pattern has ceased to change. Further investigation of this problem is in progress.

In order to avoid the effect of chemical reaction which accompanies diffusion in the procedure described, the double-diffusion technique[1] was used. The same antigen is allowed to diffuse from two parallel sources and reacting antibody from a source perpendicular to the former. As mentioned before, the antigens diffuse through the gel which is initially free of antibody. It is well known that the geometry of the precipitation line depends upon the concentration of the antigen from the opposite source. FEINBERG[12] for example demonstrated displacement of the precipitation line formed with the antigen from one source under the influence of the subthreshold concentration of the same antigen from the opposite source. These observations were reproduced with inorganic precipitating reagents and the results confirmed those obtained with specific proteins.

Studies by SABIN AND SOBOTKA[13], and FELICETTA *et al.*[14] indicate that the interstitial fluid in agar gel contains water-extractable dissolved agar which has a measurable viscosity. In the case of solution-to-gel diffusion, there is simultaneous diffusion of dissolved agar from gel to solution, and impedance to diffusion of the diffusing reactant is reduced in this area[14]. It was also shown[15] that the concentration of agar does affect the rate of diffusion of the leading edge of the precipitate. These data, along with the results of this study, suggest that the mentioned combination of two zones of inward diffusion, of dissolved agar from gel into the diffusing reactant, manifests itself in the alteration of the rate of diffusion of the diffusing reactant.

This paper serves to draw attention to the common biophysical mechanism which governs gel double-diffusion experiments. Since the rate of movement of the diffusing reactant is proportional to its concentration gradient and diffusion coefficient, the observed influence of one diffusing reactant on the movement of the other emphasizes the fact that, at a given distance between the sources of the diffusing reactants, the movement of each diffusing reactant depends upon the other concentration gradient as well as upon its own, even before contact between diffusing reactants has taken place.

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