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

II. A Statistical Examination of Available Data, Including Some Previously Unpublished

By WILLIAM C. BOYD and SANFORD B. HOOKER

(From the Boston University School of Medicine and the Evans Memorial, Massachusetts Memorial Hospitals, Boston)

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In a previous publication (6) we called attention to the fact that the data then available on the ratio of antibody to antigen in neutral precipitates indicated that the ratio was importantly influenced by the molecular weight of the antigen; further, assuming the molecule of antigen to be spherical and that in neutral mixtures its surface is just about completely coated by a layer of antibody, each molecule of which is supposed to consist of a limited number of flexibly connected spheres of molecular weight equal to the "Svedberg unit" (at that time estimated to be 34,500), then it could be calculated by spherical trigonometry that the theoretical relation between ratio by weight of antibody to antigen and the molecular weight of the antigen should be approximately[1]

$$R = \frac{34{,}500\left[2 + \dfrac{90^\circ}{\tan^{-1}\sqrt{\tan(3\theta/2)\tan^3(\theta/2)}}\right]}{M} \quad . \qquad [1]$$

where R represents the ratio by weight of antibody to antigen; M, the molecular weight of the antigen; $\sin\theta = \rho/(1+\rho)$; and $\rho = \sqrt[3]{34{,}500/M}$.

Later (34), Svedberg revised his figure of 34,500 to 35,200, and

[1] This approximate formula developed by us is amply accurate for our purposes. The general mathematical problem involved was too difficult for us to solve and apparently has never been considered by any professional mathematician.

though the difference is not large, we have substituted the latter figure for 34,500 in all our present calculations.

To simplify computation the above somewhat unwieldy expression can be replaced, with sufficient accuracy in the experimental range, by the following empirical equation containing four arbitrary constants:

$$R = 37{,}800\, M^{-0.8} + 179\, M^{-0.35} \qquad [2]$$

The few data at first available (6) have now been supplemented by a relatively large amount of published material and we have examined additional systems in order to test the applicability of our theoretical relation. Here, all the data at present available are analyzed to see if the ratio is closely connected with the molecular weight of the antigen; and if so, whether our expression has a reasonably good predictive value.

Ratios for precipitates made at the constant-antibody optimum, and for precipitates made at the equivalence point (mid-point of the equivalent zone) are included. In many cases these points appear to coincide, but they may differ considerably. The theoretical relation holds much better for the "optimal" than for the "equivalent" precipitates in the case of *Viviparus* hemocyanin. Most of the published data do not afford a comparison.

Summary of Data

The following ratios, antibody $(ab)N$/antigen $(ag)N$, are for rabbit antibody except where noted. R_{op} signifies the ratio in precipitates made at the optimum, R_{eq} the ratio in equivalent precipitates. We have calculated for each antigen the mean of all the determinations, though in some cases the difference between antisera, or the results of different experimenters, makes this of doubtful utility.

Pneumococcal Carbohydrates.—S III. M (molecular weight) assumed to be about 4000.[2] R_{eq} found (12), (horse-antibody) 69, 85, 54, 76, 99, 85; (13)[3] 69,

[2] The molecular weight of these carbohydrates has not been finally determined, and in any case evidently varies with the method of preparation. In our original paper we assumed the molecular weight of S III to be about 4,000. In a personal communication, Dr. Heidelberger informs us that he still considers that the true value is somewhere between 1,000 and 10,000 although the unheated viscous preparations must be many times larger.

[3] $R = ab/ag$ obtained by multiplying the values given (abN/ag) by 6.25.

54, 69, 76, 84, 54, 68, 66, 66, 63, 79, 63, 72, 59; (rabbit-antibody) (17)[3, 4] 44, 47, 54, 46, 52, 44, 33; (horse-antibody) 54.

S I. (horse-antibody) (17)[3, 4] 34; (rabbit-antibody) 18.

S VIII. (horse-antibody) (16)[3] 46, 56, 42.

Mean, 60.1. Predicted, 59.5.

Ovalbumins (M = 40,500 (34)).

Hen-Ovalbumin.—$R_{eq} = R_{op}$, found (35)[5] 9.8, 9.7, 9.5, 9.7, 9.5, 9.8, 9.9, 12.3, 9.4, 11.3, 9.6, 9.2, 10.2, 10.1, 8.8, 10.3, 10.4, 10.2; (1)[5] 10.9, 10.6, 14.7, 12.1, 11.3, 10.7, 13.6, 11.1, 9.5, 10.2, 10.3; (15)[4] 9.0, 9.6, 13.9, 9.8, 9.8, 11.3; (21) 10.1, 9.9, 10.1, 9.9, 10.5, 9.7, 9.8, 10.0, 9.4, 9.6, 9.7, 10.0; (25) 9.3, 11.0, 9.2, 10.6, 10.6, 10.4, 10.3, 14.2, 13.2, 13.5; (8) 12.7, 13.2, 13.5, 12.0, 12.1, 11.9, 14.1, 13.0, 15.1, 13.0, 13.5, 9.8, 11.2, 16.8, 13.1, 13.2, 11.9, 12.6, 11.3, 13.2, 14.3, 11.4, 12.8, 13.1, 13.8, 11.0, 13.3, 14.1, 13.5, 14.0, 10.4, 12.0, 13.6, 13.9, 14.2, 11.9, 14.0, 12.8, 13.6, 13.7, 15.1; (3)[6] 14.4, 10.2, 11.8, 11.5, 12.7, 11.9, 12.3, 12.1, 10.3.

Duck-Ovalbumin.—(23, 25) 10.0, 11.0, 9.7, 9.4, 10.6, 10.9. Mean, 11.5. Predicted, 12.2.

Ovalbumin-Arsanilic Acid. M = 42,500.[7] R_{op}, found (31) 3.4. Predicted, 11.8.

Iodo-Ovalbumin.—M = 43,200.[8] R_{op}[9], found (31) 4.6. Predicted, 11.7; M = 43,400.[10] R_{op},[9] found (38) 7.5, 7.5, 7.9, 8.2, 10.0, 9.3, 10.9. Mean, 8.7. Predicted, 11.6.

Ovalbumin-Dye.—M = 44,600.[11] R_{eq}, found (14) > 11.6, < 10.2, > 11.5, > 9.2, 8.3, 8.6, 8.6, 9.0. Mean, 9.6. Predicted, 11.5.

Hemoglobin.—M = 69,000 (34). $R = R_{eq}$?[12, 13] found (37) 9.2, 8.7, 8.6, 10.1, 9.1; (7)[12, 13] 9.3, 7.4; R_{op}, (25) 8.2, 8.2, 9.2, 9.5. Mean, 8.87. Predicted, 8.73.

Serum-Albumin.—M = 70,200 (34). R_{op}, found (35) 7.5, 6.3; (26) 6.3, 8.6, 7.1, 7.8, 7.4, 8.3. Mean, 7.42. Predicted, 8.63.

Serum-Albumin-Dye.—M = 78,400.[14] R_{eq}, found (26) 10.0. Predicted, 8.06.

Horse-Serum-Globulin.—M = 167,000 (34). R_{op}, found (30) 4.5, 4.0, 4.6,

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[4] Obtained by averaging the values at the two ends of the equivalence zone.

[5] $R = \frac{ab}{ag} = \frac{ppt}{ag} - 1.$

[6] Omitting results with sera from young animals, which according to Baumgartner give higher ratios.

[7] Calculated from 1.53 per cent As.

[8] Calculated from 6.2 per cent I.

[9] R = 100/(per cent ag) − 1.

[10] Calculated from 6.9 per cent I.

[11] Calculated from eight introduced dye residues.

[12] Using the values near the middle of the range given.

[13] R = 100/(per cent ag) − 1.

[14] Calculated from sixteen introduced dye residues.

4.4, 3.8, 3.9, 3.0, 3.4, 3.6, 3.9, 3.0, 3.0, 3.2, 3.2, 3.9, 3.8. Mean, 3.70. Predicted, 5.83.

Horse-Serum-Globulin-Arsanilic-Acid [*A*].—M = 172,500.[15] R_{op},[14] found (31) 1.53, 1.73, 7.6. Mean, 3.62. Predicted, 5.09; [B]. M = 180,500.[16] R_{op}, found (31) 8.5, 5.5, 5.4. Mean, 6.47. Predicted, 4.97.

Iodo-Horse-Serum-Globulin.—M = 182,000.[17] R_{op}, found (31), 5.5, 6.7, 8.0, 8.6, 5.4, 6.0. Mean, 6.69. Predicted, 4.93.

Edestin.—M = 309,000 (34). R_{op}, found (25) 1.4, 1.3, 1.2, 1.2, 1.3, 1.3, 1.8, 1.9, 2.1, 1.6. Mean, 1.51. Predicted, 3.69.

Thyroglobulin.—M = 650,000 (19). R_{eq}, found (32) 2.2, 2.0, 1.8, 2.5, 2.1, 3.5, 2.4, 2.7, 2.1, 3.6, 3.0, 3.0, 3.3, 2.4, 4.0, 2.2. Mean, 2.68. Predicted, 2.50.

Homarus americanus Hemocyanin.—M = 725,000 (34). R_{op}, found (25) 1.1, 1.0, 1.0, 0.9, 1.1, 1.0; (29) 1.2, 1.3, 0.8, 0.5, 0.3, 3.3, 2.5, 3.8, 3.3. Mean, 1.54. Predicted, 2.34.

Cancer irroratus Hemocyanin.—M = 725,000.[18] R_{op}, found (25) 1.8, 1.0, 2.0, 2.0, 2.6, 2.6, 1.6, 1.6, 2.3, 2.4; *species?* 1.2, 1.3, 1.7, 1.6. Mean, 1.84. Predicted, 2.34.

Limulus polyphemus Hemocyanin.—M = *ca.* 3,000,000.[19] R_{op}, found (22) 1.4, 1.3, 0.9, 1.4, 1.3, 1.2; (25) 1.6; (28) 2.4, 2.3. Mean, 1.53. Predicted, 1.22.

Viviparus malleatus Hemocyanin.—M = 6,630,000.[20] R_{op}, found (28) 2.7, 2.5, 4.6, 4.0, 3.5, 2.5, 2.1, 1.6, 2.7, 2.4, 3.8, 3.2. Mean, 2.97. Predicted, 0.88.

Busycon canaliculatum Hemocyanin.—M = 6,760,000 (34). R_{op}, found (22) 0.7, 0.8, 0.6, 0.6, 0.6, 0.7, 0.8, 0.7. Mean, 0.68. Predicted, 0.86.

RESULTS

The above figures show that there is a strong tendency for R to be lower with the antigens of higher molecular weight. The agreement between the values of R predicted by our theory and those determined experimentally is in general close, considering the wide scatter of some of the experimental values, though there are a few conspicuous exceptions. These relations are brought out in Fig. 1, where the values of log R are plotted against the logarithm of the molecular weight of

[15] Calculated from 1.1 per cent *As*.

[16] Calculated from 2.54 per cent *As*.

[17] Calculated from 8.4 per cent *I*.

[18] Assuming it to be the same as *Homarus*.

[19] Svedberg states (33) that *Limulus* blood contains three hemocyanins with sedimentation constants of 57.1, 34.1, and 16.9 $\times$ 10^{-13}. The first would have a molecular weight of about 3,000,000. There is some evidence (24) that this was the component chiefly affected by the sera examined.

[20] Assuming it to be the same as *Helix* (34).

the antigen. The lower curve, which goes approximately through the middle of each large group of points, represents the values of R calculated from the equation.

It has been stated by Eagle (9) that "... there is as yet no experimental evidence that proteins are constructed of unit spheres...

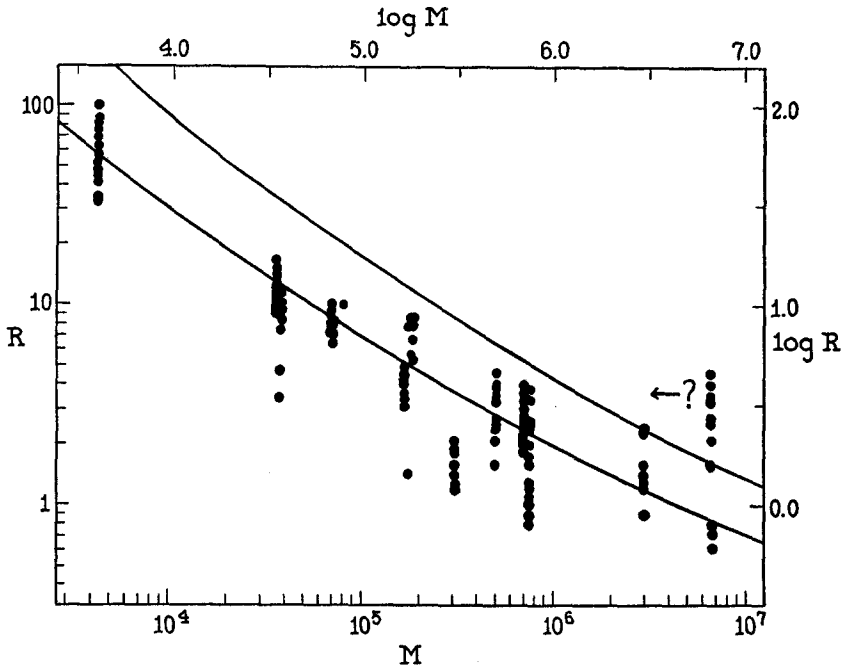

FIG. 1. Relation of molecular weight of the antigen to the ratio of antibody to antigen in precipitates.

Experimental values, solid circles.

Lower curve, relation calculated assuming Svedberg unit structure of antibody.

Upper curve, relation calculated assuming spherical antibody (9).

The queried data at right are also plotted on abscissa 5×10^5, for reasons discussed in the text.

the experimental data... are likewise compatible with the assumption that the antibody globulin is deposited as a single spherical molecule." The upper curve in the graph gives the values of R calculated according to this suggestion, using the value 150,000 (20) for the molecular weight of (rabbit) antibody. It is evident that Eagle's assumption

will give results definitely too high, unless some additional assumption is made. With the larger molecules of horse-antibody the results would be still more out of agreement.

In order to test the extent to which the experimental values of R depend upon the molecular weight of the antigen, we have calculated the regression coefficient (10), of R on M, or rather, to make the numbers involved more manageable, of $\log R$ on $\log M$. We obtained a regression coefficient of -0.529, with a standard error of 0.014. The coefficient is over 35 times its standard error, so there would seem to be no question that there is a highly significant degree of correlation between R and M.

To express this relation, in so far as it is linear, a straight line can be fitted to the data by the method of least squares. This gives

$$\log R = 3.49 - 0.529 \log M$$

The sum of the squares of the deviations of the observed values of $\log R$ from those predicted from this equation is 11.51. From our (curvilinear) relation we obtain 10.29. Thus it appears that our theoretical equation expresses the trend of the data, not merely as well, but actually slightly better, than the best fitting straight line possible. Perhaps an empirical curved line, involving several terms, might fit better than either, but since such a curve would have no theoretical significance, we have not troubled to attempt its construction. (The empirical equation [2] connecting R and M was constructed to fit our theoretical relation, not a plot of the experimental data.)

DISCUSSION

That the ratio of antibody to antigen in "neutral" precipitates is strongly influenced by the molecular weight of the antigen, seems to be established by the facts presented here. It is our purpose to call attention to the fact of this relation, and the degree of it as measured by the regression coefficient. The individual determinations, however, may vary considerably, because of experimental errors and because other factors besides the size of the antigenic molecule doubtlessly influence the ratio. Among the possible factors suggested are the relative number and spacing of reactive groups in the antigen (and antibody (15)) molecules, the quality (avidity) of antibody

from a particular animal or bleeding (28), flattening of antibody, and molecular dissociation or "depolymerization" of antigen. These are now to be discussed in connection with some advantages seemingly offered by our hypothetical model of antibody-antigen reactions.

Despite the predictive applicability of the equation to the relation between R and M, it is possibly fortuitous and we do not regard it as proving the literal accuracy of our model. Indeed, the agreement in the case of the pneumococcal anticarbohydrates is almost certainly accidental because the evidence now indicates that those carbohydrates are chain-shaped rather than spherical. Here the fit could be ascribed to an accidentally appropriate spacing of determinant groups on the antigen or perhaps to the effect of steric hindrance to the attachment of antibody, an interference that could be more marked in the case of slender molecules than spherical ones.

The larger molecules of protein may deviate from sphericity, some considerably, but this makes surprisingly little difference in the surface-volume relationships. Volumes being equal, the ratio of the surface of a prolate spheroid to that of a sphere is 1.08 when the major is twice the minor axis, and even when it is five times as long the ratio is only 1.37; with oblate spheroids the ratios are slightly higher. The appropriateness of the model depends upon two basic facts, one serological, one geometrical: (*a*) immunochemical combination takes place at the surface of the antigenic particle; this appears to be well established; (*b*) deviation from sphericity of antigenic molecules has little effect upon the amount of surface to be covered, but nonsphericity of antibody molecules enables them to cover considerably more surface. Ultracentrifugal study of antibodies (27) indicates that one axis of an antibody molecule is about a fifth as long as the others (11, ref. 12). It has been pointed out that this is a molecule having about the shape called for by our hypothesis, which postulates a chain composed of a limited number (say 4 for rabbit antibody) of contiguous, practically spherical Svedberg units. In addition to the contributions from Svedberg's laboratory relating to the physical structure of proteic molecules and their dissociation into components having an orderly range of dimensions, Bergmann and Niemann (4) offer chemical evidence that affords new reasons for accepting the fundamental reality of a unit of protein structure about the size of the

Svedberg unit, although an exactly uniform size in all proteins should not be expected. Wrinch (36) has proposed a hypothetical structure of proteins, a laminar series of cyclols, capable of leading to a unit of similar size. The idea that the unitary structure must be of limited size, in order to resist disintegration by vibrational forces, was offered by Astbury and Woods (2).

But other models can also fit the observations satisfactorily. One could assume that the antigenic molecules are coated in neutral mixtures with a layer[21] of antibody of the same thickness in all cases, saying nothing about the arrangement of the molecules of antibody; the results would be practically identical. Although it is now hardly permissible to regard the whole molecule of antibody as a sphere, if it is so regarded then the theoretical values of R become from 1.9 to 3.3 times too high (Fig. 1) if the antigen is completely coated. However, if the (spherical) antibody molecules are considered to be distorted somewhat by combination with the antigen, becoming flattened so as to cover more surface, then the assumption becomes workable in this part of the range. Distortion sufficient to make the major axis of the molecule twice the minor axis would be required to cover the surface completely.

Eagle has assumed that the antibody molecules are not distorted and that the surface of the antigen is not completely covered. He stated (9) "... it is improbable that the chance collisions between antibody and antigen which result in combination would make for the maximum possible coverage of the antigen particle. Instead, since each antibody molecule would be bound more or less where it struck the antigen, one would expect some free space between adjacent antibody molecules, less than the diameter of each, yet constituting

[21] It must be noted that this thickness would have to be considerably greater than in the protein films measured by Gorter and by Langmuir where a value of 0.8–1.0 mμ was found. If we assume the molecule of ovalbumin, which has a radius of about 2.17 mμ (30 *c*), to be coated uniformly with a layer of protein 1 mμ thick, the ratio of antibody to antigen would be calculated as $(3.17^3 - 2.17^3)/2.17^3 = 2.13$, which agrees poorly with the experimental values which average 11.5. A uniform layer of protein about 3 mμ thick on the surface of the antigenic molecule would yield figures comparable to those based on our model. Perhaps if we were dealing with minimal rather than equivalent quantities of antibody the indicated thickness of the layer would be closer to 1 mμ.

a significant proportion of the total surface." But, on this assumption, it is not possible to account for the fact that the antigen is capable of combining firmly with 2 to 3 times as much antibody as that found at the optimum or at the equivalence point. We, on the other hand, could attempt to explain the difference by assuming that in neutral mixtures the antibody chains apply themselves closely to the antigenic surface, whereas with excessive antibody, they "stand on their heads" that is, are attached by perhaps only one of their component units, leaving the rest projecting into space to contribute to the total amount of combined antibody but not to the effective coverage of the antigen (5). Such a picture is also consistent with the known higher dissociability of antibody from precipitates made with excessive antibody.

Either the assumption of distortion or of a steric limitation respecting the portion of the antigenic surface that can be covered, virtually is mathematically equivalent to introducing an arbitrary constant into equation [1] (and using 150,000 instead of 35,200). It hardly seems plausible that the forces required for a uniform degree of flattening of the antibody molecules should be so constantly and evenly distributed on the surfaces of a large variety of antigenic proteins (or carbohydrates or lipoids). It will be noted that there is no arbitrary constant in equation [1], a fact which should lend greater significance to its agreement with the experimental data.

Some objections to the lattice hypothesis have previously been discussed (24).

The agreement obtained with synthetic antigens and with edestin and *Viviparus* hemocyanin is rather poor. We have included the results with chemically modified proteins (14, 26, 31), though it might well be argued that there is a distinct possibility that the molecular size might have been altered by the rather violent processing. Also we do not know whether enough of the (artificial) determinants have been affixed to its *surface* to enable the antigenic molecule to be completely coated. This is an obvious requirement for the satisfactory working of the hypothesis and certainly influences the ratio as importantly as the molecular weight (surface) of the antigen. We found that casein-arsanilic acid must contain a minimum of about 1 per cent of arsenic in order to be precipitable with anti-ovalbumin-arsanilic acid (22 *b*) and Marrack has reported ". . . that with a given antiserum

to *p*-amino-benzene-arsinic acid, the amount of antigen equivalent to a given volume of antiserum at optimal proportions was inversely proportional to the arsenic content of the antigen" (30 *b*).

The possible influence that the quality of the antibody may have upon the ratio is not so clear. It could be expected that highly avid antibody would be less dissociable and so give a relatively high ratio. The strength of a combining group presumably depends upon the completeness and faithfulness with which it reflects the detailed pattern of the antigenic determinant. It would seem that the (assumed) multivalence of antibody could influence (increase) the ratio only when the combining groups have a relatively weak affinity for their counterparts so that several points of union are needed to prevent dissociation. Indeed if most of the antibody in a given serum were thus multivalent then the ratio might well be *lower* than that yielded by strong univalent antibody because the latter could form a thicker layer due to its more "polar" orientation to the antigen. Upon continued immunization the later bleedings do tend to give higher ratios—the zone of equivalence is widened (15)—but this is not necessarily due to increasing multivalence of antibody; it could equally well result from the increased formation of univalent antibodies directed toward minor determinants to which the animal responds only after prolonged stimulation. A similar explanation could be considered as an alternative to the assumption that only a single immunologically reactive group is possessed by the kind of antibody that "does not precipitate antigen when separated from the rest of the antibody, but is capable of adding to a specific aggregate formed by multivalent antibody and antigen" (11). It may be that such a univalent antibody is capable of uniting with adequate firmness but is directed toward a kind of determinant so sparsely represented on the antigen that the latter cannot be coated sufficiently to form a cohesive aggregate—or, the compound remains soluble.

There are plausible reasons for the discrepancies in the case of edestin and *Viviparus* hemocyanin. Because of the slight solubility of edestin in low concentrations of electrolyte our precipitates were made in 5 per cent NaCl. But considerably less (pneumococcal) antibody appears in precipitates made in such strong salt solutions (18), and it might well be that this is the explanation of the low ratio

we found. *Viviparus* hemocyanin might fairly have been excluded because we do not know its molecular weight, but have plausibly, we think, assumed it to be the same as that of *Helix* hemocyanin (34). But Svedberg has found that dissociation into smaller fragments is especially easy in the case of snail hemocyanins, occurring in the case of *Helix* at pH 8 or less. Some of our antisera, after storage in glass, have been found to be as alkaline as this. If such dissociation had taken place here, it might well have led to a higher ratio than we expected. In Fig. 1 the ratios for *Viviparus* are plotted, to show this, against $M = 503{,}000$ (a dissociation component of *Helix*) as well as against the assumed 6,630,000. The agreement for the dissociation component is seen to be good. In spite of these discrepancies, the general agreement remains striking.

SUMMARY

A statistical examination of the available data on the ratio of antibody to antigen in specific precipitates made at or near the optimum shows a definite correlation between the ratio and the molecular weight of antigen (regression coefficient $= -0.529$ (± 0.014)). The authors' assumption that at this point the antigen molecules are just about covered by a layer of antibody behaving as contiguous spherical ("Svedberg") units of weight 35,200 leads to predicted ratios that in general agree well with those found, though individual experimental determinations may deviate considerably.

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