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The Molecular Composition of Specific Immune Precipitates from Rabbit Sera[1]

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The introduction into immunology of absolute, quantitative methods satisfying the criteria of analytical chemistry[2] has resulted in the accumulation of a considerable body of exact data on precipitation reactions between various antigens (including in this term the large group of immunologically specific bacterial polysaccharides) and their homologous antibodies. Such reactions are known to immunologists as "precipitin reactions." An attempt is made in the present paper to correlate certain of the data on the basis of a new chemical theory of the reaction of specific immune precipitation[3] developed with the aid of these analytical methods and accounting quantitatively for the behavior of antigens with their homologous antibodies.

In this theory a typical immune reaction such as the precipitin reaction is considered as a series of competing bimolecular reactions between multivalent antigen and multivalent antibody, the outcome of which depends upon the proportions in which the components are mixed. The equation expressing the variation in composition of the specific precipitate

$$\text{Mg. antibody N precipitated} = 2R \times (\text{antigen}) - \frac{R^2}{A} \times (\text{antigen})^2$$

was first found empirically[2c] but was later derived on kinetic considerations from the law of mass action.[3] In this equation R represents the antibody nitrogen : antigen ratio in the precipitate at a reference point in the equivalence zone (the region of the reaction range in which neither antigen nor antibody is found in the supernatant liquid from the specific precipitate), and A the amount of antibody nitrogen precipitated at this reference point. The constants of the equation, therefore, have definite chemical and immunological significance. In protein–antiprotein systems R is taken as the antibody nitrogen : antigen nitrogen ratio.

From the effect of high salt concentrations on the reaction of pneumococcus specific polysaccharides with homologous antibodies[4] it was predicted, on the basis of the above theory, that pure antibodies could be obtained by utilizing the shift in the reaction equilibria caused by strong salt solutions. Experimental verification followed,[5] and in a number of instances it was found possible to obtain water-clear antibody globulin solutions in which 95 to 100% of the protein (nitrogen) present could be accounted for as antibody (nitrogen); that is, as precipitin or agglutinin. An ultracentrifugal study of antisera and antibody solutions prepared from them in this way[6] showed that in antisera produced in rabbits antibodies to two different antigens had the same sedimentation constant as the principal component of normal globulin, or $s = 7 \times 10^{-13}$. On the other hand, antibodies produced in the horse against Type I pneumococcus specific polysaccharide showed a sedimentation constant of about 18×10^{-13}. The actual molecular weights of these antibodies are uncertain since the shape of their molecules is not known, but it is probable that the molecular weight of the rabbit antibody is not very different from that of the principal component of normal serum globulin, namely, about 150,000.[7] Since the molecular weights are known of several of the antigens for which data are available on the composition of the specific precipitate over a large part of the reaction range, it is possible to calculate the empirical composition of the specific precipitate at certain reference points or zones in this range. Such calculations are given in the table for five immune systems.

(1) The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital, New York City.

(2) (a) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **50**, 809 (1929); (b) M. Heidelberger, R. H. P. Sia and F. E. Kendall, *ibid.*, **52**, 477 (1930); (c) M. Heidelberger and F. E. Kendall, *ibid.*, **55**, 555 (1932); (d) M. Heidelberger, F. E. Kendall and C. M. Soo Hoo, *ibid.*, **58**, 137 (1933). The micro-Kjeldahl method was first used in the study of an immune reaction by H. Wu, L. H. Cheng and C. P. Li, *Proc. Soc. Exptl. Biol. Med.*, **25**, 853 (1927); **26**, 737 (1929).

(3) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **61**, 563 (1935).

(4) M. Heidelberger, F. E. Kendall and T. Teorell, *ibid.*, **63**, 819 (1936).

(5) M. Heidelberger and F. E. Kendall, *ibid.*, **64**, 161 (1936).

(6) M. Heidelberger, K. O. Pedersen and A. Tiselius, *Nature*, **138**, 165 (1936); M. Heidelberger and K. O. Pedersen, *J. Exptl. Med.*, **65**, 393 (1937).

(7) The validity of this assumption has just been confirmed by a direct determination by Dr. E. A. Kabat in the institute of Prof. The Svedberg, Upsala, Sweden.

MOLECULAR COMPOSITION OF SPECIFIC PRECIPITATES FROM RABBIT ANTISERA

Antigen	Empirical composition of specific precipitate: At extreme antibody excess	At antibody excess end of equivalence zone	At antigen excess end of equivalence zone	In inhibition zone	Composition of soluble compds. in inhibition zone
Cryst. egg albumin[a]	EaA_6	EaA_3	Ea_2A_5	$\longrightarrow EaA_2 \longrightarrow$	(EaA)
Dye egg albumin[b]	$(DEaA_5)$	$(DEaA_3)$	DEa_2A_5	$\longrightarrow DEa_4A_3$	DEa_2A ?
Cryst. serum albumin[c]	SaA_6	SaA_4	SaA_3	$\longrightarrow SaA_2 \longrightarrow$	(SaA)
Thyroglobulin[d]	TgA_{40}	TgA_{14}	TgA_{10}	$\longrightarrow TgA_2 \longrightarrow$	(TgA)
Type III pneumococcus[e]	SA	S_3A_2	S_2A	$\longrightarrow S_4A$	S_5A

A = Antibody, **S** = Minimum polysaccharide chain weight reacting. Data in parentheses are somewhat uncertain.

[a] M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **62**, 697 (1935). [b] *Idem.*, p. 467. [c] E. A. Kabat and M. Heidelberger, *ibid.*, **66**, 229 (1937). [d] H. E. Stokinger and M. Heidelberger, *ibid.*, **66**, 251 (1937). [e] M. Heidelberger and F. E. Kendall, *ibid.*, **65**, 647 (1937).

In the case of rabbit antibody and crystalline egg albumin, Ea, for example, if the molecular weights are taken as 150,000 and 42,000,[8] respectively, their ratio is as 3.6:1. This ratio applies with sufficient exactitude to the antibody nitrogen : antigen nitrogen ratios. In the region of extreme antibody excess these were found to have a mean value of 17.3 for 14 sera ($2R$ in the equation). Dividing this by 3.6 gives roughly 5, so that the composition of the precipitate found by addition of a small amount of Ea to a large excess of A would be roughly EaA_5. Actually, in the early stages of immunization the composition EaA_4 was rarely exceeded; after several courses of injections EaA_6 was encountered frequently. In this way the approximate average empirical composition of the specific precipitate was also calculated (see table) at the antibody excess end of the equivalence zone, where only traces of antibody remained in the supernatant, and at the antigen excess end of this often broad zone, where traces of antigen first appeared in the supernatant. With increasing amounts of antigen there soon follows the "inhibition zone" throughout which the precipitate grows less and less, often with changes in its composition passing through the values indicated in the table and ending in the region of complete inhibition with a soluble compound or compounds[2a,6] of relatively simple composition.

The molecular weight of the dye egg albumin, *R*-salt-azobiphenylazo-crystalline egg albumin, DEa, is taken as 4000 greater than Ea, corresponding to the number of dye groupings in the molecule. The molecular weight of serum albumin, Sa, is considered to be 67,000[8] and that of thyroglobulin, Tg, roughly 700,000.[9] The molecular weight of the specific polysaccharide of Type III pneumococcus is not known, but the minimum chain weight reactive in the immunological sense in rabbit antisera may be computed from the antibody : polysaccharide ratio in precipitates formed in the region of extreme antibody excess, that is, where as many antibody molecules as can combine are crowded about a single polysaccharide molecule. The average ratio found in this region was 85; dividing this into 150,000, the rabbit antibody molecular weight, gives 1800, or **S**, as the minimum reactive chain weight. This corresponds to about 5 of the glucuronoglucose units[10] of which the polysaccharide is composed.

The empirical formulas given in the table are not to be considered those of definite chemical compounds, but merely expressions of the actual analytically determined composition of the specific precipitate at definite points or zones in the reaction range. If, as now appears probable,[3] specific immune precipitation (or agglutination[11]) consists in the building up of enormous aggregates by the chemical combination of multivalent antigen with multivalent antibody, the composition of the precipitate would be expected to vary continuously with small changes in the proportions of the components. This has been found to be the case, with possible exceptions in the inhibition zone. Knowledge of the limiting molecular composition of the precipitate at the ends of the reaction range and in the equivalence zone is, however, of some interest.

For example, the range of composition in the antigen-rabbit antibody system is, in general, a five- to six-fold one. The formulas given do not differ markedly from those of known compounds

(8) T. Svedberg and I.-B. Eriksson-Quensel, *Tabulae Biologicae Periodicae*, 11, 351 (1935–1936), W. Junk Verlag, The Hague.

(9) M. Heidelberger and K. O. Pedersen, *J. Gen. Physiol.*, **19**, 95 (1935).

(10) M. Heidelberger and W. F. Goebel, *J. Biol. Chem.*, **70**, 613 (1926); **74**, 613 (1927).

(11) M. Heidelberger and E. A. Kabat, *J. Exptl. Med.*, **65**, 885 (1937).

and thus appear to justify the classical treatment given. Only in the region of antibody excess do the molecular ratios in the thyroglobulin system appear somewhat fantastic, and ratios corresponding to T_gA_{60} have actually been observed. However, when one considers that the dissymmetry factor of thyroglobulin, 1.5,[9] indicates that one axis of the huge molecule has perhaps only one-fifth the length of the others,[12] the opportunity is given for a very large number of groups so placed that they could be reactive, and even a minimum of 40 to 60 combining groups does not seem excessive.

It is also evident that there are 4 to 6 combining groups on the egg albumin molecule, or some multiple of these. If 4 to 6 be taken as the minimum immunological "valence" of the egg albumin molecule, this property is found to depend to some extent on the breadth of reactivity of the antibody, and this, in turn, generally varies with the length of the immunization period to which the animal furnishing the antibody is subjected. If the minimum immunological "valence," or number of combining groups, of the antibody entering into specific precipitation is 2, it is probable that this increases during the course of immunization as the antibody becomes capable of reacting with more and more groupings on the antigen molecule.[13] Many antisera also contain antibody which behaves as if it possessed only a single immunologically reactive grouping, since it does not precipitate antigen when separated from the rest of the antibody, but is capable of adding to a specific aggregate formed by multivalent[14] antibody and antigen.

Since the groupings on the antigenic protein molecule which react with antibody are not necessarily all alike, the data offer as yet little information as to the chemical nature of these groupings. It is probable that the initial combination of antigen and antibody is ionic,[2a] but whether the subsequent building up of the huge insoluble aggregate is a continuation of a similar process cannot be stated.[15] The data here presented, however, permit a few speculations on the role of tyrosine groups in Ea and Sa. It will be noted that the introduction of large bisazo groupings into the egg albumin molecule in sufficient number to couple with all of the tyrosine groups has remarkably little influence on the combining proportions with antibody. Since the tyrosine groups of a protein are probably the first to couple with diazotized aromatic amines, and the presence of a large grouping *ortho* to the phenolic group of the tyrosine would doubtless cause considerable steric hindrance, or actual change of structure to the quinone hydrazone type, it would appear that the phenolic hydroxyl of the tyrosine is not involved in the combination of the dye protein with its homologous antibody. In the Ea–DEa series introduction of the dye is accompanied by a marked change in immunological specificity so that the constancy of the combining ratios might be taken to indicate a shift from a combining group including the tyrosine hydroxyl in Ea itself to a new one in the dye portion of the molecule in DEa. In the Sa–DSa series almost no change in specificity takes place. It is therefore probable that the phenolic groups of the tyrosine in both horse serum albumin and in the corresponding dye protein are not involved in the specific combination with antibody.

Summary

Data are summarized on the molecular composition of the specific immune precipitate at certain reference points or zones in the reaction range of five antigen–antibody systems. Several generalizations supported by the data are discussed.

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(12) Unpublished calculations by Prof. The Svedberg.

(13) M. Heidelberger and F. E. Kendall, *J. Expil. Med.*, **62**, 697 (1935).

(14) I. e., having a valence of two or more.

(15) For a discussion of some of the possibilities see J. R. Marrack, "The Chemistry of Antigens and Antibodies," His Majesty's Stationery Office, London, 1934.