Electron Microscopic Observations of Rabbit Antibodies

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ABSTRACT

Electron micrographs were obtained showing the individual, shadow-cast macromolecules from solutions of purified anti-*p*-azobenzoate rabbit antibody and of normal γ-globulin. The two materials look alike and consist mainly of asymmetrical rod-like particles about 30 to 40 A in diameter. Lengths are not constant but the weight average is about 250 A for the antibodies and about 200 A for the γ-globulin. The average observed dimensions are reasonably consistent with values deduced from physical-chemical methods, although the shape is more nearly that of a cylindrical rod rather than the ellipsoid employed in hydrodynamical theory. Mixtures of antibody and specific dihaptenic dye were examined in attempts to establish the mode of the specific aggregation. At the high dilutions necessary for electron microscopy (0.1 mg./ml.), the effect of the dye was small and tended to be masked by non-specific aggregation on drying. The evidence suggests that under these conditions the specific reaction involves an end-to-end aggregation of the elementary particles to produce a weight average length about twice that of the pure antibody.

INTRODUCTION

Recent improvements in the techniques of electron microscopy have made possible the direct observation of many of the biological macromolecules whose dimensions could be deduced previously only by indirect methods (1, 2). The deduced dimensions of antibodies and γ-globulin are such that we should be able to observe them with the electron microscope and perhaps obtain evidence regarding their mode of interaction. We report here some observations of these materials and discuss some of the difficulties arising in attempts to apply electron microscopy to these systems.

There is a large amount of evidence indicating that the γ-globulin fraction of the serum of various species is heterogeneous (3–7). Also, antibody of a given specificity is known to be heterogeneous both in immunochemical (8–12) and physical chemical properties (6, 8, 13–16). However, the nature of the heterogeneity of antibodies with respect to molecular dimensions is not well understood. Physical-chemical techniques are best suited for determination of average values of molecular sizes, and when such measurements do indicate heterogeneity the distribution of sizes must ordinarily be given as a rough approximation. It is especially difficult to estimate how the observed heterogeneity applies to each of the dimensions of the molecule. By the use of electron microscopy we have been able to measure the lengths and diameters of individual molecules of a specifically purified rabbit antibody. Also an attempt was made to observe the mode of aggregation of antibody in the presence of a small molecule capable of precipitating the antibody specifically.

Materials and Methods

Specifically Purified Anti-p-Azobenzoate Antibody.— Rabbits were immunized by repeated intravenous inoculation of an antigen made by coupling 30 mg. of diazotized *p*-aminobenzoic acid to 1.0 gm. bovine γ-globulin (Pentex Co., Kankakee, Illinois, fraction II). The coupling reaction was carried out at 0°C. and pH 9–9.5. Specifically purified antibody was made from pools of antiserum taken from several rabbits by a modification (17) of a method described earlier (18). Approximately 85 per cent of the protein in each puri-

fied preparation was precipitable by a test antigen prepared by coupling 60 mg. of diazotized *p*-aminobenzoic acid to 1.0 gm. ovalbumin. The material appeared homogeneous in the Tiselius electrophoresis apparatus. It was also examined in the ultracentrifuge at room temperature where nearly all of the protein migrated with a velocity of 7.0 S with the exception of a trace of material (less than 2 per cent of the total) having sedimentation constant of approximately 21 S.

Other Materials.—A γ-globulin fraction of normal rabbit serum was prepared by the method of Kekwick (19). When examined in the ultracentrifuge only one peak was observed, which had a sedimentation constant 6.9 S.

The dye used for specific precipitation of anti-*p*-azobenzoate antibody was the generous gift of Dr. David Pressman. It was prepared by coupling two *p*-phenylazobenzoate groups to chromotropic acid through a *p*-azo linkage. This compound will be referred to as the dihaptenic dye.

Electron Microscopy.—The preparative procedures for electron microscopy were essentially those previously described (1, 2). The polystyrene latex usually added to such preparations, however, was omitted since its addition produced an observable precipitate with antibody and γ-globulin. For a typical preparation, anti-*p*-azobenzoate antibodies or γ-globulin in a concentration of 0.10 mg./ml., suspended in 0.1 M ammonium acetate at pH 6.8 was sprayed on the surface of mica from a high pressure spray gun driven by repurified N_2 at a pressure of about 20 P.S.I. The surface was shadow-cast with 4 mg. of Pt, at a shadow-to-height ratio of 10:1, and a distance of 6 cm. This was backed normally with evaporated SiO (0.5 mg. at 10 cm.), later backed with collodion, and stripped on water. Micrographs were recorded at about 15,000 × with an RCA EMU3B at 100 kv. Other suspensions, containing various absolute concentrations and ratios of antibody to dihaptenic dye, were also examined by the above methods. Measurements were made from prints at a magnification of about 100,000.

RESULTS

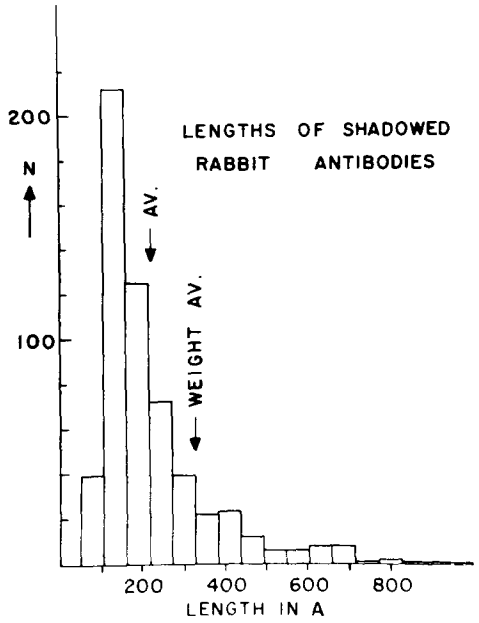
Antibodies.—A typical micrograph showing macromolecules of rabbit anti-*p*-azobenzoate antibodies is reproduced in Fig. 1. The population consists mainly of asymmetrical, somewhat nodose particles of heterogeneous length and approximately uniform width of about 30 to 40 A as evidenced by nearly constant shadow lengths. Unfortunately, the sprayed drops spread so widely on the mica surface that entire drops cannot be included in a field for good statistical evaluation of a fair sample. The best that can be done is to select for measurement a number of fields at random, where the population is sufficiently sparse that the chance of aggregation during drying is small. Considerable variability in results was obtained between such fields as demonstrated in Table I in which measurements from 5 different fields are tabulated. Obviously the sprayed and dried material is heterogeneous as to length and there are significant differences between parts of the drying pattern. Adding results from the 5 fields together (weighted according to numbers in the fields) we obtain the number and weight averages in the last line of Table I together with probable uncertainties in these figures as estimated from mean deviations among fields. A plot of the measurements represented in Table I is shown graphically in Text-fig. 1.

TABLE I

Lengths of Rabbit Antibodies

Field	No.	No. average length	Weight average length
1	99	278	397
2	70	279	363
3	105	193	246
4	196	203	268
5	112	224	358
All	582	230 ± 40	310 ± 60

We have also measured the molecular weight of these antibodies by the Archibald semiequilibrium method (20) and obtained a value of 159,000 as compared with the published values of about 160,000 (8). The heterogeneity shown in Text-fig. 1 is therefore somewhat disturbing although the centrifuge does show a very small fast peak which could represent material in the largest particles above about 500 A in Text-fig. 1. If we deduct 60 A from the weight average length obtained from electron microscopy for the cap of metal, the resultant length of 250 A is close to the length of the equivalent ellipsoid obtained from physical-chemical data (4). A direct comparison of lengths is not however possible since the particles are more nearly representable as cylinders than as ellipsoids. If, on the other hand, we assume a molecular weight of 160,000 and a length of 250 A we calculate the diameter of the cylinder to be about 30 A which is in reasonable agreement with measurements from electron micrographs. The average dimensions from electron microscopy are therefore close to dimensions deducible from



TEXT-FIG. 1. Histogram showing the distribution of lengths of rabbit anti-*p*-azobenzoate antibodies.

physical-chemical data although one might still question to what degree the size distributions seen in electron micrographs may be taken as representative of the populations in solution. The weight average length of 250 A is probably on the high side since some of the longer particles shown in Text-fig. 1 could be produced by a slight tendency for end-to-end aggregation during drying.

γ-Globulin.—As shown in Fig. 2 the size and general appearance of the normal γ-globulin fraction is very similar to that of the antibodies. From the semiequilibrium centrifugation method the molecular weight of the γ-globulin was 153,000. Measurements from fields including 233 particles gave a weight average length of 240 A (uncorrected for shadowing metal). This is a little smaller than the weight average length for the antibodies which have a slightly higher proportion of particles with lengths in excess of 500 A, although otherwise the distribution of lengths is very close to that in Text-fig. 1.

Antigen-Antibody Mixtures.—A series of experiments was carried out in which specimens for electron microscopy were prepared from suspensions of mixtures of antibody and the dihaptenic dye, in an attempt to observe modes of aggregation which might be associated with the specificity of the antigen-antibody interaction. Qualitatively, one observes: (*a*) At high concentrations (about 0.5 mg./ml.) or in crowded fields, large irregularly shaped aggregates exhibiting no significant internal regularity. (*b*) Smaller elongated aggregates that can be seen to consist of end-to-end as well as side-by-side aggregation of the elementary units. (*c*) Linear elements formed by end-to-end aggregations with negligible evidence of lateral association. This last type of morphology is sufficiently suggestive of a specificity at the ends of the asymmetrical antibodies that a number of experiments were carried out in an effort to observe the effect of precipitating dye on particle lengths.

In order to reduce the probability of non-specific aggregation during drying, mixtures were sprayed at dilutions of the order of 0.1 to 0.2 mg. of protein per ml. and fields were chosen where the population of particles was quite sparse as for the antibodies alone. Under these conditions, a qualitative examination of a great many fields, representing a variety of ratios of hapten to antibody, indicated a notable absence of the very long strands that one usually observes with systems that polymerize by end-to-end attachment, such as, for example, in the polymerization of fibrinogen (2). These experiments yielded weight averages varying between that of antibodies alone and approximately double the length. In these dilute preparations very little lateral aggregation was observed.

These results seem to indicate a tendency for end-to-end aggregation promoted by the hapten, but the observed lengths of the dried particles and aggregates are too variable for the relatively small differences in average lengths to be convincing evidence that the specific reaction is entirely responsible for the effect. It appears that the hapten-antibody attachment is weak and may easily be masked by non-specific attachments that may occur in the absence of haptenic dye.

DISCUSSION

In this work we have been able to demonstrate the individual antibody particles rather than infer their presence, as had been done in previous electron microscope studies. The observed heterogeneity of length might raise the question as to whether we are dealing with breakdown products of an initially homogeneous population. However, from the weight, average length, and estimated diameter, a molecular weight reasonably consistent

with physical-chemical data is obtained. (The population in fields measured was sufficiently sparse that we rule out aggregation as not significant.) We therefore believe that the heterogeneity is real and that the figures for dimensions obtainable from physical-chemical methods represent average values.

It is of interest to note the dimensions of the ellipsoidal models deduced previously from hydrodynamic data although by so doing we do not wish to imply that a direct correspondence between electron microscopy and results from hydrodynamic data is to be expected. Besides complications due to the fact that the particles are more nearly representable as cylindrical rods than as ellipsoids of revolution, effects of hydration could make such comparisons pointless or even erroneous. On the assumption that the molecules are prolate ellipsoids with 0.2 gm. water of hydration per gm. of protein, Boyd (8) calculates dimensions of 251 × 43 A for unhydrated rabbit antipneumococcus and 330 × 38 A for the globulin fraction of rabbit anti-ovalbumin serum. These values are apparently based on the original data of Kabat (21). The two sets of dimensions correspond to frictional ratios of 1.4 and 1.6 respectively. The differences may not be significant since, for the purified anti-pneumococcus antibody, Kabat obtained frictional ratios of 1.4 and 1.6 depending on the method used for dissociating the antibody from the precipitate. There seems to be no reason to regard the dimensions obtained from electron microscopy as being in any way inconsistent with these hydrodynamic figures.

Because the specific antibody-dihaptenic dye reaction is apparently weak, we have not been able to demonstrate the kind of attachment involved, unequivocally. Such evidence as we have, however, favors an initial end-to-end mode of aggregation. In order to establish that the structure of aggregates results from the specific reaction alone, one would have to find a system in which specific aggregates are stable at high dilutions and very much larger on the average than linear aggregates that can form non-specifically.

It is interesting to note in comparison with our observations the thermodynamic study of antibody-dihaptenic dye association made by Epstein, Doty, and Boyd (14). In concentrations of protein between 0.3 and 0.9 mg./ml., which are comparable to those we used, they found that the molecular weight (measured by light scattering) increased in most of their experiments by a factor of only about 1.5, and rarely exceeded 2. They found also that the heat liberated by the formation of the complex was negligibly small, of the order of thermal energy (kT.). In view of these results the electron microscope observations are perhaps not surprising. It appears that the association is weak at these low concentrations, and the effect is therefore difficult to separate, unequivocally, from the other random associations that can occur in drying patterns.

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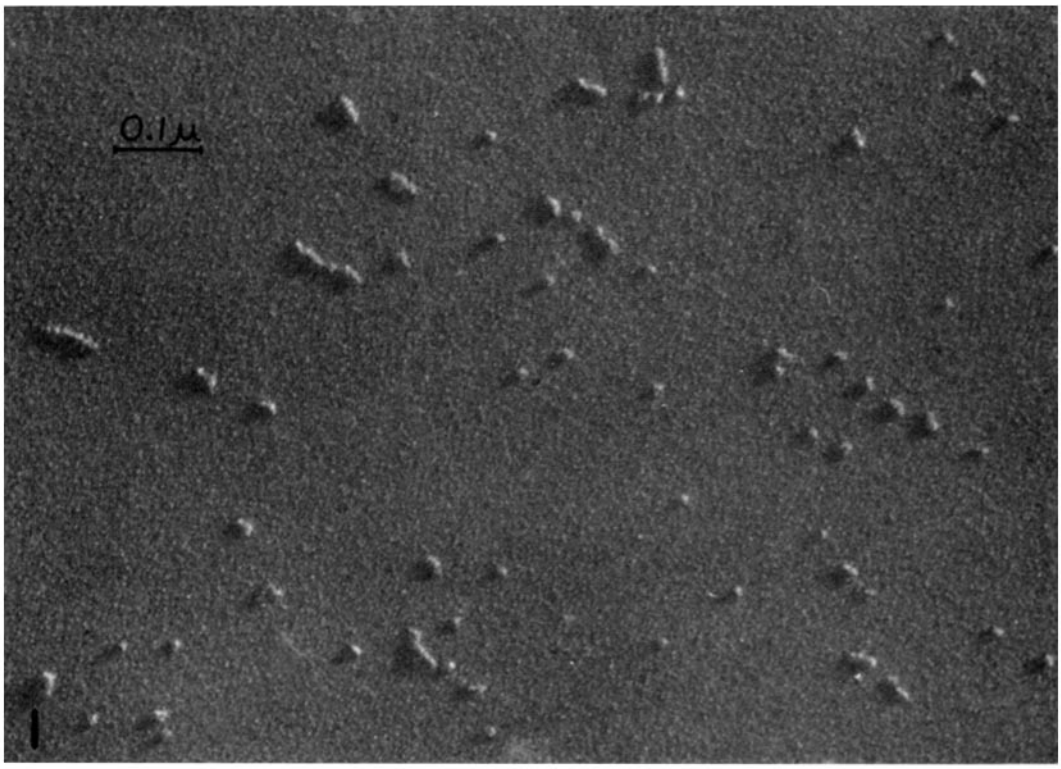
References

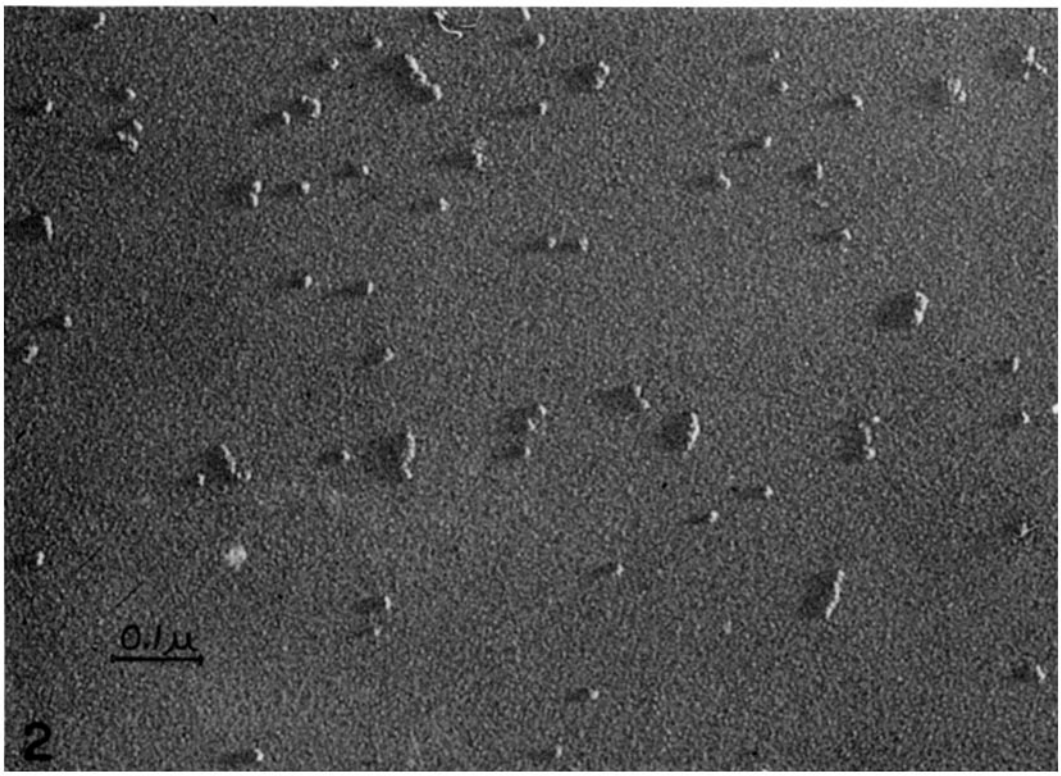
1. Hall, C. E., and Doty, P., *J. Am. Chem. Soc.*, 1958, **80,** 1269.
2. Hall, C. E., and Slayter, H. S., *J. Biophysic. and Biochem. Cytol.*, 1959, **5,** 11.
3. Campbell, D. H., *Ann. Rev. Microbiol.*, 1948, **2,** 269.
4. Hughes, W. L., The Proteins, New York, Academic Press, Inc., 1954, **2B**.
5. Kabat, E. A., *J. Immunol.*, 1943, **47,** 513.
6. Oncley, J. L., Blood Cells and Plasma Proteins, New York, Academic Press, Inc., 1953.
7. Smith, E. L., and Jager, B. V., *Ann. Rev. Microbiol.*, 1952, **6,** 207.
8. Boyd, W. C., Fundamentals of Immunology, New York, Interscience Publishers, Inc., 3rd edition, 1956.
9. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61,** 563.
10. Hooker, S. B., and Boyd, W. C., *J. Immunol.*, 1934, **26,** 469.
11. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1935, **63,** 325.
12. Pauling, L., Pressman, D., and Grossberg, A. L., *J. Am. Chem. Soc.*, 1944, **66,** 784.
13. Deutsch, H. F., Nichol, J. C., and Cohn, M. J., *J. Immunol.*, 1949, **63,** 195.
14. Epstein, S. I., Doty, P., and Boyd, W. C., *J. Am. Chem. Soc.*, 1956, **78,** 3306.
15. Humphrey, J. H., and Porter, R. R., *Biochem. J.*, 1956, **62,** 93.
16. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., Jr., *J. Am. Chem. Soc.*, 1949, **71,** 541.

17. Nisonoff, A., and Pressman, D., submitted to *J. Immunol.*
18. Nisonoff, A., and Pressman, D., *J. Immunol.*, 1958, **80,** 417.
19. Kekwick, R. A., *Biochem. J.*, 1940, **34,** 1248.
20. Kleiner, S. M., and Kegeles, G., *Arch. Biochem. and Biophysics*, 1956, **63,** 247.
21. Kabat, E. A., *J. Exp. Med.*, 1939, **69,** 103.

EXPLANATION OF PLATE 195

FIG. 1. Rabbit anti-*p*-azobenzoate antibodies. Magnification, 112,000.
FIG. 2. Normal rabbit γ-globulin. Magnification, 112,000.





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