THE INFLUENCE OF THE MOLECULAR WEIGHT OF THE ANTIGEN ON THE PROPORTION OF ANTIBODY TO ANTIGEN IN PRECIPITATES

By WILLIAM C. BOYD and SANFORD B. HOOKER

(*From the Evans Memorial, Massachusetts Memorial Hospitals, Boston*)

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Since the study by Wu, Cheng, and Li (15) on the composition of the specific precipitate in a hemoglobin-antihemoglobin system there have appeared several papers dealing with this important problem. The antigens have included iodoalbumin (16), the pneumococcus S III hapten (5), R-salt-azo-benzidin-azo-egg albumin (6), hemoglobin (1), horse serum pseudoglobulin (10), casein-diazo-arsanilic acid (8), crystalline ovalbumin (2), horse serum-diazo-arsanilic acid, and others (11, 12). These studies have revealed that the composition of the precipitate is variable, depending upon the relative amounts of the two components in a mixture; also, that, in some of the systems, the ratio of antibody to antigen at the "optimal," "neutral," or "equivalence" point is fairly constant in a given system, even with sera of widely differing potencies. The reported ratios in different systems vary considerably, from 4 to 60, and in our current study of antihemocyanin (*Limulus*) we have observed a ratio as low as 1.6.

The present note is an attempt to account for the observed ratios on a simple assumption, *that at the equivalence point the antigen molecule is just completely covered by molecules of antibody*. In the cases to be discussed we suppose that the molecules of a given antigen are of the same size and are molecularly dispersed. Artificial compound antigens are omitted from consideration at present because of the, in general, inadequate experimental data, and because of our very limited comprehension of the influence of persisting unaltered or impaired native protein specificity, the effect of the number of artificial haptens per molecule and their distribution, whether on the surface or inside the molecule; information as to the specific volumes of

artificial compound antigens, their state of dispersion, etc., is also lacking.

Eagle (4) has collected and contributed data which make it seem likely that antibody is closely similar to serum globulin. Svedberg's (13) studies of ultracentrifugal sedimentation indicate that the molecular weight of horse serum globulin is about three times that of egg albumin, *i.e.* $3 \times 34{,}500$; he has contributed much evidence that proteins are of two sorts, one including those built up of 1, 2, 3, or 6 units of molecular weight 34,500, and another composed of proteins of high molecular weight not obeying this rule (14). Many of the protein molecules studied by him behaved as spheres, and among the others deviations from sphericity were usually not great. Serum globulin molecules were found to be homomolecular (isometric) but non-spherical; we shall assume here that they are composed of three spheres of molecular weight 34,500, linked together in a flexible manner, so that when they are affixed to the surface of a molecule of antigen, each of the three component units is in contact with the antigenic surface.

The problem then is to ascertain how many spheres, of molecular weight 34,500, can group themselves around a given antigenic or haptenic molecule.

Since Svedberg's observations show that most proteins have practically the same partial specific volumes in solution, their volumes may be considered proportional to their molecular weights

The geometrical problem concerning the maximal number of spheres, all of the same radius, that can be brought into contact with a central sphere, which may or may not have the same radius, has apparently escaped the attention of mathematicians. The following formula is believed to be a very close approximation.

$$N = 2 + \frac{90^\circ}{\tan^{-1}\sqrt{\tan\left(\frac{3\theta}{2}\right)\tan^3\left(\frac{\theta}{2}\right)}}$$

where N = the number of outer spheres, ρ = the ratio of the radius of the outer spheres to that of the inner sphere, and $\sin\theta = \frac{\rho}{1+\rho}$. Then, N times 34,500, divided by the molecular weight of the a

gives the predicted ratio by weight. If the "antigen" is not a protein, suitable correction—which will be small—for the difference in specific volume should be considered.[1]

[1] The derivation of this formula is as follows: consider three outer spheres of radius r', in mutual contact, on the surface of the inner sphere with radius r and center at O. Fig. 1 represents the section by a plane through the centers of the inner and two of the outer spheres. The points of contact A, B, and C of the outer spheres with the inner sphere determine the vertices of an equilateral spherical triangle ABC on the surface of the inner sphere. It is, of course, impossible to show point C in the two-dimensional Fig. 1. The area of this spherical triangle is not completely covered by the three outer spheres, but since this is the closest packing possible, we may say that the area of this triangle, divided by the sum of the portions cut out of the three outer spheres by planes ABO, ACO, BCO (represented in the schematic Fig. 2 by shaded areas), gives the area which

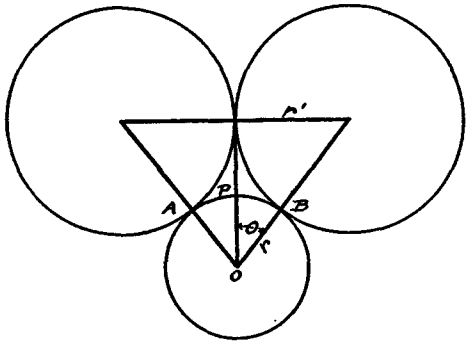

FIG. 1

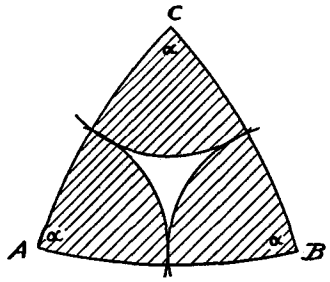

FIG. 2

one outer sphere can *effectively* cover.

This included portion will be $3 \times \alpha/360°$, where α = the angle of the triangle, which can be computed by l'Huilier's formula giving the relation between the spherical excess and the sides

$$\tan E/4 = \sqrt{\tan\left(\frac{3\theta}{2}\right)\tan^3\left(\frac{\theta}{2}\right)}$$

where E = the spherical excess of the triangle (sum of angles $-$ 180°) and θ = the angle POB, $\sin\theta = r'/(r + r') = \rho/(1 + \rho)$, where $\rho = r'/r$; so that

$$\alpha = 60° + 4/3 \tan^{-1}\sqrt{\tan\left(\frac{3\theta}{2}\right)\tan^3\left(\frac{\theta}{2}\right)}$$

(Footnote continued on following page)

When one deals with single antigens, the Dean and Webb (3) optimum, at least in some systems, indicates essential neutrality (8, 12); at this point the supernatant fluid is either devoid of demonstrable antigen and antibody, or *both* are present in small amounts, which vary presumably with the dissociation constants of the compounds. At this point, the precipitate in a crystalline ovalbumin system was found to be maximal, or nearly so (2). Precipitates formed in the region of antigen excess would not be expected to obey our rule.

The area *effectively* covered by one outer sphere ($A_{\text{eff.}}$), will be $\frac{A}{3\alpha/360^\circ}$ where A = the area of the spherical triangle = $\pi r^2 E/180^\circ$. Substituting, this becomes

$$A_{\text{eff.}} = \frac{\pi\ 8r^2 \tan^{-1} \sqrt{\tan\left(\frac{3\theta}{2}\right) \tan^3\left(\frac{\theta}{2}\right)}}{3\left[60^\circ + 4/3 \tan^{-1} \sqrt{\tan\left(\frac{3\theta}{2}\right) \tan^3\left(\frac{\theta}{2}\right)}\right]}$$

The number of outer spheres, N, will be given by $4\ \pi r^2/A_{\text{eff.}}$, where $4\ \pi r^2$ = the area of the inner sphere. Substituting for $A_{\text{eff.}}$,

$$N = 2 + \frac{90^\circ}{\tan^{-1} \sqrt{\tan\left(\frac{3\theta}{2}\right) \tan^3\left(\frac{\theta}{2}\right)}}$$

In applying this formula, we assume that the volumes of different protein molecules are proportional to their molecular weights, so that $\rho = \sqrt[3]{M'/M}$. When the antigen is not a protein the expression M'/M is multiplied by the ratio of the specific volumes. Taking the carbohydrate S III as an illustration, we have, assuming its specific volume to be the same as that of sucrose,

$$\rho = \sqrt[3]{\frac{34{,}500 \times 0.75}{4{,}000 \times 0.64}} = 2.15.$$

Therefore $\sin \theta = 2.15/3.15$, and $\theta = 43^\circ\ 2.57'$. Then

$$N = 2 + \frac{90^\circ}{\tan^{-1} \sqrt{\tan 63^\circ\ 49'\ \tan^3 21^\circ\ 16.29'}} = 2 + 4.72 = 6.72$$

and the ratio by weight of antibody to antigen would be $6.72 \times 34{,}500/4{,}000 = 58$.

The L-hemocyanin we have used was kindly furnished by Professor Redfield and is considered to be of high purity; it was repeatedly precipitated at its isoelectric point; different samples thus prepared have given nearly identical analytical figures. The equivalence

TABLE I

Ratio by Weight of Antibody to Antigen

Antigen	Molecular weight	Calculated ratio	Observed ratio	Source of data
Pneumococcus S III	4,000	58	60	(5)
Egg albumin	34,500	13.4	13	(2)
Hemoglobin	68,000	9.7	10	(15)
Pseudoglobulin	103,800	7.3	4	(10)
L-hemocyanin	2,000,000	1.47	1.57	(9)

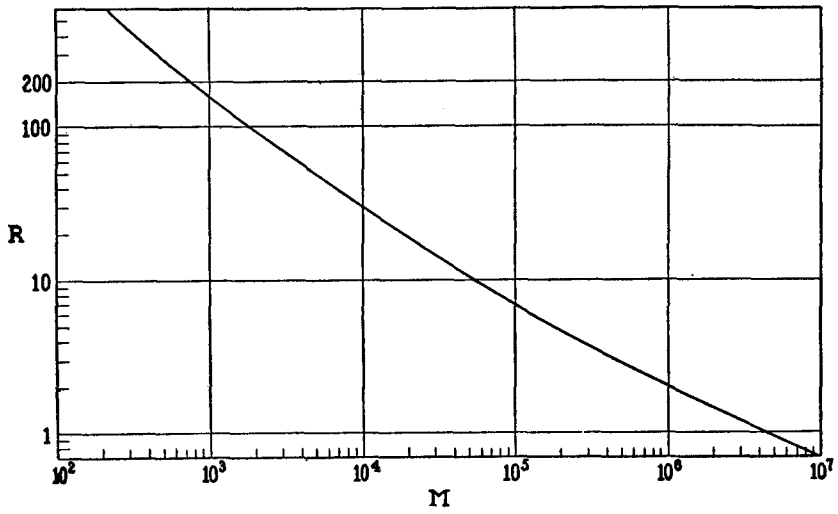

FIG. 3. Calculated relation between ratio by weight (R) and molecular weight of antigen (M).

point was determined by that optimal particulation method in which antigen is the variable, and we measured the nitrogen present in the precipitate obtained in the zone of slight antibody excess.

The table gives the comparison between calculated and observed ratios. The number of systems concerning which data are available

is small, but practically the extremes of molecular weights of antigens are represented, together with some of the important intermediates.

The graph provides a means of predicting the ratio to be expected when one is using an antigen of known molecular weight, and, if the assumption is correct, one might approximately estimate the molecular weight of any pure antigen or precipitable hapten.

DISCUSSION

Since the present accuracy obtained in determinations of antibody-antigen ratios did not seem to demand it, we have here taken no account of the fact that, in nature, N of course should always be an integer—and presumably also a multiple of 3, since globulin $= 3 \times$ 34,500; instead we have used without modification the results of the formula in which N is a continuous function of ρ. If globulin is assumed to be a single sphere there are large discrepancies between observed and calculated ratios. The latter are too high. Possibly there is some flattening of the adsorbed antibody molecules which would account for this, but we are too ignorant of the subject of molecular distortion to deal with it in this connection.

The molecular weight of 4,000 for S III is chosen as a fair mean of the values obtained by Heidelberger and Kendall by various methods (7).

The figures for hemocyanin, in spite of the close check, should not be given too great weight, as our work on that system is incomplete. Due apparently to the fact that the precipitate is more dissociable than those from other systems, comparatively large corrections had to be applied to the analytical figures. Analysis for copper in the precipitate indicated that only about 90 per cent of the hemocyanin was thrown down in this zone of slight antibody excess. The fact that L-hemocyanin was not found to be exactly spherical is probably of less importance.

The hemoglobin ratio, derived from the observations of Wu, Cheng, and Li, is assigned only tentatively. They did not exactly determine the point of equivalence but it would appear that their measurements were made in a zone closely neighboring neutrality. Some of Breinl and Haurowitz' (1) data are similarly concordant.

The greatest discrepancy occurs in the case of pseudoglobulin.

It seems possible to us that the low observed ratio could be due to failure of a part of the added protein antigen to precipitate; we had this informative experience in connection with as yet unfinished work on crystalline lactalbumin. It should be emphasized that the assumption that all of a portion of protein antigen added to an excess of antiserum enters into the precipitate is, in the absence of a chemical "marker" on the added protein, extremely perilous. One has no assurance that all of the antigenic "nitrogen" is immunologically active. Further, in view of the difficulty of separating the serum globulins and of preparing specific "univalent" sera for them, it is quite possible that the globulin preparation, although entirely active, may be but partially homologous to the antiserum employed. Indeed, in globulin systems, we have repeatedly observed two optima between which antigen was present in large excess in the supernatant fluids. We were therefore unable to establish any reliable antibody-antigen ratio.

In addition, our own calculations regarding pseudoglobulin, as antigen, are none too consistent. The ratio 7.3 is arrived at by considering the molecule to be spherical. When computations are based on the assumption that it is an aggregate of three spheres ($3 \times 34{,}500$) the ratio is 10 or 11 depending upon how the antibody spheres are supposed to orient themselves around the antigen. The situation is decidedly unsatisfactory, but demands discussion.

A further substantiatory indication of the influence of antigenic molecular weight upon the antibody-antigen ratio is afforded by the relative magnitudes of limiting titres determined by the antigen-dilution method. The notoriously active pneumococcus polysaccharides still react visibly when diluted several million fold; hemocyanin, according to our observations, had a limiting titre of about 300,000 in tests with sera of an antibody concentration (2 to 3 mg./ml.) similar to that possessed by many antipneumococcus sera. It is obvious that progressive dilution would reduce the number of molecules below the effective precipitating concentration (assuming equal dissociation constants) much sooner in the case of hemocyanin than with S III, an equivalent weight of which would contain some 500 times as many molecules.

SUMMARY

The assumption that, at the equivalence point in specific precipitin reactions, the antigen molecule is completely covered with a single layer of antibody-globulin molecules has been shown to account fairly well for the antibody-antigen ratios of some representative native single proteins, and the pneumococcus S III hapten.

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