Physical-Chemical Studies of Proteins of Squid Nerve Axoplasm, with Special Reference to the Axon Fibrous Protein

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ABSTRACT The proteins in the axoplasm of the squid, *Dosidicus gigas*, have been resolved electrophoretically into a major fraction including the fibrous protein, and possibly its structural subunits, and a minor fraction including at least two proteins with low sedimentation coefficients. A partially reversible change in the structure of the fibrous protein occurs under the action of 0.4 M salt or high pH. These experiments have been interpreted to indicate that in the intact fiber one, or a few, protofibrils are arranged helically or longitudinally along the fiber axis, and linked by electrostatic bonds. On the dissociation of these bonds the separated protofibrils assume a less extended form and sediment more rapidly than the intact fibers. Some material with a lower sedimentation rate is also released on the dissociation. This fraction may comprise smaller chain fragments. The volume fraction and the approximate refractive index of the fibers have been calculated.

INTRODUCTION

The proteins of the axoplasm of the common squid, *Loligo pealii*, have been studied in this laboratory by Maxfield and Hartley (1, 2). The studies on *Loligo* were hampered by the small quantity of axoplasm obtainable from each animal and by the seasonal nature of the supply. A team of dissectors was needed to provide material for a single physical experiment. With the generous assistance of Mr. and Mrs. Louis E. Marron, and with the cooperation of the authorities of the Universidad de Chile, a unit was established at the Marine Station, Viña del Mar, Chile. There, the larger squid *Dosidicus gigas* was

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caught and dissected. The axoplasm from a 6 foot specimen of *Dosidicus* compares in quantity with that from fifteen to twenty *Loligo*, and the larger animal is more easily dissected. Thus an adequate supply of axoplasm was available.

The advantage of quantity was partly offset by the delay entailed by the distance involved. The axoplasm was at least 3 days old when it reached Cambridge. Some of the early shipments were diluted with 0.01 M potassium chloride, since experiments by Geschwind and Taylor (3) had shown this to be a suitable medium for *Loligo* axoplasm. However, the samples from Chile arrived gelled, and although ultracentrifuge experiments showed that salt solutions could release some protein from this gel, the protein could not be completely redispersed. While all later shipments were made in 0.3 M potassium chloride, some aggregation occurred in transit; the solutions did not flow evenly through a pipette, and thickened areas could be discerned in the solution as it flowed over a flat surface. Unfortunately, this had to be tolerated since any higher salt concentration broke down the fibrillar protein component. For experiments the axoplasm solution was diluted or dialyzed, but, despite stirring, it is probable that the aggregation was never completely reversed.

Although many of the properties of the *Dosidicus* axoplasm were similar to those of *Loligo* as described by Maxfield (1, 2), the materials differed in the chemical composition of the dialyzable components (Deffner and Hafter (4)) and in behavior in the ultracentrifuge. In particular the partial aggregation of the proteins in *Dosidicus* axoplasm made it impossible to separate the particulates in the solution from the fibers by centrifugation. Geschwind and Taylor (3) clarified *Loligo* axoplasm by centrifuging at 35,000 R.P.M. for 1 hour and lost relatively few fibers. In our experiments the same treatment would have sacrificed up to 50 per cent of the fibrous protein and perhaps distorted the original length-distribution of the fibers. Thus all experiments were complicated by the presence of vesicles and particulates. These contaminants made light-scattering studies impossible.

This paper briefly describes the properties of the intact axoplasmic proteins, structural changes effected in them by various reagents, and methods of fractionation. The amino acid analysis of the fractions and physicochemical studies of the smaller protein species isolated will be described in a later paper.

EXPERIMENTAL AND RESULTS

Experimental Material The squid were dissected; the giant axons, and, from the larger specimens some collateral fibers, were carefully ligatured, removed, and cleaned and the axoplasm was extruded. The gelatinous mass of axoplasm was carefully dispersed in a loose fitting Potter homogenizer in about 10 volumes of 0.3 M KCl. The axoplasm from twenty or more animals was grouped, one drop of toluene

was added to prevent bacterial growth, and the sample was shipped by air to Boston in iced vacuum flasks of stainless steel, specially developed by the Army Medical Corps for the shipment of biologicals. This solution on arrival at Cambridge was viscous and cloudy, and contained some suspended material, presumably structural debris from the extrusion process. For physical experiments the solution was centrifuged for 30 minutes at up to 20,000 R.P.M. in a Spinco model L centrifuge. The supernate was not clearly separated from the closely packed debris, since a gel layer of varying viscosity lay between, and the amount of this layer included in the extract studied could not be clearly controlled. This gelatinous layer, experiments showed, was the partially gelled fraction of the axoplasmic protein; if the whole gel was discarded, about 30 per cent or more of the fibrous protein was sacrificed.

The supernate from the centrifugation gave a strong Tyndall effect, and the opalescence increased when the solution was dialyzed free of the smaller constituents of the axoplasm. The opalescence increased even on dialyzing against a medium including the major dialyzable constituents which Deffner and Hafter (4) identified in normal axoplasm. This behavior suggested that some stabilizing agent was dialyzable, but the problem was not pursued further.

The protein solutions studied contained about 2.5 mg. of protein per ml. After dilution these proteins were easily and irreversibly denatured at an air-water or a water-liquid interface, but at the concentration at which they were shipped such denaturation did not occur. The proteins readily precipitated from solution on the addition of acetone, alcohol, formalin, hexol nitrate, 1 per cent methylene blue, traces of heavy metals (lead, copper, zinc, or mercury), or polycations such as protamines or histones. Addition of magnesium or calcium ions in a concentration of 0.1 M to the axoplasm in 0.3 KCl had no effect, but dialysis of the axoplasm against 0.02 M solutions of these ions caused some precipitation. The proteins also precipitated when dialyzed against solutions of pH 5.7 or lower.

The solutions of axoplasm exhibited a strong positive flow birefringence, and this property provided the best test of the integrity of the proteins. After standing (2 to 10 days, depending upon the sample) at 4°C. the birefringence decreased, and the extinction angle increased as the highly asymmetric proteins were degraded, presumably by bacterial action.

The short period over which the axoplasm could be studied was inconvenient, limiting any long experiments; necessitating a continuous supply; and causing a considerable wastage of material. The solutions could not be preserved by freezing since irreversible coagulation ensued.

Some recent incomplete experiments indicate that dilute axoplasm can be frozen dropwise in liquid nitrogen and preserved in the same solution, but material has not yet been shipped in this way from Chile.

Elementary Analysis

The combined macromolecular components of the axoplasm had a nitrogen content of 15.5 per cent (determined by microKjeldahl), and a phosphorus content which ranged from 0.5 to 1.2 per cent (determined by the method of

Martland and Robison (5)). Ethanol-ether–soluble lipids amounted to less than 0.2 per cent. The ultraviolet absorption of the solution showed a typical protein spectrum with a maximum at 277 mμ. There was no maximum at 260 mμ, indicating the absence of nucleic acids and lending confidence to the belief that the axoplasm was free from contamination by other cells.

Electron Microscopy

The electron microscopes used in this investigation were the RCA EMU 2 and the Hitachi HU 10 models. Most of the specimens were placed directly on collodion-covered copper grids and fixed with 1 per cent osmic acid, pH

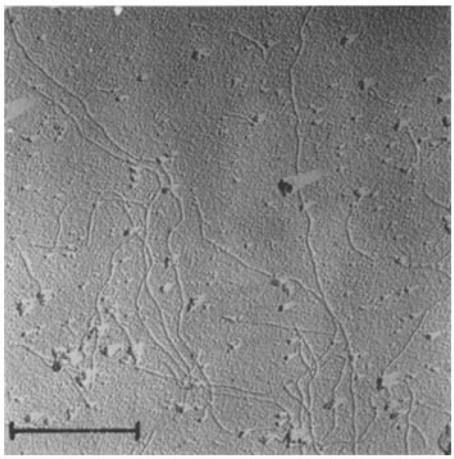

FIGURE 1. Electron micrograph of axoplasmic fibers from *Dosidicus*. Chromium-shadowed. The bar indicates 1 μ.

7.4. Polystyrene latex was added to the solutions to provide an internal indication of magnification. The preparations were shadowed with chromium.

Most preparations contained numbers of long fibers of fairly uniform diameter (about 80 A) (Fig. 1). The lengths of these fibers varied enormously; in some preparations they were so closely interwoven that the individual lengths could not be distinguished. In other preparations in which shorter fibers were seen (presumably fragments of original fibers), individual fibers were easily distinguished. Some of these measured 5 μ or more. No fibers of a diameter appreciably smaller than 70 to 80 A were detected.

In addition to the fibers, the fields contained much globular material and many vesicles. The vesicles varied in size, averaging about 0.3 μ in diameter (Fig. 2). In some electron micrographs the vesicles were plentiful. Vial (6) has reported mitochondria in the squid axons, but whether they give rise to

the large number of vesicles observed, or whether these vesicles are correlated with the vesicles in the neural synapses (7) is unknown. Occasionally many long thick fibers were observed which appeared to be formed of aggregates of fibrils. Similar structures were produced by adding 1 per cent methylene blue to the axoplasm to precipitate the classical "neurofibrils" identified by early histologists.

Attempts to count the fibers in the preparations by the method of Backus and Williams (8) failed, for, after spraying the solution on the grids or on mica, no fibers were seen. Similarly, drying a solution down onto a surface without fixation appeared to destroy the fibers.

When concentrated urea solutions were added or when the pH or the salt concentration in the axoplasm was raised, the fibers disappeared. This

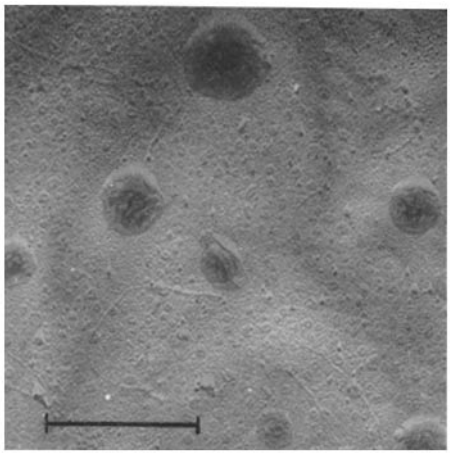

FIGURE 2. Electron micrograph of vesicles from *Dosidicus* axoplasm. Chromium-shadowed.

process will be referred to as "dissociation" (2), and the products of the dissociation will be termed "fragments." An experiment qualitatively relating the loss of the flow birefringence of the solutions with the dissociation of the fibers is described in the flow birefringence section.

Experiments are reported below which indicate that the fragments into which the fibers dissociate are markedly asymmetric. In an attempt to demonstrate these fragments, a large number of preparations were examined on collodion and on mica. In the latter technique, the solutions were applied to the surface of freshly cleaved mica, fixed by brief immersion in osmic vapor, drained, and dried. If the solution had not been previously dialyzed, it was also quickly washed. The specimens were then shadowed and stripped, as described by Hall (9). On the exceptionally smooth surface of mica, under favorable conditions, protein molecules can be resolved. In these experiments

no asymmetric molecules could be detected, but, if their diameter was small, they may have been obscured by contaminants in the water and by other molecules in the solutions. The resolution with these preparations was such that the maximum diameter of fibrous molecules which could have remained undiscerned may be set at about 30 A. These experiments indicate that the fragments of the 80 A fibers are either fibrous but less than 30 A in diameter, or that they ball up on drying and are no longer fibrous in appearance.

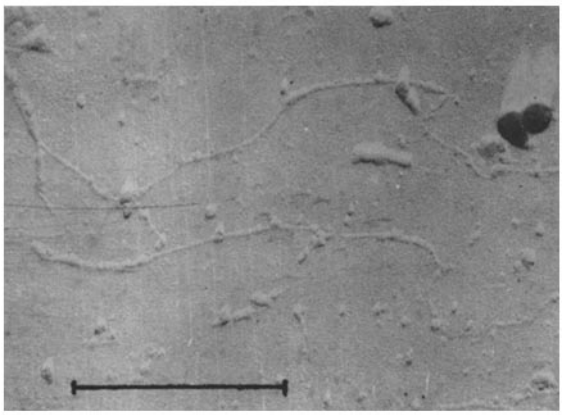

FIGURE 3. Axoplasmic fibers on mica. (The plate was unfortunately scratched.) Platinum-shadowed.

A few preparations of untreated axoplasm were successfully examined by the mica technique. They are of interest for they suggest that the true diameter of the fibers may be nearer 50 A than 80 A, the higher figure being a result of the contaminants adhering to the fibers (see Fig. 3).

Flow Birefringence

Axoplasm solutions were diluted with about 6 volumes of buffer for flow birefringence studies. The measurements were made on a Rao instrument (similar to that described by Edsall, Rich, and Goldstein (10)). It has a rotating outer cylinder, cooled by circulating water; the inner cylinder has a diameter of 4.000 cm. The annulus measures 0.23 mm., and the depth is 10 cm. The shear rates obtained ranged from 10 to 12,000 sec.$^{-1}$. Normally, experiments were made at 25°C. The light source was a 25 watt concentrated arc lamp. The speed of rotation was measured by a stop-watch or a stroboscope.

The birefringence, as a function of shear for a typical solution, is shown in Fig. 4. For comparison a similar plot for tobacco mosaic virus (taken from the data of Kausche *et al.* (11)) is included. Dilution experiments showed that the birefringence, δn, measured by the rotation, Δ, of a Sénarmont $\lambda/4$ compen-

sator, was proportional to concentration up to $\Delta = 50°$ ($\delta n = 1.5 \times 10^{-6}$) at the least, while the extinction angle, χ, was unchanged. The fact that χ was independent of the concentration at the low protein concentration used (about 0.03 per cent) made extrapolation to zero concentration unnecessary. On standing for several days, Δ decreased and the extinction angle, χ, at any shear, increased.

χ did not continue to decrease with increasing shear as predicted theo-

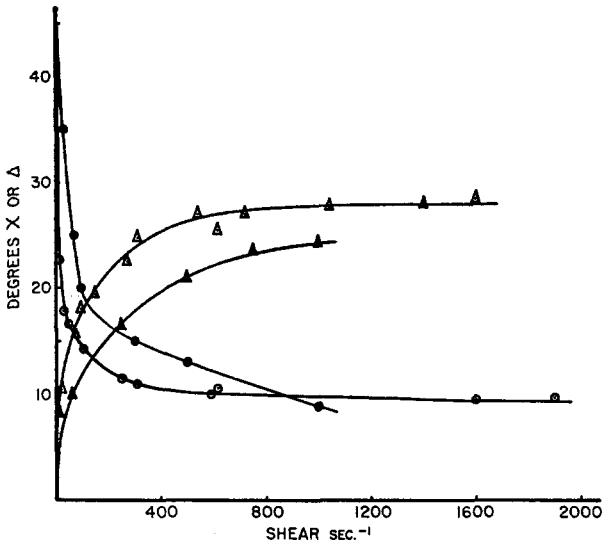

FIGURE 4. Flow birefringence as a function of shear. ⊙ and △, extinction angle and birefringence of *Dosidicus* axoplasm; ● and ▲, tobacco mosaic virus.

retically, but reached a limiting value of about 10° at 1000 sec.$^{-1}$. In most preparations, Δ continued to increase with the shear, G, as long as the former could be measured. No sign of a breakdown of the birefringent particles was detected after maintenance at a shear rate of 12,000 sec.$^{-1}$ for 15 minutes.

Precision was limited in these birefringence measurements chiefly because the solutions were opalescent, and a large fraction of the light was lost in the 10 cm. path length. In addition, since the measurements were made at 25°C., while the solutions had been stored at 4°C., bubbles of dissolved air often evolved in the annulus, though dilutions were made with degassed solutions. These bubbles, together with occasional small bubbles introduced during the filling of the apparatus, produced a scum of surface-denatured protein.

From the measured values of χ, and the known values of G, the values of $\alpha = G/\theta$ (when θ is the rotational diffusion constant) can be determined from the tables of Scheraga, Edsall, and Gadd (12). A plot of α against G (for values of χ below the limiting value at $G = 1000$ sec.$^{-1}$) is non-linear (Fig. 5),

indicating the polydispersity of the birefringent protein species. From the values of θ at 30 sec.$^{-1}$ and at 1000 sec.$^{-1}$, the lengths calculated for equivalent prolate ellipsoids (from the formula of Perrin (13),

$$\theta = \frac{3kT}{16\pi\eta a^3}\left[2 \ln \frac{2a}{b} - 1\right]$$

assuming b = 40 A) are 20,000 A and 10,400 A. This behavior shows the way in which the longer molecules are oriented at very low shear rates; progressively shorter molecules contribute to the birefringence as the shear is raised.

The flow birefringence of the solutions was decreased by as much as a factor of ten by a variety of reagents. The solutions were stable in 0.3 M KCl up to

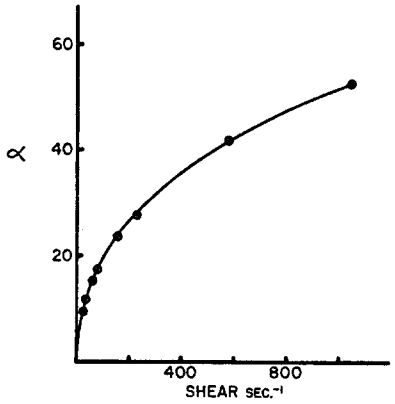

FIGURE 5. α as a function of the shear, for axoplasm.

pH 7.5 approximately; above that pH the rate of loss increased until at pH 8.1 the flow birefringence reached its limiting low value within a minute. In 0.4 M KCl the flow birefringence decreased above pH 6.3, whereas in solutions of low ionic strength the flow birefringence was unchanged up to about pH 8. Above 0.5 M KCl at any pH, the flow birefringence quickly decreased. No pH changes were detected accompanying this breakdown.

The decrease of flow birefringence under the action of high ionic strength or high pH was correlated with the rate of loss of the fibers visible in the electron microscope. Three ml. of solution were mixed with 2 ml. pH 8.12 tris 0.1 M buffer and 7 ml. water. Samples were taken at intervals for electron microscopy while the flow birefringence was measured as a function of time (Fig. 6). Since the number of fibers in solution could not be determined, it was only possible to correlate qualitatively the distribution of the lengths of the fibers measured on the grids with the gradual fall in χ. Because the flow birefringence decreased quickly to a very low value which could only be determined at high shear, and this same high shear was used throughout the experiment, the initial values of χ are the non-theoretical limiting values and cannot

be used to calculate the mean value of θ. All fibers measured appeared to have the same thickness as the original fibers, and there was no indication of longi-

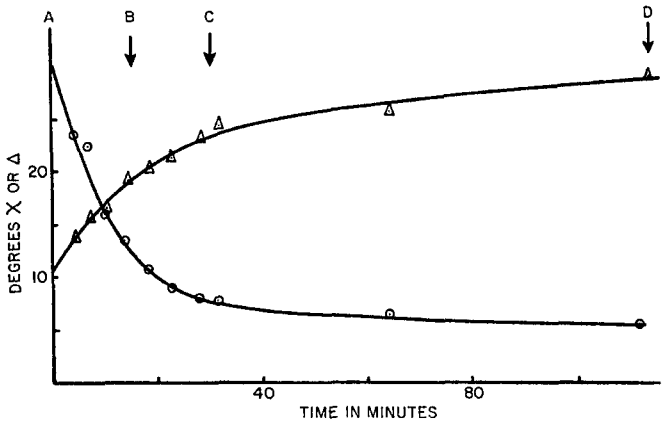

FIGURE 6*a*

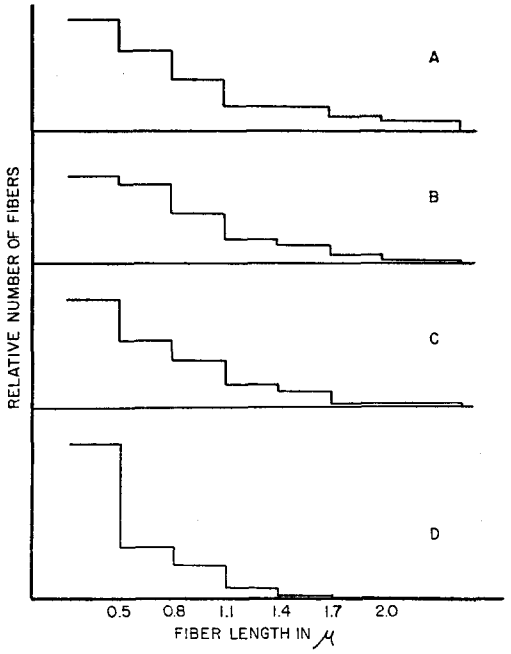

FIGURE 6*b*

FIGURE 6. (*a*) The flow birefringence (△) and extinction angle (⊙) of a dissociating solution of axoplasm (0.1 M KCl, pH 8.12), as a function of time. Measurements were made at a shear of 10,000 sec.$^{-1}$. A, B, C, and D indicate when samples were taken for electron microscopy. (*b*) Histogram showing frequency of fibers of different lengths in samples A, B, C, and D, as determined by electron microscopy.

tudinal splitting. However, the absence of narrower fibers cannot be proved because the thick background of material on the collodion might obscure such structures; similarly, some of the flow birefringence in the intact solutions might also be contributed by fibers narrower than those counted. However, the qualitative agreement between the increase in χ and the decrease in the mean length of the fibers was sufficient to give confidence that the fibers were responsible for much of the streaming birefringence, and any narrower fiber contributing to the birefringence must dissociate under the same conditions.

As the fibers are the birefringent species, the lengths ascribed to them on the basis of the Perrin formula cannot be accepted as reliable for the following reasons: (*a*) The Perrin equation applies to rigid ellipsoids while the fiber may be a flexible rod in solution. (*b*) The material is polydisperse, certainly in length and possibly in thickness, and thus the calculated length is only an average value. (*c*) The extinction angle, contrary to theory, does not continue to decrease as the shear is raised, but comes to a limiting value of $\chi \approx 10°$. Thus χ at shears below the limiting value is also, presumably, influenced by whatever factor prevents the theoretical behavior.

The action of a variety of other agents on the fibers was examined. For example, M/10 potassium thiocyanate at pH 7 caused a loss of flow birefringence in 40 minutes, while in the same concentration of KCl the solution would have remained unchanged. Dilute hydrogen peroxide or M/10 sodium thioglycollate had no influence on the rate of dissociation. Sodium citrate appeared to lower the rate of dissociation under high pH, yet this was apparently not correlated with its chelating properties, for the addition of buffered versene, or magnesium or calcium ions to the axoplasm in KCl solution did not influence the rate. Citrate, appearing to have a special influence on the axoplasmic proteins, was a useful solvent in fractionation studies. Axoplasm was stable in 0.25 M sodium citrate (pH 7.5), whereas in KCl of the same ionic strength the flow birefringence would have been lost immediately. In 30 per cent glycerol and lower concentrations of other organic solvents, dissociation occurred. The flow birefringence was unchanged or slightly increased when the salts were dialyzed from the solutions.

A temperature coefficient for the dissociation reaction was determined by comparing the rate of decrease in Δ in a solution in 0.33 M KCl at pH 6.5, at 26.2 and 35.8°C. Since the ratio of the rates was 1.52, an activation energy of 8.0 kcal./mole was calculated from the Arrhenius equation.

The decrease in the flow birefringence of the solution on standing was usually accompanied by the growth of bacteria, and it was assumed that proteolysis was the cause. Testing with trypsin, it was found that, in a 0.2 M KCl solution containing 0.2 mg. protein, 1 μg. trypsin per ml. caused the flow birefringence to decrease linearly from $\Delta = 16$ to 4° in 1 hour at 25°C. The rate of attack decreased rapidly if the salt concentration was lowered from 0.2 to 0.05 M.

The flow birefringence of the solution, δn, is given by the following equation (14):

$$\delta n = \frac{\lambda \Delta}{180h} = \frac{2\pi\phi}{n}(g_1 - g_2) \cdot f(\alpha, a/b) \qquad (1)$$

in which λ is the wave length of the light used (5400 A), Δ is the measured rotation of the Sénarmont compensator from zero, in degrees, and h is the path length (10 cm.); ϕ is the volume fraction of the birefringent protein, n is the refractive index of the solvent, f is the orientation factor, a function of α and a/b (the axial ratio of the protein) given in the tables of Scheraga *et al.* (12), and g_1 and g_2 are optical constants. In the case of very asymmetric molecules ($a/b > 10$), g_1 and g_2 reduce to a simple form

$$g_1 = \frac{n_1^2 - n^2}{4\pi}$$

$$g_2 = \frac{n^2}{2\pi} \frac{n_2^2 - n^2}{n_2^2 + n^2}$$

in which n_1 and n_2 are the refractive indices of the major and minor axes of the molecule, if the optic and geometric axes are assumed to coincide.

It may be questioned whether these equations can be applied to such long fibers, since the limit of applicability of the flow birefringence equation is usually set well below the wave length of the light used (14). This is discussed in a paper in preparation.

From equation (1) and another equation relating g_1, g_2, and the refractive increment, n_1 and n_2, can normally be determined. However, in the case of axoplasm, n_1, n_2, and ϕ are unknown. ϕ may be determined approximately from the change of Δ with the refractive index of the solvent, as shown below.

In a 2 M glycine solution the extinction angle was increased, indicating dissociation, but in M glycine χ was the same as in 0.05 M KCl solution, while Δ was reduced by 16 per cent (Fig. 7).

Since

$$g_1 - g_2 = \frac{n_1^2 - n^2}{4\pi} - \frac{n^2}{2\pi} \cdot \frac{n_2^2 - n^2}{n_2^2 + n^2}$$

then

$$(g_1 - g_2) - (g_1' - g_2') = \frac{n'^2 - n^2}{4\pi} + \frac{1}{2\pi}\left[\frac{n_2^2 - n'^2}{1 + \frac{n_2^2}{n'^2}} - \frac{n_2^2 - n^2}{1 + \frac{n_2^2}{n^2}}\right] \qquad (2)$$

in which the symbols with primes refer to the glycine solution.

Since

$$g_1 - g_2 = \frac{\Delta\lambda n}{360\, h\pi\phi f},$$

the left-hand side of equation (2) can be expressed as a function of ϕ. All terms on the right-hand side of (2) are known except n_2. As an approximation n_2 was assumed to be the same as the mean refractive index of the protein. The refractive increment of the total axoplasmic proteins, measured on a Phoenix differential refractometer, was 0.189 ± 0.004. The fibers are not the only

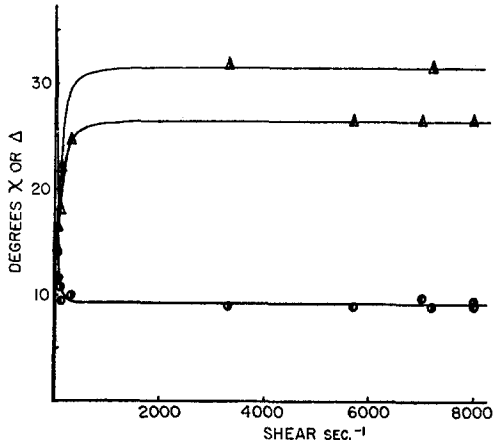

FIGURE 7. Flow birefringence of axoplasm as a function of shear. ▲ and ◑ indicate the birefringence and extinction angle measured in 0.05 M KCl; ▲ and ◐ in M glycine.

proteins present, but reasons will be given later for believing that they or their component proteins comprise a major fraction. The mean refractive index of the protein was calculated to be 1.59, from the refractive increment (assuming $\bar{v}$, the partial specific volume of the protein, to be 0.74) or 1.615, using the Lorentz-Lorenz rule. Substituting $n = 1.334$ and $n' = 1.350$ in equation (2), ϕ was found to be $4.0 - 4.4 \times 10^{-5}$. The volume fraction of the proteins in solution (0.34 mg./ml.) was 2.54×10^{-4}. Hence the protein contributing to the birefringence at $G = 1000$ sec.$^{-1}$ is 17 per cent of the total protein present. The value for the volume fraction ϕ is an approximation. The choice of the shear rate at which to measure Δ and Δ' was a compromise with inaccuracy in the rapidly increasing values of Δ for G lower than 1000 sec.$^{-1}$, and deviation from theoretical χ above this shear, leading to a false orientation factor f. The value calculated for ϕ was not much changed for n_2, ranging from 1.59 to 1.61. However, g_1 and hence n_1 was sensitive to the value assigned to n_2. Thus the intrinsic anisotropy could not be accurately determined, although it was undoubtedly high and negative (the calculated values ranged from -0.009 to -0.012).

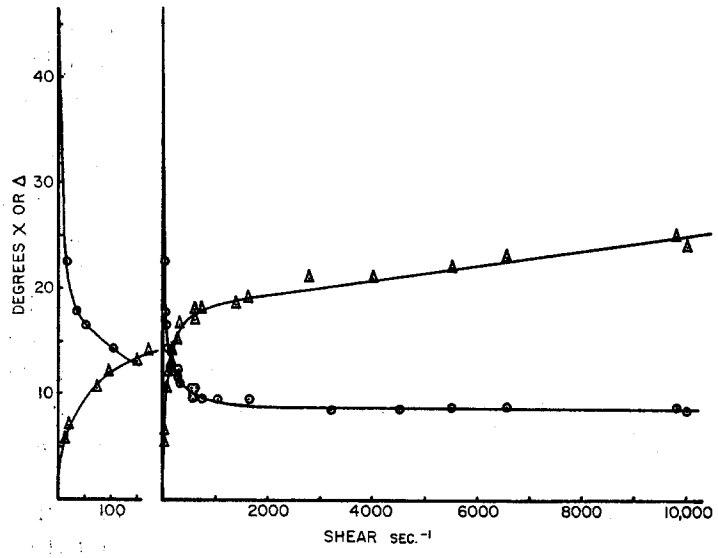

FIGURE 8*a*

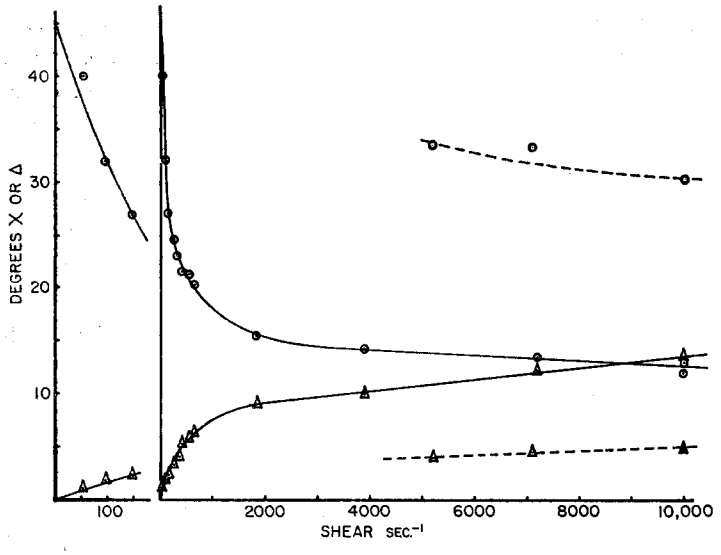

FIGURE 8*b*

FIGURE 8. Flow birefringence as a function of shear for axoplasm (*a*) before dissociation; (*b*) - - - - after dissociation and — after regeneration. In each graph the section on the left gives the data at low shear plotted against the abscissa expanded ×10.

In salt- or alkali-dissociated axoplasm the flow birefringence was not completely destroyed. Measured at a shear of 10,000 sec.$^{-1}$, about one-twelfth to one-eighth of the original birefringence remained. This could not be measured accurately in the solutions which had been diluted for the convenient measure-

ment of the flow birefringence of the intact solutions, so studies on the birefringence of the dissociated solutions were made on undiluted axoplasm solutions. Because these were very opalescent, the measurements were difficult and of low precision, particularly at low shear rates, where Δ was low. The graph of χ and Δ as a function of the shear rate G is given in Fig. 8*b*.

As in the original axoplasm solution, the values calculated for ϑ, the rotary diffusion constant, were shear-dependent, but the range of ϑ was similar for most preparations, whether dissociated by high pH or salt. The ϑ values indicated lengths of the birefringent molecules ranging from 2000 to 3000 A (assuming a diameter of 20 A) for the mean lengths of the particles present. The polydispersity of the dissociation fragments was further demonstrated by centrifuging the solution for 2 hours at 40,000 R.P.M.; this action reduced the flow birefringence to 10 per cent of its former value, and the value of ϑ was considerably raised.

The decrease in flow birefringence, on dissociation, was three times as great as would be calculated if the original long fibers all dissociated to give rodlets 2000 A long. If the optical properties of the proteins are unchanged after dissociation (*i.e.*, $g_1 - g_2$ is constant), it follows that the fragments into which the fibers break down are not all oriented under the shear applied. This observation, together with the evidence of polydispersity from the sedimentation experiments, suggests that the fibers are fragmented into polydisperse asymmetric particles. No such fibrous fragments could be detected by electron microscopy.

The flow birefringence of the dissociated axoplasm was not immediately changed by raising the pH from 8 to 10.4, but later experiments on fractionated axoplasm suggested that at pH 10 the fragments could be further dissociated into material of lower molecular weight.

When the dissociated axoplasm was dialyzed to pH 6–6.5 in 0.1 M buffer, a slow regeneration of birefringence occurred; a few details of these experiments are given in Table I. The proportion of the original birefringence (at $G = 10{,}000$ sec.$^{-1}$) recovered varied widely among different preparations and experiments (60 per cent to less than 10 per cent). Contrary to Maxfield's observations, regeneration was not improved by the inclusion of glycine in the solution, but regeneration appeared to be bettered by the inclusion of 1 per cent glycerol. However, this was not clearly demonstrated because adequate controls were not run. The graph relating χ and Δ to the shear rate for the regenerated fibers is shown in Fig. 8*b*.

Again, the variation of ϑ with G indicated a considerable polydispersity; there was also a variation between different regenerated samples. From the higher values of ϑ calculated for the regenerated fibers, it was obvious that these were shorter than the original ($\vartheta = 35$ sec.$^{-1}$ at $G = 150$ sec.$^{-1}$, and 320 sec.$^{-1}$ at $G = 7000$ sec.$^{-1}$, indicating lengths from 3900 to 8000 A). Since the

TABLE I

DISSOCIATION AND REASSOCIATION OF AXOPLASM FIBERS

Initial		Conditions of dissociation	Dissociated		Conditions of reassociation	Reassociated	
χ	Δ		χ	Δ		χ	Δ
9	39	1.3 M NaCl	*	2	M/50 NaCl, M/10 glycine		
					24 hrs.	16.5	21
					48 hrs.	16	26
9	52	pH 8.6	33	5.5	M/50 NaCl, M/10 glycine		
					24 hrs.	26.5	9
					48 hrs.	20.5	14
10	20	pH 9.2	*	2	pH 6 phosphate	17	7
9	42	pH 8.6	Not measured		pH 6.7 buffer	14	14

* Could not be measured.

higher ϑ entails a lower orientation factor, f, the high flow birefringence recovered under the best conditions must indicate a fairly complete reassociation of the fragments into a large number of shorter fibers.

Electron micrographs from the regenerated preparations showed short rodlets (1500 to 5500 A) of approximately the same diameter as the original fibers before dissociation (Fig. 9). The difference in lengths of the rodlets determined by electron microscopy and flow birefringence probably reflects the

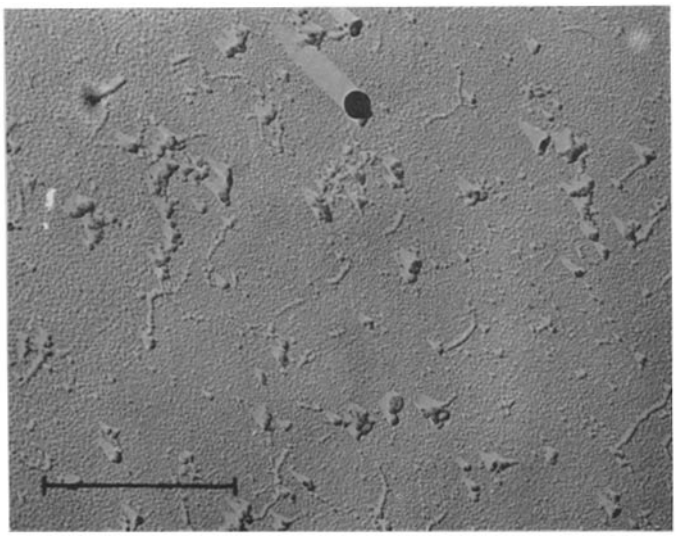

FIGURE 9. Reassociated axoplasmic fibers. Chromium-shadowed.

sensitivity of the latter technique to a small population of long fibers, and its insensitivity, at these relatively low shear rates, to short ones.

Optical Rotation

Owing to the opacity of the solution, the optical rotation of the axoplasm solution was difficult to measure, but $[\alpha]^D = -75°$ (approx.) and within the errors of the experiment there was no change in rotation on dissociation. The measurements were made on a Rudolph photoelectric instrument.

Viscometry

The axoplasm as received in Cambridge was not perfectly dispersed; hence, accurate viscosity measurements, whether by Couette or capillary viscometers, were not possible. However, some of the approximate measurements showed that the viscosity of the solutions increased steeply with the lowering of ionic strength; a plot of the viscosity of the unbuffered solutions, as a function of the concentration of KCl in the solutions, showed a noticeable inflection at 0.4 M KCl, indicating the onset of dissociation.

If the axoplasm was dialyzed against water, the viscosity increased until, if the solution were allowed to stand, it tended to gel.

The viscosity was shear-dependent; that of a 0.18 per cent solution in 0.2 M KCl dropped from an extrapolated value of $\eta_{sp} = 0.6$ at $G = 0$, to 0.25 at $G = 10{,}000$ sec.$^{-1}$. Similarly, the same solution, after dissociation, had $\eta_{sp} = 0.37$ at $G = 0$, and 0.18 at $G = 10{,}000$ sec.$^{-1}$.

Electrophoresis

The electrophoretic properties of the axoplasm were examined in a Perkin-Elmer apparatus. Most of the buffers employed were those of Miller and Golder (15). Prior to electrophoresis, the protein solutions were dialyzed at least 6 hours at 4°C.

Under electrophoresis between pH 6 and 10, the axoplasm migrated to the anode and was separated into a sharply defined fast component (60 to 70 per cent of total protein) with at least two slower components (comprising 30 to 40 per cent of the proteins present). See Fig. 10.

The hypersharpness of the leading component suggested that it contained the fibrous protein, and this was confirmed by the observation that the hypersharpness disappeared above pH 8 or after treatment with urea. When the alkali-dissociated preparations were dialyzed back to lower pH, the electrophoresis pictures resembled those of the original solutions, although the mobilities of the slower components had changed slightly.

Further confirmation that the leading component was the fibrous protein was provided by centrifuging the intact axoplasm for 2 hours at 40,000 R.P.M.; this treatment removed a considerable fraction of the birefringent protein and diminished the proportion of the fast electrophoretic component, while the amount of slower components appeared unchanged.

The concentration of protein in the axoplasm solution could not be allowed to exceed 0.3 per cent, or the solutions became so viscous on dialysis to 0.1 ionic strength that the proteins migrated as one component.

That the fibrous protein migrated in a peak which included 60 to 70 per cent of the axoplasmic protein does not imply that the fibers are present in this proportion. However, the observation that the dissociated fibers had the

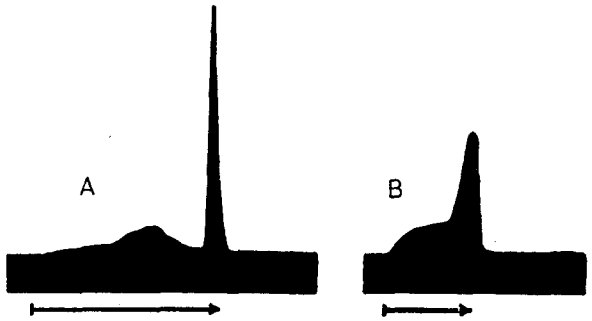

FIGURE 10. Electrophoresis diagrams (redrawn) of (A) intact axoplasm (pH 6.5) and (B) dissociated axoplasm (pH 8.4). Ascending boundaries.

same mobility suggested that the leading electrophoretic component could be composed of one protein species in various degrees of association or polymerization.

When a dissociated sample dialyzed at pH 8.2 was centrifuged for 2 hours at 40,000 R.P.M. prior to electrophoresis, the leading peak was extremely small, reflecting the greater ease of centrifuging the fragmented, as compared with the undissociated, fibers.

Electrophoresis below pH 5.8 was not possible because the proteins precipitated; hence, estimates of the isoelectric points of the proteins could not be made. The isoelectric point of the leading component was probably low, since the mobility dropped only from about 11.5×10^{-5} cm.^{2}/volt sec., at pH 9, to 10.5×10^{-5} cm.^{2}/volt sec. at pH 6.5. The mobility of the major slower component dropped from 5.5 to 4.0×10^{-5} cm.^{2}/volt sec.

The reproducibility of the mobility measurements for the different preparations was poor. The mobilities might have been influenced by traces of ions and the condition and concentration of the proteins.

Attempts by different methods of zone electrophoresis failed to resolve more clearly the number of slower components present. In electrophoresis on a

hanging paper strip or in starch gel (Smithies (16)), none of the components moved from the point of application. Zone electrophoresis in solutions by the methods of Kolin (17) concentrated the proteins into one band of insoluble gel.

Sedimentation

The behavior of the axoplasm solutions in the Spinco model E ultracentrifuge varied widely in different preparations, but there were a number of common factors. In each preparation there was a polydisperse material which sedimented rapidly. Then one or more small sharp peaks moved from the meniscus, and finally a slower component, apparently non-homogeneous, was detectable (Fig. 11).

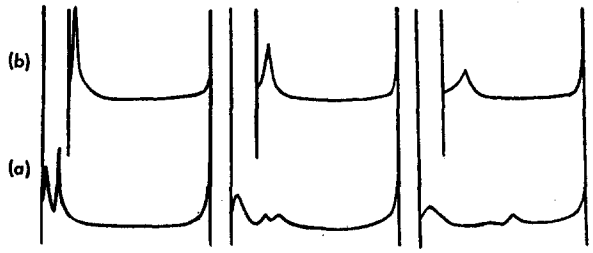

FIGURE 11. Ultracentrifuge diagrams of (*a*) in lower positions intact axoplasm (pH 6.5) and (*b*) in the upper positions (wedge cell) dissociated axoplasm (pH 8.5) respectively 4, 10, and 24 minutes after reaching 42,040 R.P.M. Bar angle 55°. s^{w}_{20} = (*a*) 4.4, 27.5, and 35.8 and (*b*) 8.2 svedbergs.

After dialyzing the solutions against buffers above pH 8, or against M NaCl, there remained a rapidly sedimenting material and the slow peak, but the intermediate sharp peaks had disappeared (Fig. 11). It was concluded that the vesicles and other particulates and the aggregated material were centrifuged down first, and that in the small sharp peaks was some of the dispersed fibrous protein. Fractionation experiments suggested that the slowly sedimenting material consisted of the material trailing the fast sharp peak in electrophoresis, and perhaps some smaller fragments from the fibers. Since the more slowly sedimenting peak appeared more asymmetric and had a higher sedimentation constant after dissociation had occurred, it appeared likely that some of the fiber dissociated products contributed to this slow peak. This was confirmed by isolating the fibrous protein by differential centrifugation into 30 or 40 per cent sucrose in 0.2 M potassium chloride. When this solution was dialyzed to pH 9 a variable percentage of the products had a low sedimentation coefficient.

Slowly sedimenting dissociation products were also demonstrated by

ultracentrifugation in synthetic boundary cells. In one experiment 65 per cent of the untreated axoplasmic proteins precipitated rapidly (as measured by the decrease in area under the peak), although only a small fraction of the proteins appeared in discrete peaks, the remainder being so polydisperse as to be undiscernible in its passage down the cell. After dissociation at pH 9 the proportion of protein sedimenting rapidly was only 40 per cent of the total, but this protein sedimented more rapidly than did the intact fibers and gave rise to no detectable peak. These proportions were confirmed by subsequent nitrogen analyses. Since this solution had been freed of most of the

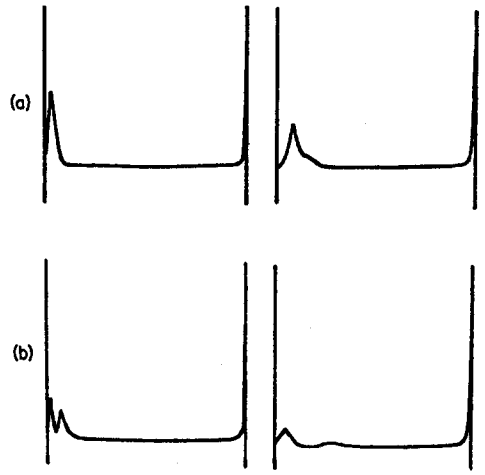

FIGURE 12. Ultracentrifuge diagram of (*a*) M/40 Na_2CO_3 extract from fibrous protein, pH 10.1, respectively 12 and 40 minutes after reaching 42,040 R.P.M., and (*b*) the same extract dialyzed to pH 6.0, 4 and 24 minutes after reaching 42,040 R.P.M. Bar angle 55°. s^{w}_{20} = (*a*) 2.7 (*b*) 3.3 and 15.8 svedbergs.

particulate components by a preliminary centrifugation at 12,000 R.P.M. it may be concluded that 65 per cent of the protein in the intact axoplasm comprises fibers or fragments. These, on dissociation, give rise to more than a third of their weight of relatively small products and a major fraction of material sedimenting more rapidly than the original fibers. When these experiments were repeated with other preparations a qualitatively similar behavior was observed, but the proportions of the large and small dissociation products and the sedimentation coefficients of the latter varied considerably. Preliminary analyses suggest that both large and small dissociation products have a similar composition.

From a concentrate of the fibers obtained by centrifugation or by chemical fractionation (to be described in a later paper), a material with a low sedimentation coefficient could be extracted by dilute sodium carbonate. Repeated extractions yielded more of this material. In the ultracentrifuge up to

50 per cent or more of each of these alkaline extracts sedimented without giving rise to a schlieren peak; a slow and frequently asymmetric peak was also present with s^{w}_{20} ranging from 2.0 to 4.5. On dialyzing back to neutral pH, a proportion of this protein extract aggregated (Fig. 12). The proteins extracted by this alkali treatment moved under electrophoresis as a single component with a mobility similar to that of the original fibrous protein, and it may be postulated that this protein is the subunit which by aggregation, gives rise to the fibers.

DISCUSSION

This investigation was intended to study the proteins present in squid axoplasm, particularly the structure and characteristics of the fibrous protein. It was realized that on its journey from Chile, the axoplasm might have undergone changes which would make the results of the investigations difficult to interpret, but for the sake of the far greater quantity of material obtainable for study, this risk was accepted. It was therefore gratifying that our results confirmed and extended many of the previous observations of Maxfield (1, 2) regarding the dissociation and reassociation of the fibers. Toward the end of the investigation, a few experiments were conducted on fresh axoplasm in Chile. The scope of these studies was necessarily limited, but from birefringence measurements and fractionation experiments, results, corroborating those obtained in Cambridge, suggested that alterations in the axoplasm *en route* from Chile were not extensive. Obviously, however, some reduction in the length of the fibers and some aggregation occurred.

The diameter of the axon filaments from different animal species has been reported to range from 65 to 200 A. In the *Dosidicus* samples on collodion, the diameter measured about 80 A. However, in two samples on mica the fibril appeared to be 50 A wide. Further work will be needed to confirm this, but the range of diameters reported possibly reflects the degree of accretion of other proteins on the fibers, as determined by the conditions of fixation.

Since the breakdown of the axoplasmic fibers is effected by a mild pH increase or salt treatment, and the breakdown occurs with no accompanying pH change, it is probably the result of the dissociation of electrostatic bonds. However, the regeneration of fibers of approximately the original diameter implies a considerable specificity in the bonding between the fragments.

The events occurring in the dissociation are obscure. Electron microscopy shows that dissociation results in an increase in the proportion of short fibers, but this is not evidence for a transverse scission of the longer fibers, since, if the chance of attack on any fiber is proportional to its length, and any single attack results in the disappearance of the fiber, the same increasing percentage of short fibers would be observed.

The residual flow birefringence appears to indicate that the dissociation produces asymmetric molecules with lengths at least 1000 to 2000 A. These are shorter than the intact fibers. A reduction in length is also suggested by the fact that the regenerated fibers never attain the lengths of the original fibers. However, the dissociation products diffuse more rapidly than the intact fibers as is shown in the electrophoresis pictures, and, as the preparative ultracentrifuge experiments showed, the fragments giving rise to the residual flow birefringence are sedimented at least as rapidly as the intact fibers. If the "dissociation" merely involved the transverse scission of the fibers, the sedimentation coefficient would be expected to fall, since the lowering of the frictional coefficient would be more than compensated by the lower molecular weight. A reasonable explanation for the increase in diffusion rate and the probable increase in sedimentation constant is a change in the structure of the molecules from an extended rod to a more compact form. The flow birefringence plotted as a function of shear does not show the positive curvature characteristic of a deformable molecule, but this might be obscured by the polydispersity of the "fragments" and the paucity of the measurements.

The following structure is postulated to explain the experimental results. The intact fiber may contain one, or several, continuous protein backbones, probably consisting of chains of globular units, constrained into a 50 to 80 A helix by electrostatic forces. This model would resemble tobacco mosaic virus with strong Van der Waal's or covalent bonds linking neighboring protein units around the helix, and electrostatic bonds between successive gyres of the helix. The dissociation reaction would simply involve the breakdown of the helical organization, allowing the component protofibrils to separate (if there are more than one) and assume a less extended form. The low molecular weight fragments released on dissociation may represent segments of the protofibrils which had been previously ruptured by proteolytic action.

The fact that the protofibrils released on dissociation, which are long enough to give rise to the "residual" birefringence, sediment more rapidly than the intact fibrils implies that the particle weight is very high and there can be few protofibrils in each fiber.

Bear, Schmitt, and Young (18) found urea and mild alkali treatment destroyed the birefringence of the axoplasm in isolated *Loligo* axons, yet the protein still precipitated in a fibrous form. These results are in accord with the suggestion that the primary effect of dissociation is the breakdown of the ordered arrangement of the constituent molecules and not an extensive rupture of the chains.

The prolonged action of dilute alkali on the dissociated fibers was shown to extract a polydisperse material which included a fraction with a sedimentation constant around 3 svedbergs. This process might reflect the rupture of alkali-labile peptide or ester bonds, but, since these products migrate with

the same electrophoretic mobility as the intact fibers, the products may represent different degrees of disaggregation of the chain of molecules constituting the protofibril. Thus, the 3 S component might be the monomer.

The values determined for the refractive index and volume fraction of the fibrous protein are comparable to some found by earlier workers. Bear, Schmitt, and Young (18) estimated for axon filaments from *Loligo* $n_1 = 1.57 - 1.6$ and $\phi = 0.004$. Thornburg and DeRobertis (19) estimated $n_1 = 1.523$ and $\phi = 0.007$ for the fibers in frog axoplasm. In our experiments the mean refractive index is 1.60 approximately, and the intrinsic anisotropy is negative; ϕ was calculated as 20 per cent of the protein in the centrifuged axoplasm. Deffner and Hafter (4) found the axoplasm consists of 4 per cent protein, but this includes particulates and vesicles, many of which were removed by the preliminary centrifugation. Since some of the fiber aggregates were also removed in this way, these effects tend to counterbalance, and the axoplasmic fibers may reasonably be assumed to correspond to at least 20 per cent of the total protein content. Thus the volume fraction of the fibrous protein in the axoplasm is roughly 0.008.

The high content of inorganic ions in the squid axoplasm and the high physiological pH (7.25, Spyropoulos (20)) suggest that *in vivo* the fibers are close to dissociation. This fact is of interest in that, although only 20 per cent of the proteins in the solution examined contributed to the flow birefringence, the fibrous protein has consistently the same electrophoretic mobility as another 40 to 50 per cent of the proteins present. And since dissociation gives fragments with the same mobility, there may be present in the axon a further large fraction of this protein in the non-fibrillar form.

This research forms part of a continuing study of nerve structure and function by Professor Francis O. Schmitt, to whom the authors express their gratitude for his constant help, interest, and encouragement.

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