dine is found in purulent pleural exudates and spinal fluids in concentrations of a half to two-thirds of those observed in the blood. In the blood of human beings a considerable fraction of the drug is frequently found in the conjugated form. Because of the irregular absorption of the drug and its tendency towards conjugation, accurate therapy with sulfapyridine is more difficult than with sulfanilamide.

10249

Note on the Mechanism of Specific Agglutination.

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One consequence of the "lattice"-theory of serological reactions* is that if 2 or more independent antigens and their respective antibodies are all mixed together, each system should aggregate independently. By using microscopically visible and distinguishable particulate antigens† (*e.g.*, bacteria and/or erythrocytes) this prediction has been recently tested.[1-5] In many cases the individual aggregates were chiefly or entirely composed of only one of the 2

* "This view differs from the hypothesis that the antibody globulin is denatured in that the aggregation of the particles of antibody combined with antigen is ascribed not only to a loss of attraction for water, but also to a *specific* attraction between the particles. *This mutual attraction is due to the link provided by further antigen molecules.*" (Italics ours.) ". . . . the formation of a continuous lattice fails progressively more and more as no further antigen molecules are available to provide links for the formation of larger structures when aggregates of larger size . . . . . are formed." (Marrack[6]).

Heidelberger and associates similarly picture the precipitate[7] (or agglutinate[8]) as a lattice-structure *in which antibody and antigen alternate.*

† Landsteiner reported similar experiments long ago (*Hand. Biochem.*, 1909, **2**, 400).

1 Topley, W. W. C., Wilson, J., and Duncan, J. T., *Brit. J. Exp. Path.*, 1935, **16**, 116.

2 Abramson, H. A., *Nature*, 1935, **135**, 995.

3 Hooker, S. B., and Boyd, W. C., *J. Immunol.*, 1937, **33**, 337.

4 Duncan, J. T., *Brit. J. Exp. Path.*, 1938, **19**, 328.

5 Wiener, A. S., in press.

6 Marrack, J. R., *The Chemistry of Antigens and Antibodies*, London, 1938.

7 Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61**, 563; **62**, 467.

8 Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, 1937, **65**, 885.

kinds of antigenic particles, but the observation has also been made, which seems difficult to account for on the basis of the lattice-theory, that in some cases the aggregates are heterogeneous, *i.e.,* contain both kinds of antigenic particles. Mixed aggregation is also indirectly indicated by the results of experiments with precipitins,[3, 4] where the rate of flocculation in mixed systems is definitely accelerated in the zones of equivalence and excessive antibody where the increased cohesive effect due to combined antibody (plus electrolyte and lipin) could be expected to exert a dominant influence.

It should be pointed out that the occurrence of homogeneous aggregates, though compatible with the lattice-theory, does not actually prove that the lattice-mechanism was in fact operative in building up the aggregates. Antibody-molecules are invisible and the postulated alternation of antibody and antigen-particles cannot actually be observed. The existing evidence would support equally any hypothesis calling for a certain amount of specificity in the second stage. Also the supposed analogy with crystal-formation is imperfect because for the lattice-structure of some crystals it is not required that *different* kinds of atoms be alternately interposed.

The essential difference between the lattice-hypothesis and the older concept concerns the mechanism by which the primary compounds of antigen and antibody combine to form aggregates (agglutinated cells or particles of precipitate). The older concept pictured the primary compound as having a nucleus (molecule or particle) of multivalent antigen more or less enveloped by molecules of antibody-globulin; such a coated particle combines with another or with an aggregate of coated particles, because of some attraction or perhaps sometimes a simple lack of repulsion between the coated surfaces. The completeness of this coating would presumably depend on the number of reactive sites (valency) of the particles; with insufficient antibody, all of these sites would not be covered. This attractive force has commonly been thought to be non-specific in the immunochemical sense.

In addition to considerations previously reviewed, this nonspecificity is also indicated by certain observations recently called to our attention by Landsteiner: precipitates (cf.[9]) and agglutinates sometimes exhibit a tendency to stick to the walls of the test-tube, and in the case of cells agglutinated by immune chicken serum, as we ourselves have seen, this tendency is sometimes very powerful, so that the agglutinated mass literally has to be scraped from the glass.

However, the well confirmed observation of homogeneous aggregates in mixed systems quite properly suggests that some sort of

[9] Hooker, S. B., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 911.

specific mechanism be considered, although the observation of heterogeneous aggregates likewise suggests that the specificity is either not of a high order or not always operative.

The newer theory pictures a particle of antigen, combined with a molecule of antibody, as now either combining through the antigenic part with another molecule of antibody, or, through the antibody-part, with another particle of antigen. Or, in either case, with a particle of antibody or antigen, respectively, already forming part of an aggregate, but having a reactive area still available. Both antibody and antigen must be assumed to be multivalent. A particle of antigen completely coated (at all available reactive areas) with antibody could only combine with a free particle of free antigen or an uncombined reactive site on a particle of antigen already in combination. Two particles of antigen, both so completely coated that no reactive groups are exposed, *should not be able to combine with each other* according to the theoretical lattice-mechanism " . . . the formation of a continuous lattice fails . . . . as no further antigen molecules are available to provide links . . . . "

This prediction of the lattice-theory can readily be tested by direct observation if we use antigenic particles visible under the microscope. We have been led by such considerations to some extremely simple experiments whose results give *no indication that lattice-formation plays any part whatsoever in the mechanism of the following typical instance of serological agglutination.*

A drop of very dilute erythrocyte-suspension was added to 3 drops of strong hemagglutinin (immune rabbit serum diluted 1:10). After centrifugation, the supernate of course still powerfully agglutinated fresh cells so it is certain that an excess of antibody was present. Nevertheless, more agglutinin was added to the original agglutinate although this made no difference in the outcome of the experiment. The tube was shaken after the addition of fresh agglutinin; the clump did not break up, but floated as one homogeneous mass in the fluid. After half an hour this clump and several others similarly prepared were placed in the same tube and centrifuged. The result was the formation of one firm clump which did not break up, even on violent shaking.

Inasmuch as at least 10,000-fold excess of serum was used there can be no doubt that every cell in these original treated aggregates, and in particular every cell on the surface, was maximally coated with antibody. According to the lattice-theory, such clumps should have not been able to combine; nevertheless they did so.

Even if the aggregates after being placed together were not centrifuged, but simply allowed to settle, they usually united, in some

cases so firmly that they could not be separated by any shaking less violent than that necessary to break up the original aggregates themselves. Thus the use of the centrifuge does not alter this experiment in any essential way, but simply accelerates the process and brings the aggregates in better contact.

It might be thought that the behavior of these relatively large aggregates would possibly be different from that of single coated antigenic particles or small aggregates. That this is not so is shown by the following experiment: a drop of very dilute suspension was mixed with a drop of strong agglutinin, and immediately placed under a large cover slip on a slide. If this preparation were not moved or shaken, microscopic observation even after half an hour revealed many small clumps of 2, 3, or a few cells, and many individual cells. This was clearly due to the poor mixing under these conditions, which prevented the encounters between coated cells and small clumps necessary for the formation of larger clumps; a similar preparation, when well mixed, agglutinated almost instantly into a few large clumps. There can hardly be any doubt, however, that the cells were one and all completely coated with antibody on all their reactive sites, for antibody was present in great excess, and the primary reaction is known to be extremely rapid. For instance, such mixtures, or others containing 1/100 as much antibody, can be centrifuged immediately after mixing, with no resultant weakening of agglutination which might indicate that sufficient antibody had not already been taken up. Also since it may be calculated (cf.[10]) that antibody-molecules at room-temperature have velocities of the order of half a meter per second, due to Brownian motion, it can be seen that in the time elapsed there must have been amply sufficient impacts between antibody-molecules and erythrocytes to coat the latter to a maximal degree, as is in any case deducible from general experience with agglutinative reactions. Also it was evident from the appearance of the clumps as the preparation stood on the slide that though they were composed of only a few, or in some cases 2, cells, nevertheless the cells were coated with an enormous excess of agglutinin, for the cells, typically, became greatly distorted, and tended to coalesce, though remaining unruptured.

When after the lapse of half an hour the contents of such a drop were thoroughly mixed, the small clumps and single cells immediately united to form 2 or 3 large clumps, and microscopic observation of the fluid showed that it was entirely clear, with no single cells or small clumps remaining. According to the lattice-theory, the

[10] Taylor, H. S., *A Treatise on Physical Chemistry*, N. Y., 1924, p. 1279.

coated single cells should not have been able to combine with anything except uncoated cell-surfaces, which were not present; but actually they did combine, obviously either with other coated single cells or with small clumps of coated cells.

These experiments clearly show that the lattice-mechanism, in the sense of aggregation through links of antigen, is not involved in agglutination, either in the later or the earlier stages under the conditions described. In fact it does not seem too much to say that if the lattice-theory were correct, then agglutination, as it is actually often observed to take place, would be impossible. There were simply no particles of antigen to serve as links, but nevertheless agglutination promptly and normally occurred. It is evident from kinetic considerations that the primary process of coating the cells would, considering the enormous disparity between the (Brownian) velocities of protein-molecules and of cells, be complete long before any large number of encounters between cells had taken place.

Similar microscopical observations cannot be made in the case of molecularly dispersed antigens. The macroscopical observation that small aggregates, even after long contact with excessive antibody, will unite, can, however, be made, and seems to us almost equally conclusive. Also, as we previously pointed out, since it is routinely observed that no excess of *antibody,* no matter how great, will prevent precipitation, if sufficient antigen to give a visible precipitate under any conditions is present, it may be concluded that the presence of molecules of antigen to serve as links is not necessary for the progress of the precipitin-reaction. For in the mixture containing the larger amounts of antibody it seems almost necessary to suppose that the primary reaction would have progressed practically to completion‡ before the secondary aggregates had reached any considerable size, and consequently, if the lattice-theory were true, flocculation would cease at this point, due to a lack of molecules of antigen to serve as links. Actually it is found that in the case of mixtures containing sufficient (2 or 3 times optimal) amounts of antibody, decrease in the amount of antigen used simply results in a proportional increase in the time that elapses before visible particles appear. In such mixtures a linear relation was found[11] between the dilution of the antigen and the time of flocculation. We concluded that the relative or perhaps absolute deficiency of molecules of antigen to act as links, which must have soon existed in the mix-

‡ This would seem to be a valid deduction from kinetic theory, allowing for the great excess of one reagent (antibody), and the greater velocities of the uncombined molecules (see above).

[11] Hooker, S. B., and Boyd, W. C., *J. Gen. Physiol.*, 1935, **19**, 373.

tures containing the more dilute antigen, did not exercise any influence on the flocculation, but instead the slowing of aggregation was adequately explained, to use Marrack's own words, by " . . . the reduction in the number of antigen molecules which serve as centers of aggregation."

Experiments similar to those outlined above can be carried out when antibody is not in great excess, but it is then harder to be quite certain that the antigenic particles are completely coated, and this is vital to the argument. We have, however, previously found that "in mixed preparations containing much less than the optimal amount of antibody . . . . there seemed to be a tendency for one or the other kind of cell to preponderate" (in the aggregates). We have also noted that when independent precipitin-systems, containing an excess of antigen, are mixed, acceleration is either less than expected or lacking. It is perhaps not inconceivable that in mixtures where antigen is in excess the lattice-mechanism, or something like it, may be partly operative, though we have as yet no convincing proof that this is the case.

The older theory may have to be altered to allow for a certain order of specificity in the stage of secondary aggregation. However, it is by no means yet proved that this specificity is at all comparable in kind or degree with the specificity of primary immunochemical combination. In any case, the older theory will be here under no disadvantage compared with the lattice-theory, which explains this specificity only by postulating a mechanism that can be shown experimentally not to be operative in the sense that particles or small aggregates of antigen, fully coated with antibody insofar as the antigenic structure of their surface permits them ever to be coated, can and do combine with each other without the intermediation of fresh particles of antigen to serve as links.

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