DISSOCIATION OF THE FIBROUS PROTEIN OF NERVE*

MYLES MAXFIELD** AND ROBERT W. HARTLEY, JR.
Biology Department, Massachusetts Institute of Technology, Cambridge, Mass. (U.S.A.)

The proteins of peripheral nerve axoplasm as found in the squid giant fiber have been investigated in this laboratory during the last decade. One of these proteins, that which composes the axon filaments, has been isolated and characterized from axoplasm extruded from giant nerve fibers of the common squid, *Loligo pealii*[1]. Physico-chemical studies by electron microscopy, ultracentrifugation, diffusion, viscosity, electrophoresis, light scattering and spectrophotometry showed that axon filaments are composed of a very highly charged protein and that the filaments are 100–200 A in width and indefinite in length, with very high particle weight, of the order of 10^8. Their width is uniform except for nodules which appear throughout their length. No definite periodic spacing has been observed between these nodules and on occasion preparations may be obtained in which the filaments are essentially free of nodules. Filaments behave in solution as somewhat flexible rods. Filaments constitute about 10% of the protein of nerve axoplasm.

That axon filaments are present in nerve axoplasm under physiological conditions is concluded from the following facts. The starting material for the isolation of filaments is axoplasm extruded mechanically, and free from contamination, from giant nerve axons. The only conceivable source of contamination with this procedure is the inside of the axon sheath. Electron micrographs (see for example SCHMITT AND GEREN[2] or FERNANDEZ-MORAN[3]) show that axon filaments are present in the axoplasm of nerves. Polarized light studies[4] have shown that the fresh axon contains asymmetric submicroscopic particles oriented parallel with the fiber axis. Filaments can exist in solution under the same conditions of pH and ionic strength that obtain is axoplasm.

It is apparent that axon filaments are highly unstable and undergo changes in size and shape in solution when pH and ionic strength are varied. This was first suggested by unpublished experiments of Drs. M. A. JAKUS and A. J. HODGE which showed a reversible decrease in the viscosity of aqueous solutions of whole axoplasm as the ionic strength was increased with potassium chloride. This result was confirmed and extended by the experiments (also unpublished) of Dr. B. B. GEREN which showed a steady decrease with time of the viscosity of aqueous solutions of whole axoplasm. These results on whole axoplasm have been amply confirmed, with some quantitative differences, on solutions of purified axon filaments, in which the viscosity decreases with time, increasing ionic strength and with simple dilution[1]. At high pH,

* These studies were aided by a research grant (B-24) from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, U.S. Public Health Service; by a contract between the Office of Naval Research, Department of the Navy, and the Massachusetts Institute of Technology (NR 119–100); and by a grant from the Trustees under the wills of CHARLES A. KING and MARJORIE KING.

** Present address: Medical Department, Brookhaven National Laboratory, Upton, New York.

References p. 87.

filaments break down into much smaller particles of molecular weight of the order of 70,000. This is incompletely reversible. This breakdown occurs in several steps, one of which will be described in detail below.

PREPARATION OF MATERIALS

The procedures involved in obtaining and dissecting the squid have been described in a previous paper[1]. Each day's axoplasm obtained from about 40 squid was extruded into 1 ml of 0.25 *M* sucrose solution. This solution was satisfactory in that axon filaments maintained their integrity in it for at least a week and, further, in that the mitochondria of the axoplasm were preserved. The mitochondria were removed by fractional centrifugation by Dr. JOHN FOSTER for enzymic study in this laboratory[5]. The supernate from this fractionation contained most of the soluble components of the axoplasm, including the axon filaments. It was diluted to about 13.5 ml with 0.1 *M* sucrose and centrifuged for 1 hour at 30,000 r.p.m. in the #40 rotor of the Spinco Model L preparative ultracentrifuge to remove all remaining particulate matter. The supernate was then centrifuged in the same rotor for 9 hours at 40,000 r.p.m. The sediment, in the form of a clear gel, was then redispersed in the appropriate amount of 0.1 *M* sucrose and, after 2 to 3 hours, was centrifuged to remove undissolved sediment. The macromolecular composition of this solution was estimated to be about 90% axon filaments from analysis in the Spinco Model E analytical ultracentrifuge. Further purification was not considered desirable in view of the considerable loss of material entailed in further centrifugation.

In order to obtain enough material for the light-scattering experiments, the axon filaments from four such runs were pooled. Day-to-day storage for this purpose was at 4°C in 0.25 *M* sucrose with particulates removed.

The electrophoresis run on whole axoplasm was made in the Spinco Model H instrument. For this purpose axoplasm from a single day's run was extruded into 10 ml potassium phosphate buffer pH 7.7, $\mu = 0.1$. This preparation was centrifuged to remove insoluble material and dialyzed overnight *vs.* the same buffer.

EXPERIMENTAL PROCEDURE AND RESULTS

It has been known for some time that the sedimentation velocity of axon filaments in the ultracentrifuge is markedly different at pH 7.7 and at pH 6.0, both being run at ionic strength 0.1 in potassium phosphate buffer. The sedimentation velocity is so concentration-dependent that it is not feasible to extrapolate the sedimentation velocities to infinite dilution. In order to compare the size and shape of filaments at pH 7.7 and 6.0 a single sample of purified filaments was prepared as above and divided into two portions. One portion was dialyzed against potassium phosphate buffer, $\mu = 0.1$, pH 6.0, and the other against potassium phosphate buffer, $\mu = 0.1$, pH 7.7, for an equal time. The relative viscosities at 0°C (see Table I) of both portions were determined in an Ostwald viscometer designed for 0.5 ml volume. The samples were removed from the viscometers and immediately examined in the ultracentrifuge simultaneously, one sample in a regular cell and one in a wedge cell in the same rotor. With this technique both samples can be photographed together giving superimposed patterns. The experimental conditions for each sample are as nearly alike as possible. The sedimenta-

TABLE I

pH of sample potassium phosphate buffer $\mu = 0.1$	*Relative viscosity before u.c. run* η/η_0	*Sedimentation velocity (in Svedbergs)* $\frac{dx/dt}{w^2r}$	*Relative viscosity aftsr u.c. run* η/η_0
6.0	2.13	15	1.40
7.7	2.02	7	1.49

References p. 87.

tion velocities so obtained are presented in Table I. At the completion of the ultracentrifuge run the sediments were redispersed, the solutions were removed from the cells, clarified by centrifugation and the viscosity of each of the samples was again determined as shown in Table I. The viscosities of both samples were, of course, reduced because the sediments can only be partially resuspended in these buffers, but the viscosities were still essentially equal.

The sedimentation velocity, which is most sensitive to changes in the diameter of fibrous particles in solutions of equal viscosity indicates a marked reduction in the diameter of axon filaments in the more alkaline solution. The relative viscosity, which is most sensitive to the length of fibrous particles, indicates no change in length, or at most only a small decrease, at the higher pH.

To test the reversibility of this dissociation a sample of axon filaments was prepared as described above in sucrose. The entire sample was dialyzed for 12 hours with stirring in a small dialysis bag in the cold against potassium phosphate buffer pH 6.0, $\mu = 0.1$. From the experience gained in dozens of experiments a sample in this state is known to give an ultracentrifuge pattern typical of pH 6. Because of the unfailing reproducibility of this result and in order to conserve material, the sample was not examined in the ultracentrifuge at this point but was immediately dialyzed for eleven hours against potassium phosphate pH 7.7., $\mu = 0.1$. At this point the relative viscosity was found to be 3.61, and the sedimentation velocity was typical of a pH 7.7 preparation. The sample was then split into two equal parts, one of which was dialyzed against the usual pH 6.0 buffer. The other part was dialyzed against pH 7.7 buffer. Both parts yielded only traces of filaments but with typical sedimentation velocities. This evidence indicates that the dissociation of axon filaments is indeed reversible. A considerable portion of the filaments are, however, destroyed by this procedure.

Verification of this phenomenon of dissociation of axon filaments was sought by an independent method, namely light scattering. Two different samples of purified filaments were prepared as above, one in potassium phosphate pH 6.0, $\mu = 0.1$, and the other in potassium phosphate, pH 7.7, $\mu = 0.1$. The samples were clarified by centrifuging in the Spinco preparative centrifuge and then examined in the Brice-Phoenix light-scattering photometer. The intensity of the scattered light was determined as a function of scattering angle measured from the direction of the incident beam. These measurements were made on successive dilutions of both samples of filaments and a Zimm plot[6] for each sample was prepared to demonstrate the data. Since only relative values of the parameters were of interest, the concentration of axon filaments was measured by the optical density of the solution at 278 mμ the absorption maximum of filaments. A small amount of much lower molecular weight material (including breakdown products of filaments) is always present in these solutions. Therefore the optical density of the scattering solution was measured; the filaments were removed by centrifugation, and the optical density of the remaining solution determined. The difference of these measurements was taken as the optical density of the filaments and proportional to the concentration of the filaments in solution. Fig. 1 shows the results of this experiment. The experimental curves for both samples of filaments are plotted on the same coordinate axes to make clear the marked difference between them. The ordinates of Fig. 1 are proportional, not equal, to the ordinates of the usual Zimm plot. From Fig. 1 the ratio of the particle weight of filaments at pH 6.0 to that at pH 7.7 is 3.75. From the extrapolated zero concentration curves the ratio

References p. 87.

of the lengths of the filaments can be estimated[6]. It is assumed for the purpose of this calculation that the filaments are rod-shaped, which is probably not far from reality. The ratio of the length of filaments at pH 6.0 to that at pH 7.7 is 1.14. This confirms the viscosity data given above and shows that the large difference in molecular weight must be explained by the smaller diameter of axon filaments in alkaline solution.

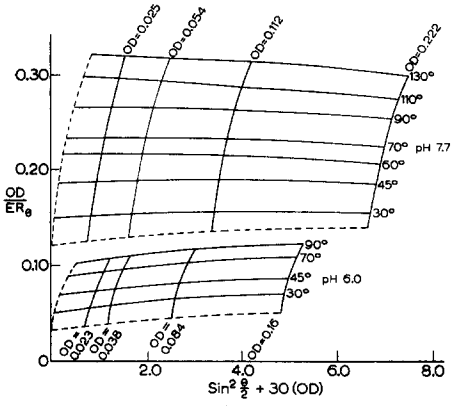

Fig. 1.

Fig. 2.

Fig. 1. Zimm plot for light scattering by axon filaments. O.D. is proportional to the concentration of axon filaments (see text).

Fig. 2. Electrophoresis pattern of whole squid axoplasm. Potassium phosphate buffer, pH 7.7, μ = 0.1. Ascending boundary. Peak marked by arrow corresponds to axon filaments.

It is of interest to inquire whether the pH at which the dissociation of axon filaments occurs in solution is near to the pH of fresh axoplasm. The pH of freshly extruded axoplasm was determined in a micro glass electrode assembly in a Beckmann pH meter. Freshly cleaned giant axons were extruded quickly and the drop of axoplasm extruded from a singly fiber was sufficient for a single measurement. The pH of axoplasm under these conditions was found to be 6.4, which does indeed lie in the region where dissociation occurs.

The dissociation of filaments is probably related to the unusually great charge density which is present. To confirm the presence of the high charge density in axon filaments an electrophoresis schlieren diagram of whole axoplasm is shown in Fig. 2 where the peak due to axon filaments is indicated by an arrow. This peak was identified with axon filaments by withdrawing small samples of solution from various levels of the electrophoresis cell for examination with the electron microscope. The filament peak is seen to move with great rapidity compared to the other components of axoplasm and therefore have a comparatively high charge density.

DISCUSSION

The conclusion that the diameter of axon filaments is greater at pH 6.0 than at pH 7.7 seems inescapable. The mechanism of this phenomenon is, however, completely unknown. It is unlikely that the filaments unravel, because their length at pH 7.7 is certainly not greater but is probably slightly less than at pH 6.0. There may be a longitudinal splitting of filaments at the higher pH into narrower filaments of similar length. The decrease in diameter might be due to removal of smaller adsorbed material on the surface of filaments. Unfortunately it is not possible to distinguish between the two possibilities on the basis of the ultracentrifuge pattern because filaments are rather unstable and are constantly breaking down, giving rise to a small amount of slowly

References p. 87.

sedimenting material which makes the identification of small adsorbed molecules not feasible. A swelling of the filaments, owing to absorption of solvent at the lower pH, would not be consistent with the data, which indicate an increase in the cross-sectional weight.

The physiological role of axon filaments remains unknown. It is curious that filaments dissociate under conditions which are so nearly physiological.

ACKNOWLEDGEMENT

The authors wish to express their appreciation for the encouragement and advice of Professor F. O. SCHMITT.

SUMMARY

The dissociation of axon filaments, the major fibrous protein of nerve axoplasm, has been studied by two independent methods, ultracentrifugation and viscosimetry, and light scattering. Both methods agree in demonstrating a marked decrease in molecular weight of filaments when the pH is raised from 6.0 to 7.7. During this process the length of the filaments decreases only slightly, while the diameter decreases markedly. It is not possible at present to distinguish a lateral splitting of filaments into nearly equal parts from a splitting off of much smaller material from the filaments. This dissociation of filaments is reversible and occurs in the region of physiological pH of axoplasm which was found to be 6.4. Axon filaments are shown by electrophoresis to be very highly charged compared to other macromolecular components of axoplasm.

REFERENCES

1 M. MAXFIELD, *J. Gen. Physiol.*, 37 (1953) 201–206.
2 F. O. SCHMITT AND B. B. GEREN, *J. Exptl. Med.*, 91 (1950) 499–504.
3 H. FERNANDEZ-MORAN, *Exptl. Cell Research*, 3 (1952) 5–83.
4 R. S. BEAR, F. O. SCHMITT AND J. Z. YOUNG, *Proc. Roy. Soc. (London)*, 123 (1937) 505–520.
5 J. FOSTER, *J. Neurochem.*, 1 (1956) 84–90.
6 B. H. ZIMM, *J. Chem. Phys.*, 16 (1948) 1099–1116.

Received October 22nd, 1956

MECHANISMS IN THE INTERCONVERSION OF RIBOSE-5-PHOSPHATE AND HEXOSE-6-PHOSPHATE IN HUMAN BLOOD*

I. ISOMERIZATION OF RIBOSE-5-PHOSPHATE IN HUMAN HEMOLYSATES

ZACHARIAS DISCHE AND HAROLD SHIGEURA
Department of Biochemistry, College of Physicians and Surgeons
Columbia University, New York (U.S.A.)

It has been shown that when ribose-5-phosphate (R-5-P) is added to a human hemolysate, sedoheptulose phosphate and alkaline-labile phosphate ester are formed after a short incubation at room temperature[1]. It was also found that the amount of the alkaline labile phosphate produced from R-5-P during a 30 minute incubation at room temperature at a concentration of $M/150$, was greater than that equivalent

* This investigation was supported by a grant from the Rockefeller Foundation.

References p. 99.