

VELOCITY OF COMBINATION OF ANTIBODY WITH SPECIFIC POLYSACCHARIDES OF PNEUMOCOCCUS*

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Aggregation of staphylococci by immune serum was observed by Eagle (1) to take place at room temperature in 15 seconds but much more slowly at 4°. It was shown in this laboratory by means of quantitative chemical analysis (2) that combination of Type I pneumococci with homologous antibody in horse serum, without regard to aggregation, was 80 per cent complete in a sample removed after 5 minutes at 0.5°, although the reaction period was lengthened to a possible 10 to 15 minutes by the necessity of centrifugation (also in the cold¹). Similar experimental difficulties prevented Follensby and Hooker (3) from establishing at much less than 8 minutes the initial rapid reaction between diphtheria toxin and antitoxin postulated earlier by Pappenheimer and Robinson (4). A more exact calorimetric timing of the reaction between hemocyanin and antibody in horse serum (5) showed that heat evolution was 80 per cent complete in 2 minutes after mixing.

In the meantime application had been made in this laboratory of the principle of competitive reactions which has rendered such service in the kinetic study of inorganic and organic chemical reactions. It was found (6) that addition of rabbit anti-egg albumin to a suitable mixture of egg albumin (Ea) and horse anti-Ea serum at 0° within about 20 seconds produced no greater reversal of the soluble reaction product of the immune horse serum reaction than did addition of rabbit anti-Ea after a week. Since addition of Ea to a mixture of horse and rabbit anti-Ea sera had shown that both forward reactions proceeded at similar rates, this was interpreted to mean that combination of Ea and horse anti-Ea to form soluble compounds was essentially complete within 20 seconds at 0°.

Additional studies of this type are now reported for certain homologous and cross-reactions of horse antibody and pneumococcus polysaccharides. The cross-reaction between Types III and VIII pneumococci was chosen

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¹ In an International Equipment Company refrigerated centrifuge.

because it has been studied extensively by the quantitative precipitin technique (7, 8).

Antipneumococcus Type VIII horse sera contain antibody fractions which react with Type III specific polysaccharide (S-III) as well as with the homologous Type VIII specific polysaccharide (S-VIII). Experimental conditions were so chosen that S-III and S-VIII competed for the cross-reactive antibody molecules in the resulting precipitin reaction. The outcome of this competition could then be determined by analysis of the supernatants for S-III and S-VIII.

EXPERIMENTAL

To 1.0 or 2.0 ml. of antipneumococcus Type VIII serum No. H-909 (1938),² diluted with the desired amount of saline, 1.0 ml. portions of solutions of S-III and S-VIII were added with thorough mixing at measured intervals in the order stated (Table I) at 0-10°. The same proportions of serum to S-III and S-VIII were used in every instance. Approximately one-half of the experiments was done with 1.0 ml. of serum, 0.076 mg. of S-III, and 0.150 mg. of S-VIII, while 2.0 ml. of serum, 0.152 mg. of S-III, and 0.300 mg. of S-VIII were used in the remainder in order to provide larger amounts of supernatants for analysis. Doubling the experimental quantities did not affect the outcome of the competition (Experiments 12 and 13). A unit volume of 3.5 ml. was chosen for a single quantity run and 7.0 ml. for double quantities except in several earlier experiments with double amounts at 4.5 ml. (Experiments 3, 8, 11, 18) in which volume was not a decisive factor. Two experiments (Nos. 4 and 17) were carried out at 14 ml. with double quantities in order to test the effect of dilution. Polysaccharide additions were made rapidly from calibrated 1 ml. tuberculin syringes. With the cooperation of several persons it was possible to make the minimum interval between the polysaccharide additions as short as 3 seconds. A further decrease would have involved appreciable errors in timing with the technique used. All reaction mixtures flocculated before centrifugation was started. At the end of the reaction time given in Table I the tubes were centrifuged in the cold. Aliquot portions of supernatant were analyzed for S-III by addition to an accurately measured volume of a calibrated sample of Type III antipneumococcus horse serum No. H-792² from which the antibody reactive with S-VIII had been removed (9). Only a few of the supernatants were analyzed for S-VIII (with S-III-absorbed Type VIII antipneumococcus horse serum), since the effects observed were more clearly reflected by the variation in S-III than

² These sera were obtained through the kindness of Dr. Ralph S. Muckenfuss and the late Dr. William H. Park of the New York City Department of Health Research Laboratories.

in S-VIII. The latter reacts with all of the antibody, while S-III combines only with the cross-reactive antibody fractions which are competed for by the two polysaccharides.

TABLE I
Combination of Antibody with Specific Polysaccharides of Pneumococcus

Experiment No.	Antipneumococcus VIII serum volume		S-VIII added	Total volume	Order of polysaccharide addition to antiserum	Interval between polysaccharide additions	Reaction time between addition of second polysaccharide and centrifugation	Total reaction time*	S-III in supernatant†	S-III in ppt. (by difference)‡
	ml.	mg.								
1	1.0	0.076	0.150	3.5	Simultaneous	0	5	10	0.049	0.027
2	1.0	0.076	0.150	3.5	"	0	60	65	0.060	0.016
3	2.0	0.152	0.300	4.5	"	0	1000 (Ca.)	1000 (Ca.)	0.075	0.001
4	2.0	0.152	0.300	14	"	0	5	10	0.057	0.019
5	1.0	0.076	0.150	3.5	S-VIII first	3	5	10	0.079	0.000
6	2.0	0.152	0.300	7.0	" "	7	5	10	0.073	0.003
7	1.0	0.076	0.150	3.5	" "	20	5	10	0.072	0.004
8	2.0	0.152	0.300	4.5	" "	10	1000 (Ca.)	1000 (Ca.)	0.077	0.000
9	1.0	0.076		3.5	S-III only		5	10	0.007	0.069
10	1.0	0.076		2.5	" "		45	50	0.009	0.067
11	2.0	0.152		4.5	" "		2500 (Ca.)	2500 (Ca.)	0.009	0.067
12	1.0	0.076	0.150	3.5	" first	3	5	10	0.033†	0.043
13	2.0	0.152	0.300	7.0	" "	3	5	10	0.034	0.042
14	2.0	0.152	0.300	7.0	" "	12	5	10	0.016	0.060
15	2.0	0.152	0.300	7.0	" "	60	5	11	0.011	0.065
16	1.0	0.076	0.150	3.5	" "	180	2	10	0.009	0.067
17	2.0	0.152	0.300	14	" "	3	5	10	0.047	0.029
18	2.0	0.152	0.300	4.5	" "	10	1000 (Ca.)	1000 (Ca.)	0.075	0.001
19	1.0	0.076	0.150	3.5	" "	180	1000 (")	1000 (")	0.040	0.036
20	1.0	0.076	0.150	3.5	" "	90,000 (Ca.)	1000 (")	2500 (")	0.020‡	0.056

* This includes the first 5 minutes of centrifugation, since inspection at that time showed that the precipitate had settled. Centrifugation was, however, continued for another half hour in order to clear the supernatant thoroughly for the subsequent analysis.

† Calculated to 1.0 ml. of serum.

‡ Single analysis only.

0.150 mg. of S-VIII (a slight excess), when added alone, precipitated 1.00 mg. of antibody N from 1.0 ml. of Type VIII antipneumococcus horse serum No. H-909. 31 per cent of the total antibody N was precipitable in the cross-reaction by 0.076 mg. of S-III (a slight excess).

S-III and S-VIII were prepared according to the methods previously described (10, 7). Samples for the preparation of quantitative solutions

were dried to constant weight *in vacuo* over P_2O_5 at room temperature. Quantitative precipitin analyses were carried out as described (11) and nitrogen analyses by a modification of the Pregl micro-Kjeldahl method.

DISCUSSION

It has become customary in this laboratory to consider specific immune precipitation (12) and the closely related specific agglutination of bacteria (2) as due to the combination of antigen and antibody which are multivalent with respect to each other; that is, molecules of antigen may become attached to molecules of antibody through one or more of several linkages on either type of molecule. The initially formed antigen-antibody complex may, by virtue of the plurality of reactive groupings, combine with additional antigen or antibody molecules or with already formed antigen-antibody compounds until gigantic aggregates are built up and separate from solution, or, in the case of bacteria, clump together and settle. This conception of the reaction mechanism appears to account most satisfactorily for the main body of knowledge regarding this immune reaction, and possesses the additional advantages of resting on and being in accord with modern concepts of protein and carbohydrate structure and being susceptible to expression in quantitative form in most instances in which sufficiently precise data are available. Not only has a somewhat similar view been proposed by Marrack (13), but there is an increasing tendency to view the industrially important polymers in much the same light (14-16), since these also arise by combination of polyfunctional substances. In accord with such a mechanism the speed of this series of reactions should diminish as the interacting immune aggregates grow larger (*cf.* (17)).

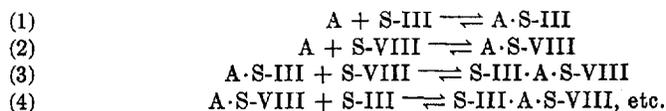
The process of immune combination has been shown to be reversible (18, 17, 4). Under suitable conditions aggregates may dissociate into uncombined antigen and antibody molecules, but the dissociation tendency of most immune reactions is relatively small. Since dissociation of an aggregate involves the breaking of many immune linkages the over-all speed of reversal should depend, at least in part, on the extent of aggregation, for the larger the immune aggregate the more linkages must be broken in its dissociation.

The data now assembled may be interpreted with the aid of these considerations and without new assumptions.

Simultaneous addition of S-III and S-VIII to anti-S-VIII and centrifugation after 5 minutes (Experiment 1) resulted in precipitation of 0.027 mg. of S-III, or 39 per cent of the total with which the antibody is capable of combining in the absence of S-VIII. The forward cross-reaction therefore takes place with a velocity roughly of the same order as that of the homologous S-VIII-anti-S-VIII reaction. When the simultaneously

added polysaccharides were permitted to react for 60 minutes before centrifugation (Experiment 2) only 0.016 mg. of S-III (23 per cent) remained in combination. After 17 hours (Experiment 3) only 0.001 mg. of S-III (1.5 per cent) was left in the precipitate. A slow liberation of S-III from combination evidently occurred. Experiments 9, 10, and 11 showed that, in the absence of S-VIII, S-III was not eliminated from the precipitate.

If one considers a single antibody molecule (A) in contact with S-III and S-VIII, it may take part in the following initial reactions which are represented as reversible, but with the equilibrium point far to the right.



Antibody molecules or reactive antibody sites set free by reversal, as from right to left, may enter into combination with either polysaccharide. However, the slow liberation of S-III observed would indicate that the course of the reaction in the case of anti-S-VIII tends toward combination with S-VIII. In terms of the reversible equilibrium reactions formulated such a shift indicates a greater dissociation tendency of the heterologous compound. This explanation had already been given (7) for the presence of both antigen and antibody in equivalence zone supernatants in this cross-reaction.

When S-VIII, the homologous antigen, was given a head start over S-III (Experiments 5 to 8), no S-III was found in the precipitate even when the interval between additions was as short as 3 seconds. If one omits, for the moment, consideration of the possibility of appreciable reversal in so short a period, the conclusion seems justified that combination of S-VIII and anti-S-VIII was essentially complete in less than 3 seconds even at the low temperature used.

It is less easy to interpret the results of Experiments 12 to 20 in which S-III was given a head start over S-VIII. When S-VIII was added to the serum only 3 seconds after S-III (Experiments 12 and 13), 0.043 and 0.042 mg. respectively of S-III were found in the precipitates compared with 0.069 mg. of S-III when no S-VIII was introduced (Experiment 9). In Experiments 14, 15, and 16 in which S-VIII was added to the serum 12 seconds, 1 minute, and 3 minutes after S-III, 0.060, 0.065, and 0.067 mg., respectively, of S-III were found in the precipitate. At first sight this would appear to indicate that approximately 1 minute was required for complete reaction of S-III with anti-S-VIII. Alternatively, since S-VIII reacted in less than 3 seconds and since it was found that S-III and S-VIII reacted with velocities of roughly the same order, one may assume that S-III also reacted completely within 3 seconds in Experiments 12 and 13 but

that liberation of S-III through reversal took place to a measurable extent during the 10 minutes after addition of S-VIII. Thus, instead of 0.069 mg. of S-III, as in Experiment 9, only 0.042 and 0.043 mg. were found in the precipitates, corresponding to a liberation during 10 minutes of about 40 per cent of the weight of S-III originally bound. In a similar calculation applied to Experiment 1 account is taken of the fact that only one-third of the antibody present is cross-reactive. Therefore of the 0.150 mg. of S-VIII added, roughly 0.050 mg. competed with 0.076 mg. of S-III in Experiment 1, since roughly 0.100 mg. of the S-VIII would be expected to react with the two-thirds of the total antibody which does not cross-react. If the molecular weights of the two polysaccharides and their initial reaction velocities are similar, two-fifths of the cross-reactive antibody should enter into combination with S-VIII and three-fifths with S-III in proportion to these quantities of polysaccharides. Then $\frac{3}{5} \times 0.069$ or 0.041 mg. of S-III should be bound. Instead 0.027 mg. of S-III was found in the precipitate, corresponding to a release through reversal of 34 per cent of the amount calculated as combined (0.041 mg.), in good agreement with the percentage of reversal found above. These admittedly crude calculations show that about one-third of the weight of S-III originally in combination may be released during 10 minutes. On this basis it is possible to estimate the effect of S-III liberation on the outcome of Experiments 5 to 8 in which S-VIII was added before S-III. Since the largest quantity of S-III found in the precipitate was 0.004 mg., no more than 0.006 mg. of S-III ($0.004 \times \frac{3}{2}$) could have reacted, or less than 9 per cent of the weight of S-III with which the cross-reactive antibody fraction can combine (0.069 mg.). Therefore at least 91 per cent of the cross-reactive antibody reacted with S-VIII in less than 3 seconds. Since the cross-reactive antibody is indistinguishable from the rest of the antibody as far as S-VIII is concerned (7, 8), this reaction velocity may be considered to apply to the homologous reaction as a whole.

In Experiments 1, 2, and 3, in which the polysaccharides were added simultaneously, it was shown that liberation of S-III could be completed in less than 17 hours. Release of all S-III was also demonstrated within the same period in Experiment 18 in which S-VIII was added to the antiserum 10 seconds after S-III. It is evident from Experiments 12 to 20 that the longer the interval before addition of S-VIII the slower is the reversal. This is as expected, for as stated before, the over-all speed of S-III liberation would depend at least in part on the state of aggregation at the time of S-VIII addition. Longer head starts permit aggregation and possibly other molecular rearrangements to proceed far enough to prevent liberation even over prolonged periods. For example, S-III-anti-S-VIII which had been allowed to aggregate for 2 days in the cold and which had then been centrifuged and washed was shaken mechanically in

the ice box for 4 weeks with a solution of S-VIII and a drop of toluene. No release of S-III was observed.

Experiments 4 and 17 were run in double volume in order to obtain further evidence on the mechanism of S-III reversal. While dilution would be expected to retard both forward reactions to about the same extent, the resulting delay in aggregation should accelerate S-III release through reversal. Less S-III was indeed found in these precipitates than in those of the corresponding experiments, Nos. 1, 12, and 13, in more concentrated solution.

The rapid initial S-III release (elimination of one-third of the bound S-III in 10 minutes) may be related to the existence in the cross-reactive antibody of a fraction which behaves toward S-III like univalent antibody (8). This fraction, which is multivalent toward the homologous S-VIII and which comprises approximately 30 to 50 per cent of the cross-reactive antibody, may release combined S-III more rapidly than the remaining polyvalent cross-reactive antibody fractions. It might be expected that retention of S-III in many of the precipitates would be accompanied by a decrease in the amount of combined S-VIII. In Experiments 8, 13, 14, and 15 the supernatants were accordingly analyzed for S-VIII as previously indicated. 0.002, 0.004, 0.005, and 0.006 mg. were found, respectively, showing that 0.148, 0.146, 0.145, and 0.144 mg. of S-VIII were present in the precipitates. It is thus seen that in the presence of only a very slight excess of S-VIII (0.150 mg. total) the quantity of S-VIII combined with the antibody is not significantly affected by the extent of combination with S-III and that the liberation of S-III is not due to exchange with additional S-VIII in solution. However, Experiments 9, 10, and 11 show that the S-III is not liberated unless S-VIII is present. An explanation of the difficulty may be sought in the demonstrated multivalence of specific polysaccharides (19, 7). This renders it possible for a molecule of S-VIII already in combination to react still further with antibody by virtue of uncombined reactive sites or groupings and thus effectively to displace the more easily dissociated cross-reacting polysaccharide, S-III. If this interpretation is correct, it would indicate that quite extensive intramolecular rearrangements may take place before specific polysaccharide-antibody precipitates attain their final gel-like form. It would certainly appear inadequate to attribute a dynamic process of this nature to a mere physical aggregation due to the presence of salt, as was the older view.

SUMMARY

Chemical combination of S-III and anti-S-VIII and of S-VIII and anti-S-VIII is at least 90 per cent complete in less than 3 seconds at 0°. Subsequent polymerization leading to the formation of insoluble aggregates

takes place with progressively diminishing velocity. In the presence of homologous polysaccharide the cross-reacting S-III may be liberated from combination with antibody. The velocity of this effect also diminishes as aggregation progresses. Complete elimination of S-III within a finite time occurs only when S-VIII is added during the earlier stages of the reaction.

BIBLIOGRAPHY

1. Eagle, H., *J. Immunol.*, **23**, 153 (1932).
2. Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, **65**, 885 (1937).
3. Follensby, E. M., and Hooker, S. B., *J. Immunol.*, **37**, 367 (1939).
4. Pappenheimer, A. M., and Robinson, E. S., *J. Immunol.*, **32**, 291 (1937).
5. Boyd, W. C., Conn, J. B., Gregg, D. C., Kistiakowsky, G. B., and Roberts, R. M., *J. Biol. Chem.*, **139**, 787 (1941).
6. Heidelberger, M., Treffers, H. P., and Mayer, M., *J. Exp. Med.*, **71**, 271 (1940).
7. Heidelberger, M., Kabat, E. A., and Shrivastava, D. L., *J. Exp. Med.*, **65**, 487 (1937).
8. Heidelberger, M., Kabat, E. A., and Mayer, M., *J. Exp. Med.*, **75**, 35 (1942).
9. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **55**, 555 (1932).
10. Heidelberger, M., Kendall, F. E., and Scherp, H. W., *J. Exp. Med.*, **64**, 559 (1936).
11. Heidelberger, M., Kendall, F. E., and Soo Hoo, C. M., *J. Exp. Med.*, **58**, 137 (1933). Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **61**, 559 (1935); **62**, 697 (1935).
12. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **59**, 519 (1934); **61**, 563 (1935).
13. Marrack, J. R., *The chemistry of antigens and antibodies*, London, 2nd edition (1938).
14. Carothers, W. H., *Chem. Rev.*, **8**, 353 (1931).
15. Kienle, R. H., *J. Soc. Chem. Ind.*, **15**, 229 T (1936).
16. Flory, P. J., *J. Am. Chem. Soc.*, **63**, 3083, 3091, 3096 (1941), and earlier papers.
17. For reviews, cf. Heidelberger, M., *Chem. Rev.*, **24**, 323 (1939); *Bact. Rev.*, **3**, 49 (1939).
18. For example, Arrhenius, S., *Immunochemistry*, New York (1907); Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **50**, 809 (1929); Healey, M., and Pinfield, S., *Brit. J. Exp. Path.*, **16**, 535 (1935).
19. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **57**, 373 (1933).