DIFFUSION-PRECIPITIN STUDIES OF THE COMPLEXITY OF ANTIGEN MIXTURES[1]

II. The Number of Zones Formed by One Antigen

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As antigen molecules diffuse from a solution superimposed on a column of agar supported saline solution, a gradient of concentrations is established, ranging from the concentration within the solution to zero at the advancing border. If antibody also is incorporated in the agar, the precipitin reaction will establish a zone of visible precipitate which appears to advance as though it marked the location of a particular antigen concentration within that gradient (Becker *et al.*, 1951). It is reasonable to suppose that the antigen concentration thus indicated visually is the one required for equivalence with the concentration of antibody present.

It has been suggested that antigen enters a region, combining with the antibody until equivalence is reached, as heralded by precipitation. Only then will the region be free of antibody and antigen permitted to penetrate farther into an adjacent region, satisfying the antibody there and causing the zone to advance. According to this view, the gradient which would be established in antibody-free saline is cut off sharply by the presence of the antibody at a concentration greater than zero. Since lower concentrations of antigen cannot be established than that representing equivalence between an antigen and the more concentrated of two antibodies for which it bears specific determinant groups, equivalence could never be established with a second, less concentrated antibody. In consequence, Oudin reasoned that the number of zones in an Oudin tube could not exceed the number of antigens responsible for them.

Testing this hypothesis, Oudin (1952) examined the precipitates formed when ovalbumin diffused into rabbit antiserum containing two antibodies specific for the ovalbumin employed. As predicted, only one zone was observed, located as expected for the system involving the more concentrated antibody. On this basis, he formulated the principle that the number of zones in an Oudin tube represents the minimum possible number of antigens involved and, hence, is a clue to complexity of an unknown antigen solution.

The principle has been accepted by leading investigators (Becker and Munoz, 1949; Pope *et al.*, 1951; Telfer and Williams, 1953; Oakley and Fulthorpe, 1953). It should be recognized however, that it was derived from reasoning based on assumptions and supported by negative experimental evidence.

In the following report it will be shown that a basic premise in the reasoning is invalid. Positive experimental evidence will be presented, indicating that the visible precipitate cannot represent an interface between effective concentrations of the specific reactants. An alternative line of reasoning, based on accepted immunochemical theory and compatible with the present observations, will be suggested. If it is agreed that the reasoning is theoretically sound, and that the interpretations offered as to the significance of the experimental evidence are admissible, then the hypothesis arrived at must be considered to have as firm a foundation as does the currently accepted principle. While the approach is not one which permits a positive conclusion that the Oudin principle is necessarily incorrect, it does lead to a reasonable doubt. Under these circumstances, the number of zones in an Oudin tube cannot be accepted as evidence of the complexity of the antigen in an unknown preparation.

MATERIALS AND METHODS

Precipitin systems. The conclusions reached in the following study could have been derived from patterns illustrated in the previous article. Unfortunately, the equivocal nature of the antigen-

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antibody systems studied weakens the acceptability of the evidence. The tetanus antigens and antitoxin, used to produce the precipitates described below, are scarcely less open to the criticism that unknown precipitin systems were present as impurities in the reagents, but the nature of the patterns observed demonstrates that this cannot be a factor in the results obtained.

The antibodies were employed as whole immune horse serum,[2] rather than a globulin concentrate, and were therefore relatively dilute. No more than two zones were observed in any experiment. While two antibodies may have been present in sufficiently high concentration to produce visible precipitates, it is also possible that antitoxin proper was the only antibody actually entering into visible reactions. Support for this statement is beyond the scope of the present report, however, and the distinction is not critical in the interpretation of results.

The antigens were supplied as crude filtrate from tetanus cultures in Mueller's medium and used without further purification.[3] Both toxin and toxoid contained about 45 Lf units of antigen per ml. Except for experiments designed to show the effect of relative concentration, these reagents, as well as the antiserum, were used without dilution other than that incident to addition of the 2 per cent agar as described below.

Double-diffusion technique. The apparatus employed possesses certain advantages in the matter of speed and accuracy which are the subject of current studies. A detailed description of the method will be presented when such studies are complete. The principles of operation and interpretation pertinent to the present thesis may all be derived from the discussion of double diffusion in the recent review by Oudin (1952).

The vessel, illustrated in figure 1, is a shallow triangular dish formed of plastic. The lumen is an equilateral triangle with 50 mm sides, 6 mm deep. Four ml of one per cent agar in buffered saline with preservative are placed in this dish and allowed to solidify. Notches midway on each wall then serve as guides for a sharpened strip of thin sheet metal, acting as a knife to divide the agar into four smaller triangles. With the knife in place, the agar is cleaned out of one corner triangle at a time, leaving the central triangle extending into the notches as shown in the illustration.

This central triangle will be referred to as the reaction arena. The reagent wells of the Ouchterlony technique are represented in this preparation by the three vacant corner triangles. Antibody solution, mixed with an equal volume of two per cent agar in buffered saline, is placed in one of these depots and allowed to solidify. By permitting the agar to set before proceeding to the other reagent depots, the seal against leakage into other depots provided by the extensions of the reaction arena into the notches may be checked. Antigen solutions to be studied are placed then in the other two depots, mixed with agar as in the case of the antibody.

The arrangement is quite analogous to the usual Ouchterlony plate, except that the reagents diffuse out, forming gradients with rectilinear concentration isobars rather than circular ones, and establishing equivalence almost immediately where the appropriate conditions are first established in the notches. The zones develop toward the center of the reaction arena, along the path of points of intersection of the advancing equivalent concentration isobars of antigen and of antibody. When they meet, coalescence or crossing is governed by the same factors and has the same significance as in the Ouchterlony method (see Oudin, 1952).

The preparations are placed in inverted petri dishes at room temperature. Distilled water poured into the dish provides a seal and keeps the atmosphere within the dish saturated so that the agar does not dry out during observation periods of a week or more.

Direct shadowgraphy was not satisfactory with the plates used because of the thickness of the plastic bottoms. However, the precipitin systems employed in the tests described below gave dense enough precipitates to permit direct photography with backlighting against a matte black paper background. The housing of the circular fluorescent light supplying the illumination is visible through the agar of the reagent wells in some of the illustrations.

EXPERIMENTAL RESULTS

Pattern produced by two sources of tetanus toxoid. The pattern formed by the crude toxoid

[2] Available through the generosity of Jensen-Salsbery Laboratories, Inc.

[3] Gift of Sharp and Dohme, Inc.

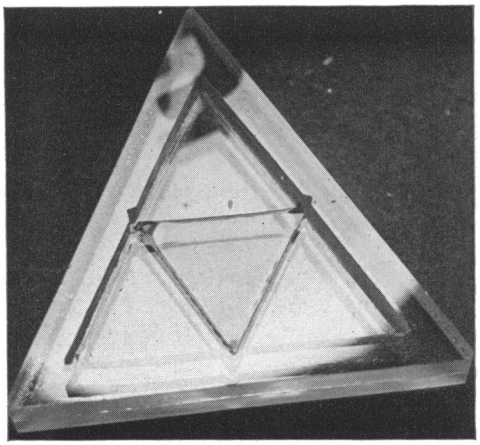

Figure 1. Triangle diffusion plate ready for addition of reagents.

in one case and by the toxoid mixed with an equal volume of buffered saline in another, further diluted by addition of agar solution, is shown in figure 2. In both cases, reaction with antibodies, made available by diffusion from the serum diluted only by agar solution, gave rise to but one zone. In this form of double diffusion, zones must begin within the notch and are separated only by subsequent migration, as in the Oudin tube. It cannot be said with certainty, therefore, that the single zones prove that only one antigen-antibody system contributed to the precipitate.

Pattern produced by two sources of tetanus toxin. A similar experiment, using toxin rather than toxoid, leads to the pattern shown in figure 3. In view of unpublished experiments, some reservations must be made regarding the interpretation of this pattern as a demonstration that two antibodies and two antigens are represented. It is possible that this pattern could have been produced by antitoxin alone, reacting with two molecularly dissimilar antigens with the toxin specificity. The distinction is not a matter of concern in the present report, however.

Pattern produced by toxoid diffusing across toxin. Using the reagents diluted only by the addition of an equal volume of 2 per cent agar, toxoid in one depot produced the typical single zone which coalesced with both toxin zones as shown in figure 4. The similarity of this pattern to that produced by zones 2 and 3 of the scarlatinal toxin antigens with one of the "proteose peptone antigens" (see figure 2 of preceding article) is apparent. It may be possible to interpret this

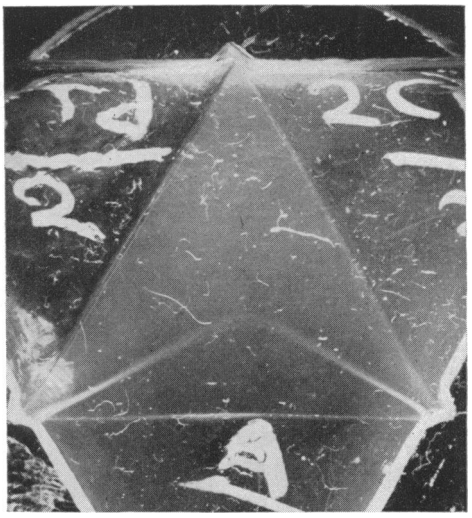

Figure 2. Double diffusion pattern obtained with two sources of tetanus toxoid. Upper right and left, sources of toxoid; bottom, source of antitoxin.

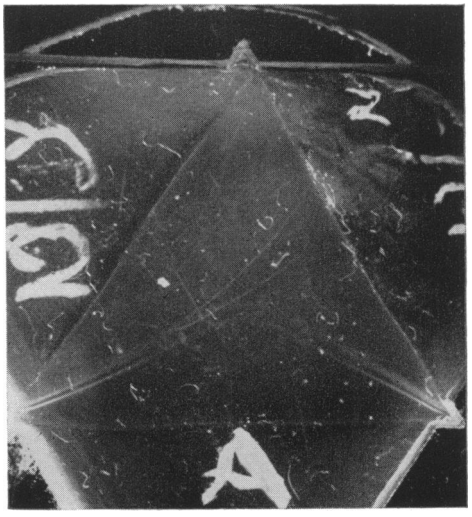

Figure 3. Double diffusion pattern obtained with two sources of toxin. Upper left and right, sources of tetanus toxin; bottom, source of antitoxin.

dual coalescence in a number of ways, but all lead to the conclusion that, however many antigens are involved, no antigen reacting visibly with antibody supplied by the serum is present in one preparation but not the other.

Pattern produced by toxin diffusing across mixed toxin and toxoid. Coalescence of zones is

Figure 4. Double diffusion pattern obtained with toxin versus toxoid. Upper left, source of toxin; upper right, source of toxoid; bottom, source of antitoxin.

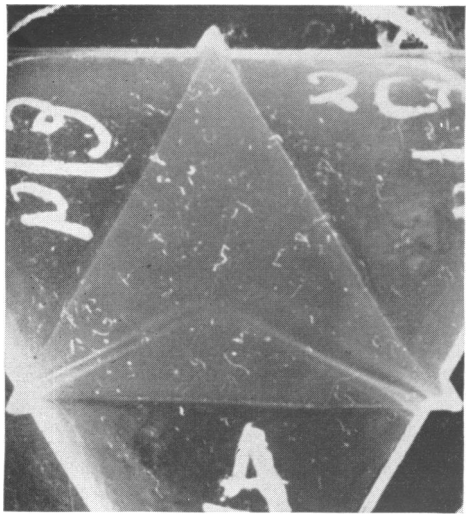

Figure 5. Double diffusion pattern obtained with toxin versus mixed toxin and toxoid. Upper left, source of toxin; upper right, source of mixture; bottom, source of antitoxin.

accomplished by the establishment of antigen excess through the additive concentrations contributed by the two sources of diffusion. If the antigen specific for the antibody in question is the same molecularly on both sides of the plate, this additive effect will be reflected also in the diffusion from the doubly supplied area. The zones, therefore, appear to bend toward each other as they approach. This behavior is illustrated in figure 3 in the case of both toxin zones. In figure 5, resulting from toxin in one depot and mixed toxin and toxoid in the other, the coalescence and bending of the two toxin zones are again apparent.

However, there is a third zone visible on the side of the mixed antigen depot. That this zone was due to toxoid was made certain by the relative location of the zone in different plates containing the toxin and toxoid in different concentrations and by controls in which similar concentrations of toxoid without toxin were employed.

This toxoid zone developed to the point where it would have met the toxin zones if they had not been deflected by coalescence with the toxin antigen zones from the mixed source. At this point, it ceased to develop, probably showing the competition of antigen from the toxin depot for the antibody forming the zone.

DISCUSSION

In the preparation illustrated in figure 2, a thin strip of agar parallel to the left or right wall of the vessel bears a close resemblance to an Oudin tube. At the outset, a column of uniformly distributed antigen is nearly in contact with a similar column of antibody. Diffusion of the two reagents quickly bridges the gap. The meeting is heralded by formation of the precipitate in the notch, and its subsequent behavior, in terms of migration along the strip, is controlled by the same factors as in the Oudin tube.

When this precipitate is formed, antigen and antibody are brought into contact along the immediately adjacent agar strip which thus becomes analogous to a second Oudin tube prepared a moment after the first, and so on across an area defined by the length of the visible zone.

The analogy of the double diffusion triangle plate to a series of Oudin tubes breaks down in one respect. The later "tubes" in the series would have an area on either side of the antigen-antibody interface in which the reagents were arranged as a gradient rather than a uniform atmosphere. However, this would affect the rate of migration of the zone rather than the mechanics of its formation.

According to the currently accepted view of these mechanics, the zone of visible precipitate in the double diffusion plate would have to repre-

sent an interface between free antigen and free antibody. Oncoming "isobars of concentration" of antigen would meet equivalent concentrations of antibody at this line and be used up, so that lower antigen or antibody concentrations would not be established further on.

The coalescence of the two toxin zones from the mixture of toxin and toxoid with the two zones from the unmixed sources proves that, in the region of the zones, equivalence has been established between antigen and all the antibody capable of reacting with material from either source. Regardless of the number of precipitin systems concerned, figure 4 demonstrates that no antibody capable of reacting with toxoid antigens fails to react with toxin antigens.

Nevertheless, a toxoid zone was formed on the antigen side of the region of primary equivalence. It follows that antibody, at least acting as though it were free, must necessarily be present in the region of antigen excess, permitting establishment of equivalence with a second antigen.

Conversely, the continued development of the toxin zones, as shown by the greater density of precipitate near the point of origin, shows that antigen penetrated the toxoid zone and, therefore, into a region of excess antibody capable of reacting with it specifically. It follows that the basic premise on which the interpretation of the significance of the number of zones in an Oudin tube now rests is in error.

It is virtually certain that free antigen and antibody or complex capable of acting as free antigen or antibody is to be found in regions well beyond the limit defined by the leading edge of the visible Oudin zone. The explanation of this phenomenon is a matter of speculation. The following explanation is offered tentatively as one compatible with the evidence.

Antigen diffusing into the agar of an Oudin tube meets antibody and combines to form complex. In order to permit aggregation of complex particles to a size visible as precipitate, the complex must diffuse from its point of origin to permit collisions with other complex particles. A gradient of concentrations of complex should exist to either side of the region of maximum formation—the region of antigen-antibody equivalence. The extent of this gradient should be influenced to some degree by the time required for aggregation.

A good rabbit precipitin system, such as that employed in Oudin's experiments, may produce precipitates within a few minutes. Horse antitoxin systems, such as the tetanus system studies in this report and the scarlatinal system dealt with in the previous one, often require several hours to accomplish the same result. It may be supposed, therefore, that both the heart infusion antigens system and the tetanus system presented conditions in which diffusion of the complex could result in greater penetration of the region of antibody excess than was true with Oudin's ovalbumin experiment.

Again, the well known effect of excess horse antiprotein antibody, as contrasted to the effect of rabbit antibody, may have been a factor in determining the physical state of the complex in this region.

Having established such a gradient, it is possible that if antigen combined with antibody by virtue of one determinant group possessed a second group specific for a more dilute antibody, enough of the second determinant might remain exposed to establish equivalence with the second antibody. The effect might be accomplished either as a result of release of the antigen to the second antibody or perhaps by development of a lattice initiated with the more concentrated antibody through subsequent combinations with the more dilute. In other words, the complex itself might act as though it were an antigen bearing the specificity not involved in the first reaction, and diffusing from a source located at the region of the primary equivalence zone.

The conditions required for the formation of two zones by one antigen under the suggested hypothesis would be as follows: (1) The presence of two determinant groups on the antigen molecule. (2) The presence of the two appropriate antibodies in the agar. (3) Penetration of the antigen, in a form permitting combination with the more dilute antibody, beyond the zone formed by the first system. (4) The inclusion, in the concentration gradient of antigen in the region of primary antibody excess, of a concentration equivalent to the concentration of the secondary antibody. (5) The location of this concentration at a sufficient distance from the first zone to permit visual resolution of the two reactions.

It is postulated that these conditions were not fulfilled by the rabbit ovalbumin system employed by Oudin but were with the heart infusion antigens system described in the previous report.

SUMMARY

The demonstration that two zones involving the same antibody may be formed in patterns of precipitate obtained by double diffusion is interpreted as being incompatible with the belief that essentially free reactants cannot penetrate the line of visible precipitate. It is suggested that antigen, after reacting with one antibody for which it bore a specific determinant group, might still diffuse to form a gradient of concentrations beyond the line of visible precipitate from the first reaction. Thus, equivalence with a second, more dilute antibody, by virtue of a second specific determinant group, might lead to the formation of a second Oudin zone. The evidence supporting the currently accepted contention that a single antigen cannot form two zones is discussed, and comparison with the evidence now presented is invited. If the two views prove to be equally acceptable, the hypothesis that the number of zones in an Oudin tube does not exceed the number of antigens involved cannot be relied upon in demonstrating the complexity of unknown preparations.

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