JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

(Registered in U. S. Patent Office)

VOLUME 80 MARCH 31, 1958 NUMBER 6

PHYSICAL AND INORGANIC CHEMISTRY

[CONTRIBUTION FROM THE BIOLOGY DEPARTMENT, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, AND THE DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

A Comparison between the Dimensions of Some Macromolecules Determined by Electron Microscopy and by Physical Chemical Methods

BY CECIL E. HALL AND PAUL DOTY

RECEIVED AUGUST 19, 1957

By means of an improved method of electron microscopy photographs have been obtained of individual molecules of three macromolecular substances (collagen, nucleic acid, poly-L-glutamic acid) known from physical chemical investigations to have rod-like configurations in solution. The results show that the rod-like structures are preserved upon deposition permitting a determination of average diameters, average lengths and length distribution. The results of such determinations of diameter and length are found to be within 0–40% of those obtained from physical chemical measurements (flow birefringence, viscosity, light scattering and sedimentation rate) and the distributions of length are in agreement with those deduced from the various averages determined by the physical chemical methods. In each case the average lengths are related to molecular weights to a good approximation by means of the mass-to-length ratio characteristic of the Ramachandran–Crick–Rich structure for collagen, the Watson–Crick structure for deoxyribose nucleic acid and the Pauling–Corey α-helix. Thus these structures appear to have been observed directly in electron microscopy. The over-all results indicate that this technique can serve as a reliable method for investigating the dimensions and size-distribution of asymmetric macromolecules.

Weights and dimensions of large molecules have been determined mostly through the use of indirect physical chemical methods such as sedimentation and viscosity or diffusion, osmotic pressure and light scattering, although the electron microscope, since it has for many years offered resolving power down to the order of 10 Å., should have permitted the determination of these parameters by direct observation. The delay in realizing this potentiality has been due to technical difficulties arising mainly from the fact that such molecules have very low electron scattering power, and so lack contrast in the electron image, and to the confusing structure of conventional supporting surfaces on which the molecules are placed for observation. Recently, there was described[1] a method whereby particles to be examined with the electron microscope are first placed on the atomically smooth surface of freshly cleaved mica and shadow-cast with platinum. The metallized layer is then backed with a supporting film and stripped for observation. This method enables the reliable observation of molecular dimensions down to the order of 10 Å. which means that the full capabilities of the electron microscope may be realized. In addition, this procedure appears to circumvent previous difficulties in such a way that electron microscopy may be expected to become increasingly important as a means for the determination of weights and dimensions of a large class of macromolecules.

In view of the novelty of electron microscopy for the observation of macromolecules with molecular weights less than several million, it is highly desirable in the beginning to compare results with those obtained by other methods on identical materials. In this paper we present such data obtained on macromolecules of soluble collagen, fragmented deoxyribose nucleic acid and polypeptides.

Materials and Methods

Collagen.—The soluble collagen was ichthyocol prepared by citrate extraction of carp swim bladder tunics. The preparation and characterization by a number of methods has been carried out by Boedtker and Doty.[2] The particular sample used in this investigation was prepared by Mr. E. H. L. Chun and was found to exhibit the same extinction angle *versus* gradient in flow birefringence as that found by Boedtker and Doty.[2] Since this property is considered to be the most sensitive measure of length and length distribution, the properties of this sample were taken to be the same as those reported.[2]

The following sequence of operations was carried out.

(1) C. E. Hall, *J. Biophys. Biochem. Cytology*, **2**, 625 (1956); *Proc. Natl. Acad. Sci.*, **42**, 801 (1956).

(2) H. Boedtker and P. Doty, THIS JOURNAL, **78**, 4267 (1956).

The citrate extract was precipitated by dialyzing against dilute phosphate. The precipitate was redissolved in 0.15 *M* citrate buffer (*p*H 3.7) and centrifuged at 100,000 × *g* for 90 minutes. The extinction angle as a function of gradient was then measured and the solution was dialyzed against 0.05% acetic acid. In this condition it was sprayed on mica. Citrate buffer was added to a part of this solution to return it to the original condition. It was then measured and found to have the same extinction angle—gradient behavior as before, indicating that the sample had not been altered by the dialysis.

Sonically Produced Fragments of Deoxyribose Nucleic Acid (DNA).—The preparation and properties of sonically degraded DNA are discussed in a current paper by Doty, McGill and Rice.[3] In this work it is shown that exposure of dilute DNA solutions to 5 or 9 kc. frequency for periods from 10 seconds to 1 hr. degrades DNA from an initial molecular weight of about 8,000,000 to values in the range of 3,000,000 to 300,000. Evidence was presented to show that the degradation was exclusively due to double chain scission and that the hydrogen bonding characteristic of the Watson–Crick structure remained intact. Since electron micrographs of the original DNA seldom permitted observation of both ends of the DNA molecules, these shorter, sonically produced fragments were used.

The study of these fragments[3] had led to a calibration of intrinsic viscosity and sedimentation rate in terms of weight average molecular weight. Consequently, the determination of either of these quantities permitted the assignment of molecular weight. For this study two sonically fragmented samples of salmon sperm DNA[4] that had been characterized by Mr. M. Litt were used. One, having been exposed to 9 kc. for 1 minute, was found to have an intrinsic viscosity of 7.9 and hence a molecular weight of 970,000 as determined from the above-mentioned calibration. The other, having been exposed for 60 minutes, had a sedimentation rate, S_{20w}^0, 6.1 *S*. Its molecular weight was 240,000. The absolute probable error in these weight average molecular weight assignments is estimated at ±15%.

Poly-L-glutamic Acid and Poly-γ-benzyl-L-glutamate.—Poly-L-glutamic acid samples prepared by Drs. E. R. Blout and M. Idelson have been investigated in solution by Doty, Wada, Yang and Blout[5] and found to exist as rod-like α-helices in the *p*H range between insolubility (~ *p*H 4) and about *p*H 5.0, the latter limit being ionic strength dependent. A more recent study[6] involving sedimentation rates, intrinsic viscosity and light scattering and osmotic pressure has provided a calibration of weight average molecular weight in terms of intrinsic viscosity in several solvents and has shown that the samples examined had a polydispersity ranging upward to that characterized by a value of 2 for the ratio of weight to number average, M_w/M_n.

Most of the poly-L-glutamic acid samples available for this work displayed a limited but significant drop in molecular weight when exposed to *p*H 12.[6] Such treatment was generally carried out because the viscosity of the solutions was much more stable as a result. The particular sample employed here had a degree of polymerization of 620 ± 60 before exposure to *p*H 12 and 335 ± 30 afterwards. It was examined in the electron microscope under both conditions.

The poly-γ-benzyl-L-glutamate sample has been characterized in a previous study[7] in which it was shown that the configuration was helical in solvents such as ethylene dichloride.

Electron Microscopy.—The specimens for electron microscopy were sprayed from a Nebulizer (throat sprayer) on to the surface of freshly cleaved mica, shadow-cast with platinum (2.5 cm. of 0.1 mm. Pt wire) at a distance of 7 cm. and usually at a shadow-to-height ratio of 10:1. The metallized surface was coated with SiO (0.5 mg. at 10 cm.) at normal incidence which was subsequently backed with collodion from a 0.5% solution in butyl acetate. The film was stripped on a water trough and picked up over 200-per-inch grids. In practically all instances polystyrene spheres of average diameter 880 Å. were added to the solutions before spraying as an aid in focussing.

The ichthyocol preparation dissolved in 0.05% acetic acid (*p*H 3.6) was kept carefully refrigerated and sprayed on the mica in a cold room at 5°. The most satisfactory concentration of protein was found to be about 0.005%.

The DNA samples were diluted with buffer containing 0.05 *M* ammonium carbonate and 0.1 *M* ammonium acetate and sprayed at room temperature. Some salt is necessary to prevent denaturation and this pair of volatile salts was chosen since they gave better results than either used alone. This presumably is due to the maintenance of a more nearly neutral *p*H during evaporation, thereby preventing acid or base denaturation. A concentration of 0.002% gave satisfactory results.

The poly-L-glutamic acid in water acidified with HCl to *p*H 4.5 and in a concentration of 0.02 g./100 cc. was sprayed at room temperature.

The success of this method seems to depend for the most part on obtaining clean suspensions of the macromolecules in volatile salts and buffers. Non-volatile salts cannot be used because if the sprayed surface has to be washed, impurities deposit from the wash water and obscure the structures sought. The sprayed droplets spread over such a large area that the concentration of impurities per unit area is much less than it would be for a washed and drained surface. This spreading of droplets over wide areas makes it impossible in general to record the image of entire droplets, which is a preferred method of sampling. Instead, sampling is carried out by recording fields at random and measuring all discernible particles in a given field.

Except for the preparative steps, the measurement of lengths of relatively long macromolecules involves only the routine techniques of calibration and statistics common in electron microscopy. Micrographs were recorded at magnifications between 10,000 and 15,000 × with an RCA Type EMU-3B electron microscope. Measurements were made with rulers from prints enlarged to about 100,000× and the accuracy is judged to be within ±2%. Diameters of particles in the 10 to 25 Å. range cannot be obtained with high accuracy because of the shortness of shadows and uncertainties associated with the granularity and possible distortions produced by the metallic deposit. In this study, however, we are confining our quantitative comparisons to length measurements although the diameters of the observed macromolecules are estimated to be in the 10 to 25 Å. range, which estimates are consistent with other data.

Another problem in the interpretation of electron micrographs can arise in connection with apparent internal structure of macromolecules. Such appearances, for example, as nodosities frequently seen in long, thin molecules can be real, or they can be caused by granulation of the metal or adhering particulate material and appearances can be affected by conditions of focus and photography. One cannot generalize easily on these interpretive problems. Each case has to be examined individually and fields should be recorded as through-focus series as an aid to interpreting optical artifacts.

Poly-γ-benzyl-L-glutamate (PBG) in ethylene dichloride did not spray satisfactorily. When the solution was applied with an eye dropper to the mica surface which was drained and dried, a very high dilution factor was found necessary and this in turn raised the ratio of impurities to sample to undesirably high levels. A major advantage of aqueous sprays on mica results from the fact that water droplets spread so readily over the surface that relatively high concentration can be used. Fairly satisfactory micrographs of PBG finally were obtained by putting the two faces of cleaved mica back together under a weight and introducing a drop of the ethylene chloride solution at the edge. The solution penetrated the narrow space between but was kept sufficiently thin that reasonable concentrations could be used. After the ethylene chloride had evaporated the two mica sheets were separated.

Results

Ichthyocol.—In the initial report[1] of the electron microscopy method used here, a sample of ichthyocol was examined and found to consist of long filaments with an estimated diameter of about

(3) P. Doty, B. H. McGill and S. A. Rice, *Proc. Natl. Acad. Sci.*, **44**, in press (1958).

(4) This sample had been prepared by M. Litt by first isolating the sperm heads and then using the Simmons procedure employing sodium xylene sulfonate at *p*H 4.3 for removal of proteins.

(5) P. Doty, A. Wada, J. T. Yang and E. R. Blout, *J. Polymer Sci.*, **23**, 851 (1957).

(6) A. Wada and P. Doty, unpublished results.

(7) P. Doty, J. H. Bradbury and A. Holtzer, THIS JOURNAL, **78**, 947 (1956).

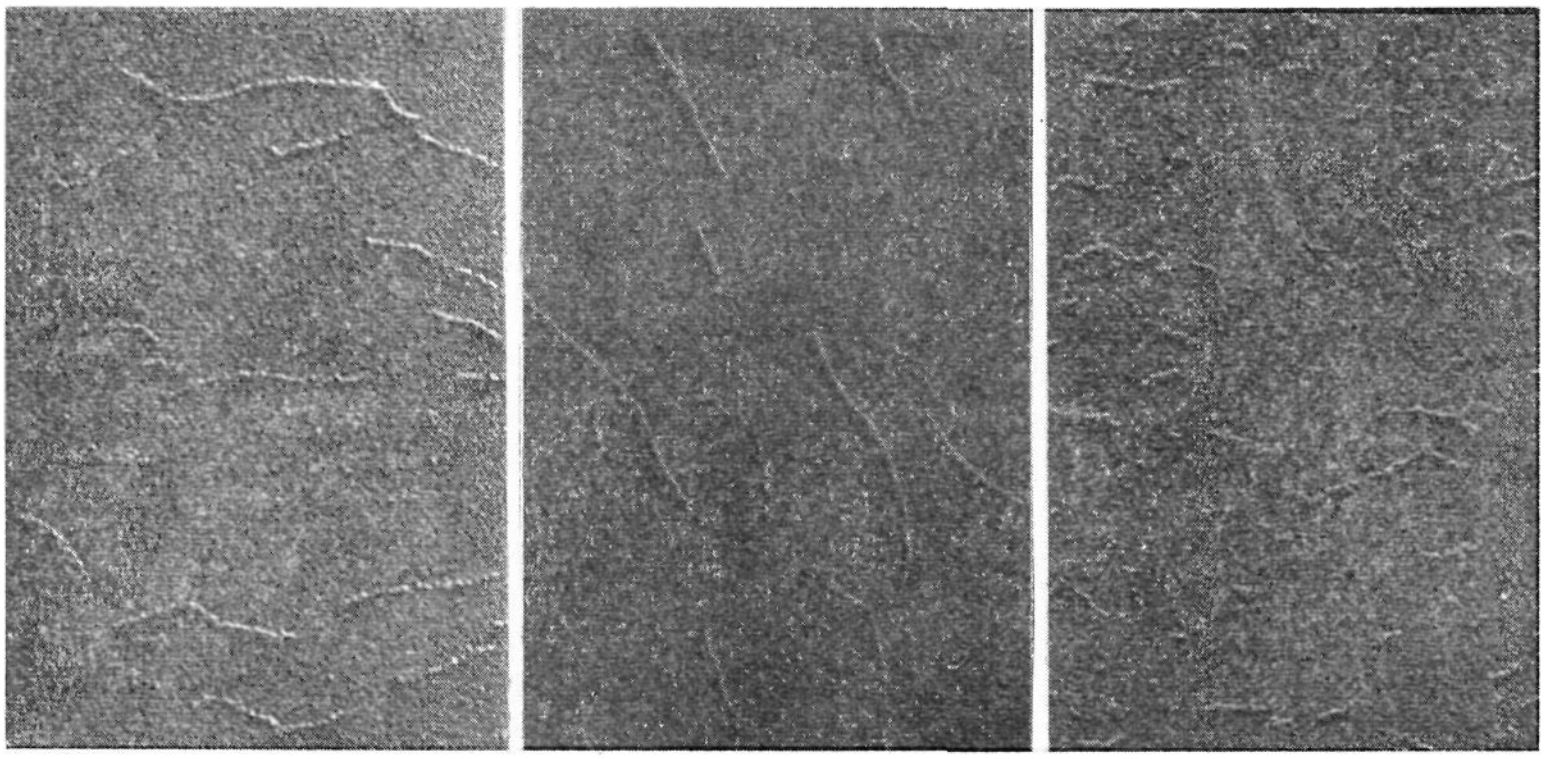

Fig. 1.—Electron micrograph showing ichthyocol macromolecules whose widths obtained from shadow lengths are about 15 Å. Magnification 70,000 X.

Fig. 3.—Electron micrograph showing desoxyribose nucleic acid macromolecules which have been fragmented by sonic vibrations. Shadow lengths indicate diameters of about 20 Å. Magnification 100,000 X.

Fig. 5.—Electron micrograph showing macromolecules of polyglutamic acid. Magnification 120,000 X.

15 Å. The diameter agreed with the physical chemical data,[2] but the weight average length was significantly smaller. Subsequently, after the electron microscopy technique had been improved, a new sample of this material was examined immediately after flow birefringence measurements had been made and care was taken to keep the material cold throughout the preparative technique by spraying in the cold room at 5°. A part of an electron micrograph showing typical filamentous macromolecules of collagen is reproduced in Fig. 1. The diameter of these particles is estimated to be about 15 Å. and the weight distribution of lengths is shown in Fig. 2. The number average length is 2450 Å. and the weight average which is the most appropriate average to compare with the physical chemical results is found to be 2820 Å. The weight average length now was computed in the usual fashion by performing the summation $N_iL_i^2/N_iL_i$, where N_i is the number of molecules of length L_i. This same ichthyocol sample was reconstituted in the fibrous long spacing form[8] by Professor F. O. Schmitt. In the electron microscope the reprecipitated fibrils show the typical periodicities of this form of collagen and the average value was found to be 2700 Å. Thus the length observed in the fibril is nearly the same as weight average determined for the individual molecules.

In the study of ichthyocol by Boedtker and Doty[2] it had been shown that the collagen molecules were long, rod-like in shape and had a diameter of 13.5 Å. The electron microscope observations are in agreement with this, although they show a beaded or nodose structure that could not have been picked up by the solution studies. It remains to be proven that these are not imposed on the collagen during the electron microscope procedure. This is, however, illustrative of the kind of detail that can be detected electron microscopically but not by the usual physical chemical methods. A comparison of the measurements of length is perhaps best done by translating the data of Fig. 1 into a continuous weight distribution. This is done in Fig. 2. Three direct measurements of length were available from solution studies.

(1) Light scattering provides a highly weighted average value of the length of rod-like particles. To be precise it is the square root of the product of the z-average and the next higher average, $z + 1$: that is[6] $\sqrt{L_zL_{z+1}}$. The mean was taken as 3100 Å. On the basis of the values measured for the number and weight average molecular weights (310,000 and 345,000, respectively) the molecular weight distribution was known to be relatively narrow. Consequently it was estimated that the weight average length would only be about 100 Å. less than the higher average obtained from light scattering. In this manner the weight average length had been assigned[2] a value of 3000 Å. It is seen that this is less than 7% higher than the electron microscope value and hence within probable experimental error these two methods have led to the same result.

(2) With variation of gradient a range of values of length is picked up in measurements of extinction angle (flow birefringence). In the range of 500 to 5000 sec.$^{-1}$ this range is 2600 to 2950 Å. From very limited experience[9] with polypeptides in the α-helical configuration, one can deduce that the length distribution is relatively narrow[10] and that

(8) F. O. Schmitt, J. Gross and J. H. Highberger, *Proc. Natl. Acad. Sci.* **39**, 459 (1953).

(9) J. T. Yang and P. Doty, unpublished results.

(10) By relatively narrow we mean a distribution having a weight to number average ratio of length or molecular weight less than about 1.3. The "most probable" distribution that results from the random scission of very long molecules has a value of 2 for this ratio.

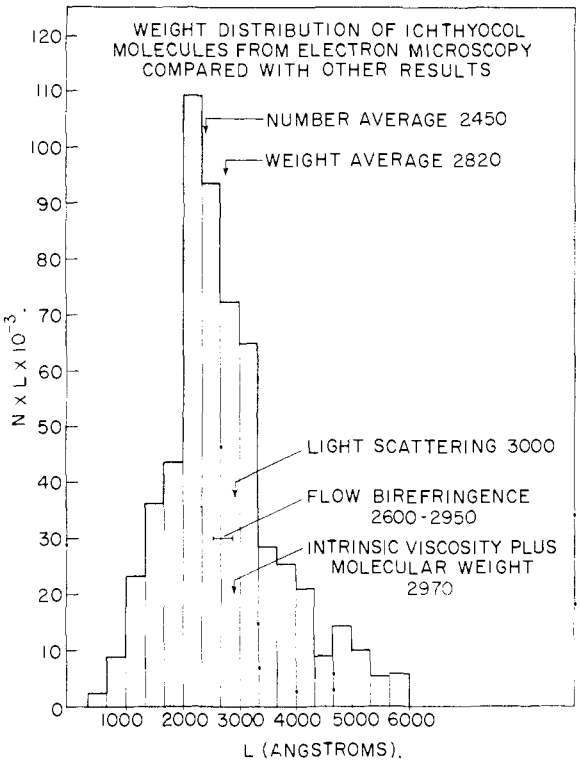

Fig. 2.—Weight distribution of lengths of ichthyocol macromolecules as measured from electron micrographs compared with results from other methods. Total number represented is 238.

the weight average falls within the range measured. The weight average was estimated[2] to be 2900 Å. This is only 3% higher than the electron microscope value and must be accepted as being in complete agreement within probable experimental error.

(3) Length measurements are also available from intrinsic viscosity combined with knowledge of the molecular weight derived from light scattering. This method could provide a precise value for the length that would be slightly higher than a weight average. However, the intrinsic viscosity was not determined with high accuracy (11.5 ± 1.5) and consequently the present uncertainty in the length from this method is rather large (see Fig. 2). The mean value, however, is 2970 Å. This again is in quite satisfactory agreement with the electron microscope result.

Fragmented DNA.—Electron micrographs of undegraded DNA showed filaments, many of which had lengths too great to be determined with good statistical accuracy because individuals were difficult to follow when they became entangled. The diameters are estimated to be about 20 Å. and in this respect are indistinguishable from electron micrographs of DNA from calf thymus previously reported. There is a notable difference between the two preparations, however, in that in the earlier micrographs free ends of molecules were rarely seen. The molecules generally terminated in flat patches which we now believe are degraded portions produced by a dissociation of the double helical structure. Such degradation probably is aggravated by the fact that ammonium acetate in which the DNA was suspended approaches a low *p*H in the last stages of drying. In the new preparation, which was dissolved in mixed ammonium carbonate and acetate, free ends are the rule in both the unfragmented material and in the segments resulting from sonic fragmentation.

The individual particles in sonically degraded preparations can be distinguished sufficiently well that good statistical measurements can be obtained from micrographs. A typical result is shown in Fig. 3 for material that was degraded to a little less than one million in molecular weight. It is seen that almost all of the ends are abrupt and clean-cut indicating that the degradation was the result of double chain scission at single loci. As can be seen in Fig. 3 DNA molecules are much smoother than those of collagen and also the background in Fig. 3 is very clear.

A number distribution based upon length measurement of about 700 molecules is shown in Fig. 4. Particles less than 400 Å. in length were not included because it is not always clear whether such

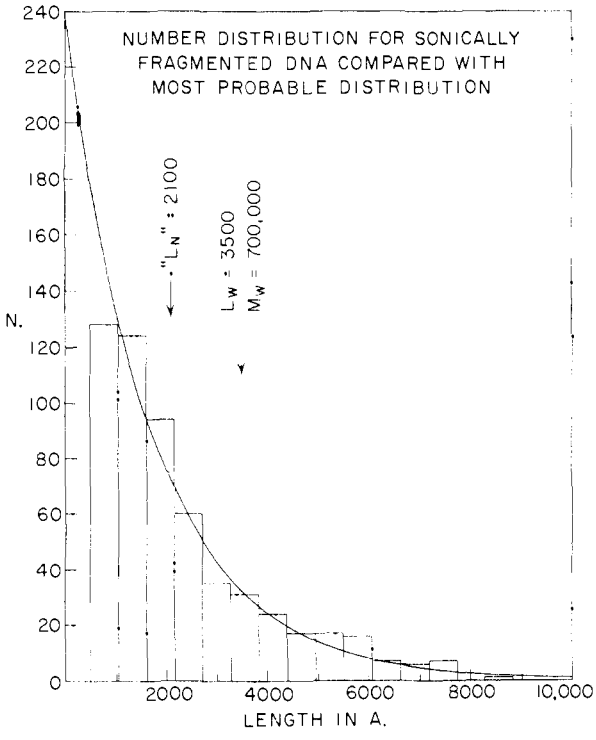

Fig. 4.—Lengths of fragmented DNA obtained from electron micrographs. Total number represented is about 700.

small material is DNA or extraneous material. The weight average length of this distribution is found to be 3500 Å. If we make the assumption that the mass to length ratio of the crystalline state (200) is preserved in these individual molecules, this can be translated into a weight average molecular weight of 700,000. This is nearly 30% lower than the value obtained from intrinsic viscosity.

The number average length was found to be 2100 Å. Since this value would have been significantly lower if a count of particles under 400 Å.

had been available, it is apparent that the actual number average length of this sample is approximately half of the weight average value. Such a relation between these two averages is characteristic of a "most probable distribution," that is, one that results from the random scission of very long molecules. To illustrate this further, the most probable distribution with a weight average length of 3500 Å. is plotted in Fig. 4. It is seen that this fits the observed distribution quite well.

A briefer study also was made of a more degraded sample. Here an attempt was made to include materials under 400 Å. in the count since so much of the sample did fall in this range. The results are included in Table I.

TABLE I

A COMPARISON OF MOLECULAR WEIGHTS OF DNA FRAGMENTS DERIVED FROM ELECTRON MICROSCOPY AND HYDRODYNAMIC MEASUREMENTS

Sample	Electron microscopy[a] No. av.	Wt. av.	Intrinsic viscosity or sedimentation rate (Approximate wt. av.)
A	420,000	700,000	970,000
B	142,000	330,000	240,000

[a] Mass-to-length ratio taken as 200.

Poly-L-glutamic Acid.—The two samples of poly-L-glutamic acid were dissolved in water and the *p*H was adjusted to 4 with HCl to bring it into the helical configuration. An electron micrograph of the lower molecular weight sample is shown in Fig. 5. It is seen that rod-like structures are clearly visible. Their diameters are approximately 10 Å. A length distribution (of the shorter sample) is shown in Fig. 6 which represents 292 particles. The results for these two samples are collected in Table II.

TABLE II

COMPARISON OF MOLECULAR WEIGHTS OF POLY-L-GLUTAMIC ACID DERIVED FROM ELECTRON MICROSCOPY AND INTRINSIC VISCOSITY MEASUREMENTS

Sample	Electron microscopy[a] No. av.	Wt. av.	Intrinsic viscosity
186C	26,000	51,000	80,000
186D	26,500	33,000	43,000

[a] Mass-to-length ratio taken as 86 (*i.e.*, 129/1.5).

The molecular weights, determined from intrinsic viscosity measurements with the aid of a correlation of molecular weights[6] determined by light scattering and intrinsic viscosities measured in dimethylformamide, 0.2 and 2 *M* NaCl at *p*H 7.3, are seen to be about 40% higher than the values obtained by electron microscopy. This difference exceeds expected probable error. The most likely explanation of the difference is suggested by the character of the electron micrograph (Fig. 5) in which the poly-L-glutamic acid rodlets can be seen to contain frequent faults or nodules, that is transverse dislocations that may have resulted from one part of the helix unrolling somewhat from the other. A sufficient number of such faults could lead to a significant shortening in comparison to the undamaged helix. Thus we conclude tentatively that there has been some shortening due to the creation of imperfections as the helix is deposited in the mica surface. This is perhaps not unexpected since this helix being composed of a single strand is structurally the weakest of the three examined here.

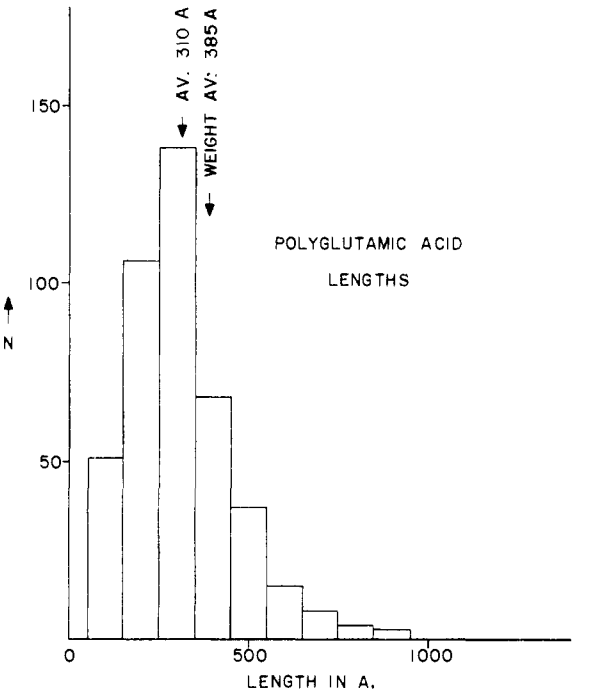

Fig. 6.—Lengths of poly-L-glutamic acid molecules obtained from electron micrographs. Total number represented is 292.

Polybenzyl-L-glutamate.—The electron micrographs of polybenzyl-L-glutamate showed clearly visible rodlets about 15 Å. in diameter. However, they were sufficiently entangled to prevent an accurate statistical count. The strands that were distinguishable were in the range of 500 to 2000 Å. with a number average of about 1000 Å.

Discussion

The main purpose of this paper has been to demonstrate that recent progress in electron microscopy has brought the technique to the point where it can provide useful information regarding certain aspects of macromolecular morphology which could hitherto be studied reliably only by physical chemical methods. Insofar as different techniques frequently provide conflicting results on similar materials, we have attempted to reduce this kind of uncertainty by comparing results from the new technique with the physical-chemical results on the same materials. The differences and corroborations that we find are therefore more meaningful than they might be otherwise.

The data presented here on molecules containing one, two and three molecular strands show the new technique of electron microscopy yields average dimensions for both the *diameter* and *length* that are within 0–40% of those found by physical chemical methods. It appears to us that the most significant feature of this level of agreement is its demonstration that the dimensional distortion suffered by depositing and shadowing these very

fragile macromolecular structures has been kept to below 40%. Indeed, experimental uncertainties are such that probably only about half of the differences observed are due to distortions produced in macromolecules.

In addition to achieving a precision in the determination of average dimensions of asymmetric macromolecules nearly comparable with other methods, the electron microscope procedure provides much more detail of the distribution of lengths than heretofore available. Although this too may suffer some from residual distortional effects, it offers direct access to that type of information that is most difficult to obtain by other methods. It is therefore of interest to note that the comparisons made in this study have shown general agreement not only in average values but in breadths of distributions as well: the ichthyocol being quite narrow and the other two being close to the most probable distribution as predicted. With this check on the reliability of the distribution determination as well, the new electron microscope method appears ready to contribute to a number of problems where detailed knowledge of size-distribution of asymmetric macromolecules is desired.

It seems desirable to add a note concerning a quite different method of measuring average molecular weights of macromolecules with the electron microscope that is applicable, independent of shape and capable of much higher accuracy. This method which involves counting the number ratio of standard to unknown particles in a given volume has been adapted to electron microscopy by Williams and Backus[11] to determine the molecular weight of tomato bushy stunt virus (mol. wt. $\sim$ 10,000,000). This is potentially the most accurate method for the determination of molecular weights by electron microscopy, but there are technical difficulties in the application of the method to particles with molecular weights in the range 10^4 to 10^6, although such particles can be seen clearly by the methods used in the present study. These difficulties are mainly: (1) the production of droplets small enough to be entirely included in the field of the microscope at suitable magnifications, (2) availability of standard particles not too different in size from that of the unknowns and (3) adsorption or interaction between standard and unknown species.

(11) R. C. Williams and R. C. Backus, THIS JOURNAL, **71**, 4052 (1949).

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[CONTRIBUTION NO. 1456 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

Physical-Chemical Studies of Soluble Antigen–Antibody Complexes. IX. The Influence of *p*H on the Association of a Divalent Hapten and Antibody[1,2]

BY SAMUEL I. EPSTEIN[3] AND S. J. SINGER

RECEIVED AUGUST 5, 1957

A quantitative study has been made of the light scattering from mixtures of a divalent benzenearsonic acid hapten and anti-benzenearsonic acid (anti-R) antibody (Ab), over a wide range of *p*H. Analysis of the data at alkaline *p*H reveals that a single ionizable group with pK 9.9 $\pm$ 0.2 is present in each Ab site, which must be ionized for association to occur appreciably. Ultracentrifuge experiments with these mixtures are consistent with this interpretation. Acetylation of anti-R Ab, under conditions specific for the blocking of the ϵ-NH_3^+ group of lysine, inactivates it, while an excess of a specific antigen, benzenearsonic acid-azo-bovine γ-globulin (RBG), protects anti-R Ab against this inactivation. Taken together, these experiments demonstrate that a single ϵ-NH_3^+ group of lysine is critically present in each anti-R Ab site and therefore that a salt-linkage between this group and the negatively charged hapten is an essential feature of the specific binding. The light scattering data also suggest that at acid *p*H, anions, especially phosphate, effectively compete with benzenearsonic acid hapten for combination with the Ab sites and that this competition obscures any effect that the titration of the benzenearsonate ion might be expected to have on the association of hapten and Ab. The parallelism in the properties of this hapten-Ab system, and that containing the natural protein antigen bovine serum albumin and its specific Ab is discussed.

It has long been assumed that an important feature of the bonds formed in many antigen (Ag)–antibody (Ab) systems is the coulombic interaction of one or more pairs of oppositely charged groups located in the specific reactive regions of the two molecules. This assumption has derived partly from the fact that those haptens, such as benzenearsonic acid, benzenesulfonic acid, etc., which on being coupled to proteins, are most effective in eliciting antibody production, bear electric charges at physiological *p*H. The inference has been drawn that oppositely charged groups are present in the Ab sites directed against these haptens. Some support for this inference is provided by the experiments of Nisonoff and Pressman,[4] who found that the substitution on a hapten of an uncharged nitro group for a negatively-charged carboxyl group resulted in a marked reduction of the interaction of the hapten with antibodies directed to the *p*-(*p*-azophenylazo)-benzoate ion. While this demonstrates that the negative charge on the hapten is critical, it does not prove, however, that positively charged groups occur in the antibody site.[5] One of the ob-

(1) These studies were supported in part by grants from the National Microbiological Institute, United States Public Health Service, and from the Rockefeller Foundation. Parts of this paper were presented before the meeting of the American Chemical Society in Atlantic City, September, 1956.

(2) The previous paper in this series is F. A. Pepe and S. J. Singer, THIS JOURNAL, **78**, 4583 (1956).

(3) United States Public Health Service Postdoctoral Fellow of the National Microbiological Institute, 1954–1956. Dept. of Chemistry, Tufts University, Medford, Mass.

(4) A. Nisonoff and D. Pressman, THIS JOURNAL, **79**, 1616 (1957). See also D. Pressman, A. L. Grossberg, L. H. Pence and L. Pauling, *ibid.*, **68**, 250 (1946).

(5) It is possible to interpret this result without requiring a complementary charge in the Ab site. If the negatively charged hapten polarizes, or "binds," water molecules, then on formation of the hapten–Ab bond this water might be released, irrespective of the nature of the groups in the Ab site. This would result in a considerable entropy increase favoring the reaction. On the other hand, the uncharged hapten, binding less water to it, would react less favorably with Ab.