Measurement of Globular Protein Molecules by Electron Microscopy

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PLATE 318

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ABSTRACT

A series of molecular species with approximately spherical shape and with molecular weights between 35,000 and 250,000 were shadowed with platinum while resting on a cleaved mica surface. They were backed, stripped from the surface, and examined by electron microscopy. Materials examined were: pepsin, liver alcohol dehydrogenase, yeast alcohol dehydrogenase, glutamic dehydrogenase, polyhedral virus protein (insect), fibrinogen substructure, alkaline phosphatase, and microsomal particles from *Escherichia coli*. Measurements were made of widths perpendicular to the shadowing direction and heights were deduced from shadow lengths. For those molecular species with well established molecular weights the average heights correlate very well with the diameter of the theoretical sphere but the average widths are too great by 50 to 80 A due to the lateral growth of the deposited metal. Although the distortion in shape of shadowed particles is relatively large, with standardized conditions for shadowing, it is possible to make allowance for the distortion and to obtain reasonably reliable estimates of the dimensions of spherical organic particles down to a molecular weight of about 35,000.

INTRODUCTION

The shadowing of objects for electron microscopy (1) when used in conjunction with a suitably smooth substrate (2) makes possible the observation of molecules with heights as little as 10 A. Studies of asymmetrical molecules or long-chain molecules with this method can be particularly fruitful since individual particle lengths can be measured with good accuracy (3). Globular molecules, *i.e.* those which are "approximately spherical," can be rendered clearly visible; but the problem of obtaining significant dimensional data from electron micrographs is complicated by a number of difficulties:

1. The granular nature of the shadowing metal and of the film support is more confusing in the identification of very small spherical particles than it is for asymmetrical particles with one dimension much greater than the "noise level" of the background.

2. In general, shadowed particles will appear to be wider than they actually are in a direction perpendicular to the shadowing direction owing to the cap of metal, but it has not been demonstrated under practical conditions just how much wider they will appear.

3. The accumulation of metal may also be expected to increase the height of a particle but to what extent this would distort the shadow length is again uncertain.

For these reasons the quantitative significance of measurements made on small shadowed particles is questionable, and since a molecular volume or weight involves the third power of a measured diameter, accuracy is very much affected by these uncertainties.

An elegant method for determining average molecular weights (MW) by counting the number of unknown particles relative to the number of a standard species has been used by Williams and Backus (4) to determine the molecular weight of virus particles. The method requires only the identification of the particles and does not require a measurement of their dimensions. This would appear to be applicable to the smaller observable molecules but again there are serious difficulties:

1. Recognizable standard particles of known weight which are not too different in size from the

ones to be determined must be available. The smallest uniform polystyrene latex spheres obtainable commercially average 880 A in diameter, which is much too large to be useful for the measurement of molecules of molecular weight of a few hundred thousand and less. We have made smaller spheres but have not been able to produce satisfactory uniformity with average diameters of say 100 to 200 A as yet.

2. There should be no interaction between the two species and clumping on the drying pattern should be negligible. When large particles clump, say in a monolayer, they can still be counted, but when small molecules clump they usually lose their identities in the aggregate. Most proteins adsorb to admixed polystyrene spheres which would result in a false count, but this difficulty could probably be overcome by making counts at two or more relative concentrations and extrapolating the results.

3. The contents of an entire drop must be counted. With the mica method the drops spread so widely on the hydrophilic surface that it is difficult to contain an entire droplet in one field. This is, however, a technical problem and suitably small droplets have on occasion been produced.

Although these difficulties conceivably might be overcome, the determination of molecular weight of small spherical particles by the counting method does not appear particularly promising at present in comparison with conventional techniques. The chief value of electron microscopy would appear to be in the determination of molecular dimensions and in the observation of heterogeneities rather than average values such as one obtains from the counting method or physical chemical techniques.

In the course of the 3 or 4 years in which mica surfaces have been used in this laboratory for the preparation of shadowed macromolecules for electron microscopy, we have observed a number of globular molecules, particularly enzymes, and although the particles can be seen clearly we have refrained from making definite dimensional analyses because of the uncertainties involved. A sufficient amount of experience has accumulated, however, that now it is possible to draw some conclusions on empirical grounds that will aid in the interpretation of such images in general.

Materials and Methods

Materials.—Samples were suspended in volatile buffers, principally ammonium acetate or mixtures of this salt with ammonium carbonate (5). Values of pH were adjusted with acetic acid or ammonium hydroxide. (With certain materials some non-volatile salt up to concentrations of about 0.002 M have been used without obvious image deterioration.) Table I shows a list of some of the materials examined together with typical conditions producing good results. The protein concentrations are rather low which in some instances may produce depolymerization. Suspensions were sprayed onto the surface of freshly cleaved mica from a high-pressure spray gun as previously described (2).

Shadow-Casting.—Platinum has been used exclusively as the shadowing metal because it is dense, chemically inert, has a reasonably fine grain, and strips readily from mica. Platinum wire 0.1 mm. in diameter is wound helically on 0.030 inch tungsten wire as shown in Text-fig. 1. Although a number of variations in the parameters shown in the figure were tried at first, we have tended to standardize the conditions for the type of particles discussed here in order to obtain better comparisons. Two of the most commonly employed sets of parameters for shadow-to-height ratios of "5:1" and "10:1," chosen as a result of qualitative inspection of micrographs, are shown in Table II. Pressures in the vacuum chamber were reduced to 0.2×10^{-4} mm. of Hg (as read on a Philips gauge) and generally rose to 1×10^{-4} during evaporation. The Pt first melts on the application of sufficient heater current and alloys with the tungsten. As the filament current is raised higher, up to 50 amperes, the Pt evaporates leaving a dull etched helix in the tungsten wire. Probably some tungsten evaporates too. A filament is reused about 6 times before replacement.

When one considers the interpretation of the dimensions of small shadowed particles there are a number of factors which should be taken into account. On purely geometrical grounds there are possible variations in local shadow-to-height ratios, unsharpness of shadows due to the finite diameter of the tungsten wire, and possible shortening of the shadow due to finite lengths of the evaporating source. Alterations in local shadow-to-height ratios can be corrected for by incorporating polystyrene as an internal standard (6). The geometrical conditions applying to the source geometry used here can be easily calculated and turn out to be small (< 10 per cent) compared with the other experimental uncertainties encountered. On physical grounds, there is a distortion in the width of objects normal to the shadowing direction due to the build up of metal and also presumably an uncertainty in shadow-to-height relationship due to a build up of metal in a direction normal to the substrate. If one attempts to calculate metal thickness from the amount evaporated there is also the complication that metal is not radiated with spherical symmetry from sources such as are used, or, indeed, from any of the common evaporation filaments (7). In making measurements of shadow lengths there is uncertainty in locating the end of the shadow and judgment of this location may vary some-

TABLE I

*Partial List of Materials and Typical Conditions for Spraying**

Material	Source		Salt	pH
		mg./ml.		
Alkaline phosphatase (AP)	C. Levinthall, M.I.T.	0.1	0.01 M NH_4Ac	6.8
Glutamic dehydrogenase (GDH)	J. S. Adelstein and B. L. Vallee, Harvard Medical School	0.185	0.1 M NH_4Ac	7.6
Liver alcohol dehydrogenase (LADH)	B. L. Vallee and F. L. Hoch, Harvard Medical School	0.1	0.01 M Ammonium succinate	6.0
Yeast alcohol dehydrogenase (YADH)	"	0.1	0.01 M NH_4Ac 0.01 M $(NH_4)_2CO_3$	7.4
Yeast alcohol dehydrogenase (YADH)	"	0.3	0.1 M NH_4Ac 0.05 M $(NH_4)_2CO_3$	7.5
Pepsin, ×2 crystalline	Nutritional Biochemicals Corp., Cleveland 28, Ohio	0.25	None	7.0
Polyhedral virus protein (PVP)	C. H. Bergold, Dept. of Agriculture, Sault Ste. Marie, Ontario, Canada	0.10	0.01 M $(NH_4)_2CO_3$	9.5

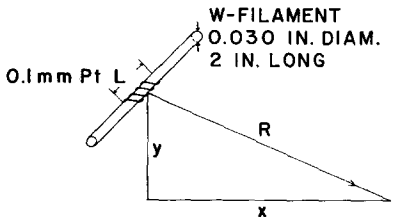
* All suspensions contained about 0.05 mg./ml. of polystyrene latex of average diameter 880 A. Primary source of GDH, LADH, and YADH was C. F. Boehringer and Sons, Mannheim, W. Germany.

what between observers. In view of these variables and uncertainties, measurements have been made from micrographs and conclusions drawn therefrom on a purely empirical basis.

Most micrographs have been recorded on an RCA type EMU-3 at 100 kv. and at × 15,000. The higher than usual voltage is not essential but it obviates removal of the collodion backing which might otherwise have to be dissolved away for good imaging at 50 kv. Plates were recorded as through-focus series at × 15,000. This magnification is chosen as a compromise between size of field and limitation of resolution due to plate grain. Actually, at this magnification the plates cannot resolve all the detail in the shadowed Pt film, but the micrographs probably appear better to the eye as a consequence, since the finest detail in the Pt is meaningless in terms of the structures under investigation. Measurements were made from prints at about × 100,000 with an ocular micrometer graduated to 0.1 mm. Different observers were likely to vary a little in judging shadow lengths on similar prints but this variation tended to be compensated for by corresponding variations in their judgment of the shadow-to-height ratio from the admixed polystyrene spheres. With the small angles of shadowing used, the center of the particle is assumed to be at the edge of the metal cap toward the shadow, the particles themselves being invisible (except for the polystyrene spheres).

OBSERVATIONS AND RESULTS

In Figs. 1 to 4 are shown typical micrographs of four enzyme molecules, pepsin, yeast alcohol dehydrogenase (YADH), alkaline phosphatase (AP), and glutamic dehydrogenase (GDH). From prints such as these, widths and shadow lengths were measured with an eyepiece micrometer to 0.1 mm. A typical plot of data for YADH is shown in Text-fig. 2 where shadow lengths have been converted to heights by using the shadow-to-height ratio measured from polystyrene spheres. It is seen that the peak frequency of widths is about 65 A greater than the peak for the heights. This could be due to a real difference in these dimensions or to a distortion of spherical shape by the deposit of a metal cap. In the case of YADH the experiment was repeated 4 times, including 3 with a shadow ratio of 5:1 and 1 at 10:1, with practically the same results. Similar graphs were obtained for other molecular species for which the average heights and widths are listed in Table III. The molecular weights from conventional methods are in the second column and in the third column are listed the calculated diameters, D, which these particles would have if they were perfect anhydrous



TEXT-FIG. 1. Diagram showing geometry for shadowing with platinum from a straight tungsten filament. See Table II for typical parameters.

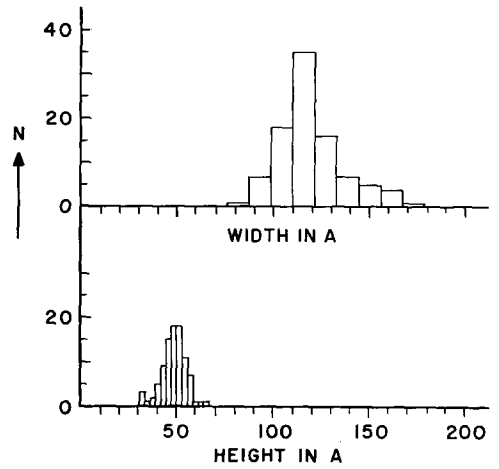
TABLE II

Parameters for Shadow-Casting

See Text-fig. 1 for meaning of the symbols.

x/y	Polystyrene shadow/ diameter	x	y	R	Platinum 0.1 mm. diameter	
					Wire length	L
		cm.	*cm.*	*cm.*	*cm.*	*cm.*
5:1	3.5–4.5	5.8	1.2	6	1.8	0.4–0.5
10:1	6.5–8.5	5.97	0.59	6	2.4	0.5–0.7

spheres with a density 1.35 gm./cm³. In the fourth and fifth columns are listed the average heights (h) and the widths (w) from electron microscopy and in the last column the difference w-h. If the theoretical figures are compared with the EM measurements, it is obvious that h is generally close to the theoretical D and that w is consistently too large. The height is, in fact, not significantly different from D except for the LADH, PVP, and the 50 S RNP. In the last case it is reasonably certain that the particles have been slightly flattened during drying which produces an abnormally large width. When the volume of the 50 S RNP is calculated on the basis of an oblate ellipsoid the volume is in good agreement (22) with the volume obtained from physical chemical methods (21). The observed height in all cases is slightly less than the calculated D. The fluctuation in the difference between the apparent width and the height is probably to be expected since distances from the filament are not precisely the same and the amount of metal radiated may vary somewhat from one experiment to another. Except for two or three extreme cases, however, the exaggeration in width is 60 to 70 A. Since the experiments listed in Table III were performed over a period of time and by different operators, one would not expect the



TEXT-FIG. 2. Histograms showing measurements of widths (perpendicular to the shadowing direction) and heights of shadowed molecules of yeast alcohol dehydrogenase. The widths are greater than the true width due to the cap of metal. Heights shown are computed from the shadow-to-height ratio (4.03:1) measured from admixed polystyrene spheres.

TABLE III

Material	MW from other sources	Theoretical sphere D	h	w	$w-h$
		A	*A*	*A*	*A*
Pepsin	35,000 (8)	44	40	90	50
LADH	83,000 (9)	58	52	105	53
AP	80,000 (10)	57	60	147	87
YADH	150,000 (11–15)	70			
	75,000 ?	57	47	122	75
GDH	10^6 (16) (11)	130			
	250,000 (16–18)	84	80	145	65
PVP	380,000 (19)	97	83	159	76
Fibrinogen* nodule (larger)	138,000 (20)	60–65	60	114	54
50 S RNP‡	1.8×10^6 (21)	153	137	220	80
				Average	68 ± 12

* Estimated from the known MW of the whole molecule and relative sizes of the three nodules.

‡ This is an approximately spherical fragment of the 70 S unit. The 30 S fragment is asymmetrical (21, 22).

figures for exaggerated width in the last column to be too close to one another. It is possible on occasion to have a little platinum left on the filament from a previous experiment to increase the amount deposited. This was probably the case with the AP which has an unusually large width.

Ultracentrifugal sedimentation indicates a molecular weight of 1,000,000 for beef liver GDH

TABLE IV

Measurements of Platinum Deposited on Ends of TMV (In Angstroms)

Shadow Ratio		No.	Width of cap	Extra width	Depth of cap	Calculated depth*
Theoretical	Measured					
10:1	8.2:1	41	210	60	91	42
5:1	3.6:1	43	212	62	97	32

* Assuming spherical symmetry.

and of 150,000 for yeast ADH (11). The GDH is dissociated into particles of ¼ that size in dilute solutions (16) or by DPNH (diphosphopyridine nucleotide) or zinc-binding agents (17, 18); the particle sizes shown in Table III correspond to those of the dissociation products of GDH. Yeast ADH, on the other hand, does not give ultracentrifugal evidence of dissociation upon dilution. However zinc-binding or —SH-binding reagents can produce more slowly sedimenting molecular species (12–15). This enzyme shows particles of 75,000 MW size, one-half that of the native enzyme, when examined electron micrographically, and some dimers are observed. The relation between these half size particles of YADH and those observed after treatment with the reagents mentioned (12–15) requires further investigation.

As a further check on the increase in width due to shadowing, tobacco mosaic virus (TMV) was shadowed as for the spherical particles and rods were sought in the field which were lying approximately parallel to the shadowing direction so that the cap of metal over the ends could be measured. TMV is an excellent test object since it is remarkably uniform in diameter, close to 150 A (23), and resistant to distortions on drying. Measurement of the width of ends facing the evaporating source should, therefore, give a good check on growth in apparent width. The results are shown in Table IV where it is seen that the width of the metal cap is about 60 A greater than the true particle diameter which is consistent with the results on spherical particles shown in Table III.

At these shadow ratios, the thickness of the metal cap parallel to the shadowing direction is a measure of the amount of metal radiated in this particular direction. In the fifth column of Table IV are listed the average measurements of this thickness and in the last column the thickness which would be expected on the basis of spherical symmetry of radiation from the source. It is seen that in this direction (perpendicular to the filament) the amount of metal radiated is much greater than one would expect on the basis of symmetry, which result is in agreement with measurements reported by Preuss (7). The thickness of the cap in this direction is probably also increased by some tendency to creep over the top of the particles.

DISCUSSION

These results show that although the shadow-casting technique produces considerable distortion in the shape and size of isolated small molecules, fairly good estimates of the true dimensions can be obtained of particles with diameters down to about 40 A. Shadow lengths apparently can be a good measure of particle heights and the distortion in width can be estimated under standardized conditions. The electron microscope method cannot be expected at the present time to compete with conventional methods of molecular weight determination since small errors in measurements of diameter become quite large when the diameter is used to calculate a volume. The electron microscope method should be most useful in conjunction with other techniques when there is doubt or ambiguity regarding particle shape. Electron microscopy has the advantage that heterogeneity is directly observable. It should be of value in counting relative numbers in populations of mixed systems or in following polymerization reactions.

Although all the materials described were of relatively high purity as rated by the usual criteria of activity, crystallinity, sharpness of the sedimentation peak, etc., the measurements when plotted always showed a more or less normal distribution as typified by Text-fig. 2. This seems to be due to experimental errors in measurement, some real differences in dimensions, and also to the fact that the particles are not truly spherical and are probably presenting slightly different aspects to the shadowing beam. It is perhaps surprising that the measurements correlate as well as they do with the hypothetical spheres. In the studies described here plots of h *versus* w have been made to see if there was any systematic relation between variations in these dimensions from one particle to another. The plots are random. When the difference between average h and w is appreciably greater than what is to be expected on the basis of metallic distortion

alone, it seems fairly certain that the particle is asymmetrical (or is made so by drying forces) and it can then be better represented by an oblate or prolate ellipsoid.

The smallest average particle diameter listed in Table III is 40 A (pepsin). Smaller particles can be observed and measured by the method, but it is not clear how the apparent size will be related to the actual size as the latter approaches zero. This will have to be investigated empirically. Some materials with lower molecular weights than those in Table III (*e.g.* ribonuclease, MW 13,000) have been examined but the shadowed particles lack the uniformity and distinctness to enable confident identification. The smallest particles that are well defined and distinct above the "background" have widths of about 60 A and an apparent height of about 20 A. This would suggest a molecular weight of about 10,000 based on the height and would indicate that the growth of metal laterally is less than that which occurs at larger particle dimensions.

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Explanation of Plate 318

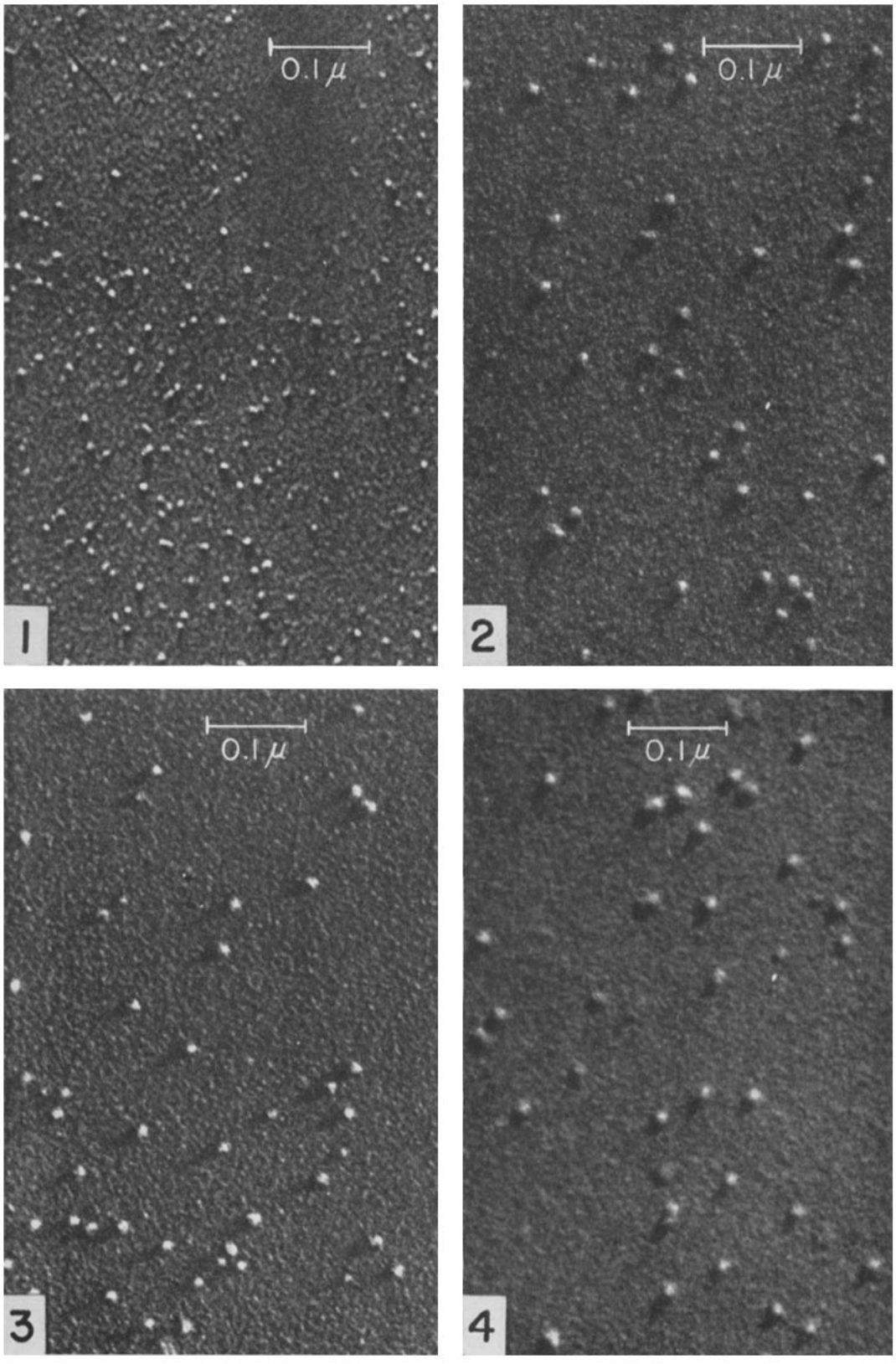
Enzyme molecules on a mica substrate shadowed with platinum. Magnification × 150,000.

Fig. 1. Pepsin. MW 35,000.

Fig. 2. Yeast alcohol dehydrogenase. MW 75,000.

Fig. 3. Alkaline phosphatase. MW 80,000.

Fig. 4. Glutamic dehydrogenase. MW 250,000.



(Hall: Protein molecules)