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IDENTIFICATION OF A CALCIUM-BINDING, BRAIN SPECIFIC PROTEIN IN THE AXOPLASM OF SQUID GIANT AXONS

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Abstract—An acidic protein has been isolated from the optic lobes of two cephalopods, *Sepia officinalis* and *Loligo vulgaris*. The protein has been obtained in pure form by fractionation with ammonium sulphate and chromatography on DEAE-cellulose and Sephadex G 100. Its apparent molecular weight is 13,000–15,000. Glutamic and aspartic acids account for 35 per cent of the amino acid residues. The protein binds Ca^{2+} ions with an apparent dissociation constant of $2{\cdot}5 \times 10^{-5}$ M at physiological concentrations of KCl. Antibodies have been prepared against the protein purified from *Sepia officinalis*. By the microcomplement fixation technique it has been shown that the protein is highly concentrated in the nervous system of cephalopods and that the amount in the axoplasm of squid giant axons is eight to nine-fold higher than in the optic lobes of the same animal.

SEVERAL proteins unique to the vertebrate nervous system have been recently identified and characterized in terms of their physicochemical and biological properties (MOORE, 1965; BENNETT and EDELMAN, 1968; MOORE and PEREZ, 1968; GRASSO, MOORE and CICERO, 1969). The availability of specific antibodies has allowed analysis of their regional distribution in the nervous system of various species with very sensitive immunochemical methods (MOORE, PEREZ and GEHRING, 1968; MOORE and PEREZ, 1968). Some insight into the presumed role of one of these proteins (S-100) has been gained by *in vivo* injection of specific antibodies (HYDÉN and LANGE, 1970). Indications on its possible function have also been gathered by its interaction with Ca^{2+} ions, whose binding induces a conformational change in the protein (CALISSANO, MOORE and FRIESEN, 1969) and enhances the permeability to monovalent cations of artificial lipid membranes (CALISSANO and BANGHAM, 1971).

Despite intensive efforts, however, the functions of the brain-specific proteins of mammalian species remain essentially unknown. Presumably the great complexity of mammalian brain represents a major factor in hindering advances in this field. The nervous system of cephalopods, notably octopus and squid, offers distinct advantages from this point of view for the great simplicity of some of its regions, e.g. the giant axon of the squid (BULLOCK and HORRIDGE, 1965), and the homogeneity of some of its neuronal populations, e.g. the vertical lobe of octopus (YOUNG, 1971). We have therefore undertaken an analysis of the brain specific proteins present in cephalopods, in the hope that the relatively simpler structures of these nervous systems may facilitate the elucidation of their functions. In this paper we report the purification and some properties of an acidic protein present in high concentration in the axoplasm of the squid giant axon.

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Abbreviation used: SDS, sodium dodecyl sulphate.

MATERIALS AND METHODS

Optic lobes from squid (*Loligo vulgaris*) and cuttlefish (*Sepia officinalis*) were dissected from frozen specimen purchased from a local fishing company or were generously given by Dr. CONTI, University of Genoa. Animals were decapitated, their heads washed and the optic lobes carefully dissected out and stored at − 30°C. Tissue samples for immunological studies were obtained from animals freshly caught off the coast of Naples and dissected at the Zoological Station of Naples.

Determination of protein concentration. This was by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951) with bovine serum albumin (Sigma Chem. Co.) as a standard. Extinction at 280 nm was used as a measure of protein content during chromatographic procedures.

Analytical gel electrophoresis. Electrophoresis was performed in a continuous system keeping the pH and ionic strength of the buffer the same in the reservoir and in the gels (CALISSANO *et al.*, 1969). At the end of the runs, the gels were stained with 0·8% Amido Black in 7·5% acetic acid.

SDS-electrophoresis. Electrophoresis in 10% acrylamide gels containing sodium dodecyl sulphate (SDS) was performed using 0·1% SDS, 0·1% mercaptoethanol and 0·1 M-sodium phosphate buffer, pH 7·0 as described by WEBER and OSBORN (1969). Samples were heated in 1% SDS, 1% mercaptoethanol either at 95°C for 3 min or at 37°C for 60 min without appreciable difference in results. The gels were run at 8 mA/gel column (0·6 × 10·0 cm) for 4–5 h. Then they were fixed and stained in Coomassie Blue–7% acetic acid.

Acrylamide isoelectric focussing. Synthetic carrier ampholites with isoelectric points between pH 3 and 6 were purchased from LKB Instruments. Determinations were carried out according to the method described by DALE and LATNER (1968).

Amino acid analysis. Amino acid analysis was performed with the Beckman automatic amino acid analyser. Triplicate samples were hydrolysed with 6 N-HCl for 20, 40 and 60 h. Tryptophan content was not determined.

Ca^{2+} binding assay. The Ca^{2+} binding activity was determined by gel filtration assay (FAIRCLOUGH and FRUTON, 1966). Sephadex G-25 coarse columns (0·5 × 25 cm) equilibrated with 20 mM-tris–Cl buffer, pH 8·3, containing 150 or 300 mM-KCl and the desired concentration of $CaCl_2$ plus ^{45}Ca in trace amounts, were used at room temperature. Protein concentration in the eluate was determined by extinction at 280 nm and by the method of LOWRY *et al.* (1951). The Radiochemical Centre, Amersham supplied ^{45}Ca (specific activity 25 mCi/mg Ca^{2+}). Radioactivity was measured in a Nuclear Chicago Counter using BRAY (1960) scintillation fluid.

Preparation of antiserum. The anti-cuttlefish serum was prepared from rabbit injected with the purified protein. Two subcutaneous injections, each containing 2 mg of protein emulsified with an equal volume of Freund's adjuvant, were made at weekly intervals, followed by two intravenous injections of 200 μg of antigen. Sera were heated at 56°C for 25 min to inactivate complement and stored at − 25°C. They gave a single precipitin live when tested with the purified antigen and total extracts from optic lobes on immunodiffusion plates.

Immunochemical assay. The samples of nervous tissue and other organs were weighed just after dissection and frozen. Homogenization was carried out in a glass homogenizer with 25–50 vol. of cold veronal–NaCl buffer (MOORE *et al.*, 1968). The homogenate was centrifuged at 105,000 *g* for 60 min, the supernatant fluid collected and kept at −25°C until used. Antigen content in supernatant fluids was determined by a modification of the microcomplement fixation technique (MOORE *et al.*, 1968) Purified cuttlefish and squid proteins were used as standards to quantitate the amount of the antigen in the soluble extracts of each animal. The results were expressed in μg of antigen/mg of soluble proteins. Control assays were always run for anticomplementary activity in the extracts.

RESULTS

Electrophoresis in acrylamide gels of aqueous extracts obtained from the optic lobes of squid or cuttlefish revealed the presence of several protein bands with high electrophoretic mobility which were absent in the soluble extracts prepared from liver, kidney, muscle or gills (Fig. 1). This was considered presumptive evidence for the exclusive or prevalent localization of these components in nervous structures. Our attention was then focussed on the protein with the highest electrophoretic mobility toward the anode and this property was used to identify its presence during the purification procedure, in a similar fashion to that used by MOORE (1965) to isolate the S-100 protein from beef brain.

Purification of the protein. Optic lobes from squid or cuttlefish were homogenized in 5 vol. of 10 mM-tris–KPO_4 buffer, pH 7·1, containing 5 mM-EDTA (Buffer A) and

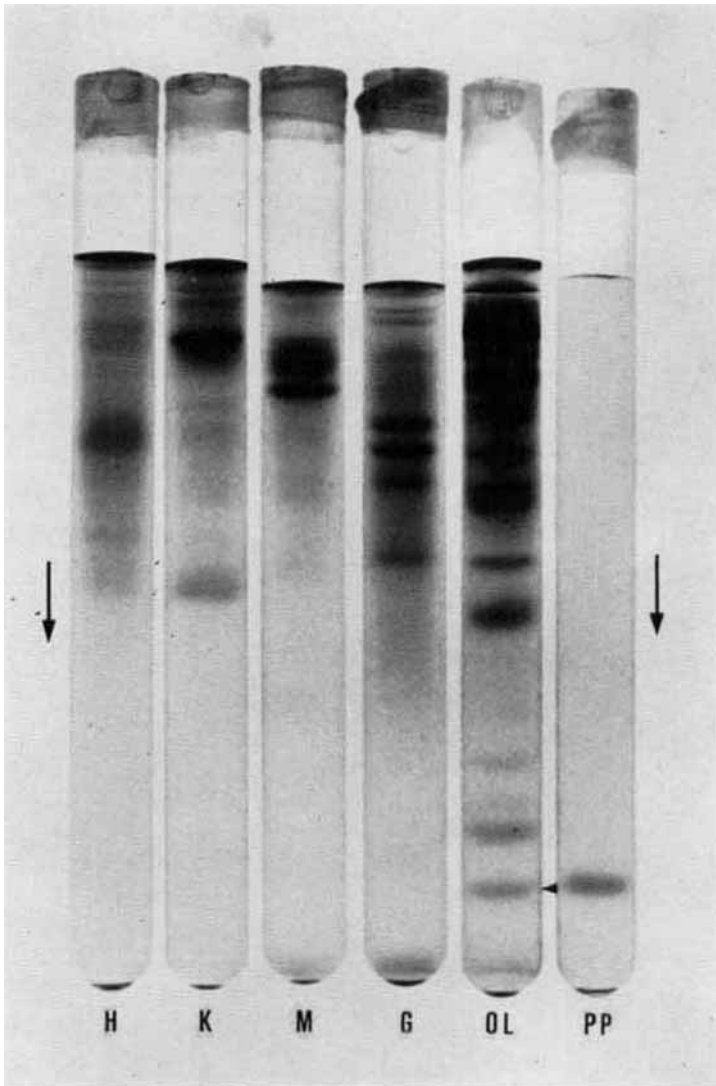

FIG. 1.—Electrophoretic patterns of total soluble proteins from various squid organs. H, hepatopancreas; K, kidney; M, muscle; G, gills; O.L., optic lobe; PP, purified protein from squid optic lobes. The amounts of protein applied were respectively 400, 410, 200, 270, 300 and 30 μg. Migration was from top to bottom.

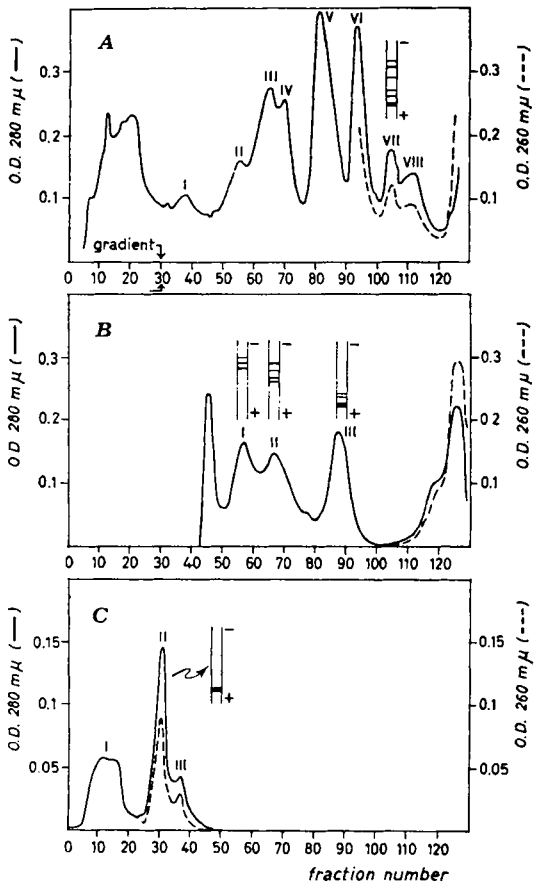

FIG. 2.—Purification steps of the squid protein. *A*: DEAE-cellulose chromatography of the 100% ammonium sulphate fraction. A sample of each peak was analysed by acrylamide electrophoresis as described in Materials and Methods. The inset shows the electrophoretic pattern of peak VII + VIII containing the fastest moving component. *B*: Sephadex G-100 chromatography of peak VII + VIII from the DEAE-cellulose column. *C*: DEAE-Sephadex chromatography of peak III from Sephadex G-100.

centrifuged at 25,000 *g* for 120 mm. The clear supernatant fluid was subjected to fractionation with ammonium sulphate at 40, 60 and 80 per cent saturation. The final supernatant fluid was brought to 100 per cent saturation with $(NH_4)_2SO_4$ and the pH was lowered to 4·5 by dropwise addition of acetic acid. After centrifugation the resulting precipitate was redissolved in 0·1 M-K-phosphate buffer, pH 7·2 and dialysed for 48 h against buffer A. This fraction was chromatographed on a DEAE-cellulose column (2 × 12 cm) equilibrated with buffer A and eluted with a gradient of pH and ionic strength (MOORE, 1965). Peaks VII and VIII (Fig. 2A) eluted just before the nucleic acids contained the protein with the highest electrophoretic mobility. They were collected together, dialysed against distilled water and freeze-dried. The frozen-dried protein was dissolved in 1 ml of 20 mM-K-phosphate buffer, pH 7·3, containing 200 mM-NaCl and added to a Sephadex G-100 column (1 × 145 cm) equilibrated with the same buffer. The third peak of Fig. 2B, which contained the

protein with some minor contaminants, was dialysed against 100 mM-K-phosphate buffer, pH 7·2 and adsorbed on a DEAE-Sephadex A-50 column equilibrated with the same buffer. Elution was performed with a three chamber gradient containing 2 M-NaCl in the third chamber. Peak II (Fig. 2C), containing the fastest moving component, was collected, desalted through a G-25 column and freeze-dried.

With this purification procedure 4–5 mg of purified protein from 100 g of fresh optic lobes from both squid and cuttlefish were usually obtained; since the concentration of this protein in the optic lobe is 0·25 mg/g.w.w. as determined immunochemically (see Table 2), the purification yield is 20–25 per cent. However the protein under investigation is also precipitated in the 80% ammonium sulphate step. This amount which represents 30 per cent of the protein measured in the extract can be purified by the same procedure, although some difficulties may be encountered due to the presence in this fraction of other small acidic proteins. The protein fraction obtained after chromatography on DEAE-Sephadex appeared to contain essentially a single component. A single band was observed by electrophoresis in a continuous system in the presence of 1 mM-EDTA (Fig. 1) and the mobility of the protein was identical to that of the fastest moving component present in the soluble extract of the optic lobe (Fig. 1). Moreover a single symmetrical peak was obtained by chromatography on a G100 column and a single band after electrophoresis in SDS gels.

Physico-chemical properties. The molecular weight of the purified protein was determined by gel filtration on a 1·0 × 150 cm column of Sephadex G-100 equilibrated with 20 mM-K-phosphate buffer and 200 mM-NaCl, pH 7·2 at 4°C, using bovine

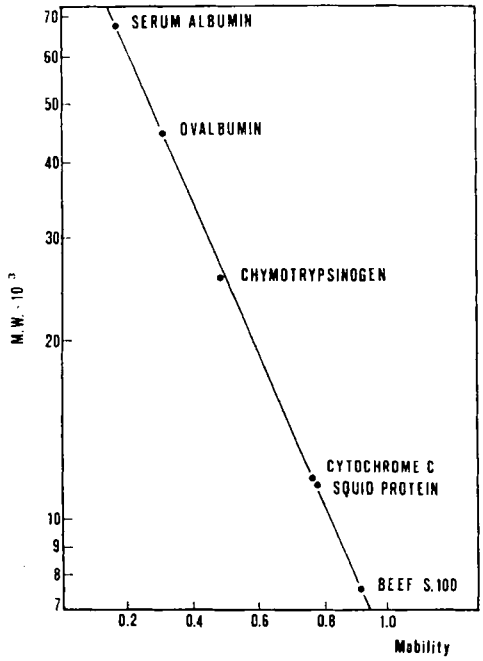

FIG. 3.—Calibration curve for sodium dodecyl sulphate gel electrophoresis. Approximately 20 μg of each protein were applied to the gels. Mobility was determined according to the method of WEBER and OSBORN (1969). Purified proteins were from Sigma Chem. Co. Beef S-100 was purified according to MOORE (1965).

serum albumin, ovalbumin, α-chymotrypsinogen and cytochrome *c* as reference standards. A molecular weight of about 15,000 (±1000) was found for both the squid and the cuttlefish protein. Polyacrylamide gel electrophoresis of the purified protein in 0·1% SDS revealed the presence of one single component with a molecular weight in the range 12,000–13,000 (Fig. 3). Standard proteins were the same as in gel filtration studies. Similar results were obtained for the squid and the cuttlefish protein. The protein is strongly acidic, as shown by its late elution from DEAE-cellulose and by its amino acid composition (Table 1), indicating a high content of glutamic and aspartic acids (up to 35 per cent of total residues). The isoelectric point of the protein from squid and cuttlefish optic lobes was measured with the technique of acrylamide isoelectric focussing and found to be 4·2 ± 0·2.

TABLE 1.—AMINO ACID COMPOSITION OF THE PURIFIED PROTEIN ISOLATED FROM CUTTLEFISH OPTIC LOBES

Amino acid	Nearest integer number of residues per 13,000 mol. wt.
Lysine	7
Histidine	1
Arginine	4
Aspartic acid	18
Threonine	8
Serine	6
Glutamic acid	24
Proline	3
Glycine	8
Alanine	8
Half cystine	—
Valine	5
Methionine	5
Isoleucine	6
Leucine	7
Tyrosine	1
Phenylalanine	5
Total	116

Tryptophan and cystine were not determined. Values for threonine, serine and tyrosine were obtained by extrapolation to zero time.

Ca^{2+} binding. Binding of Ca^{2+} to the protein purified from cuttlefish optic lobes was studied by the gel filtration technique (see Materials and Methods). Results are given as a double reciprocal plot in Fig. 4: the protein appears to have two or three binding sites with an apparent dissociation constant of 1·5 and 2·5 × 10^{-5} M at 150 and 300 mM-KCl respectively. Very similar binding constants were obtained in experiments performed with the protein purified from squid optic lobes. Varying the pH from 8·3 to 7·0 did not affect these values significantly.

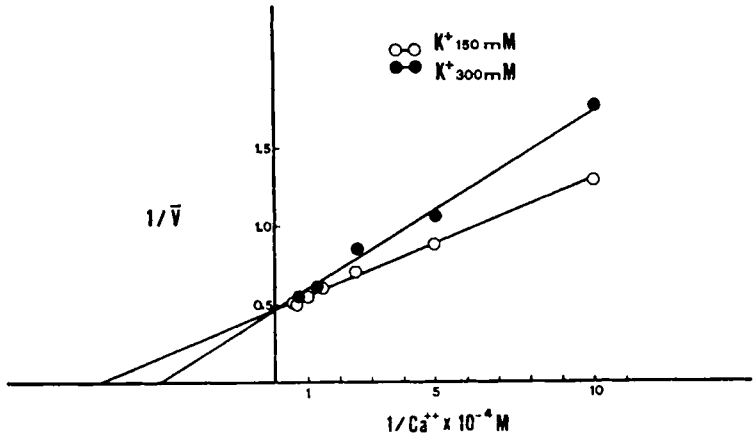

FIG. 4.—Double reciprocal plot of data for binding of Ca^{2+} by the cuttlefish protein in 20 mM-tris-Cl buffer, 150 or 300 mM-KCl, pH 8·3 at 25°C. Ordinate: average number of Ca^{2+} ions bound per molecule of protein (mol. wt. 13,500); abscissa: free Ca^{2+} concentration. Protein concentration was 0·4 mg/ml.

Immunological studies. Nearly identical complement fixation curves were obtained with the proteins purified from cuttlefish and squid optic lobes, using the anti-cuttlefish serum (Fig. 5A). Although the calibration curves for the two proteins were very similar, all assays on cuttlefish or squid samples were done with the homologous pure antigen. The pure protein from each species gave a curve very similar to that given by the crude extract from the corresponding optic lobe. Similar curves were also obtained for the axoplasm from squid giant axon (Fig. 5B). Apparently no change in the immunological properties of the protein had occurred during the purification procedure and no other antigens in the extract were reacting with the antiserum.

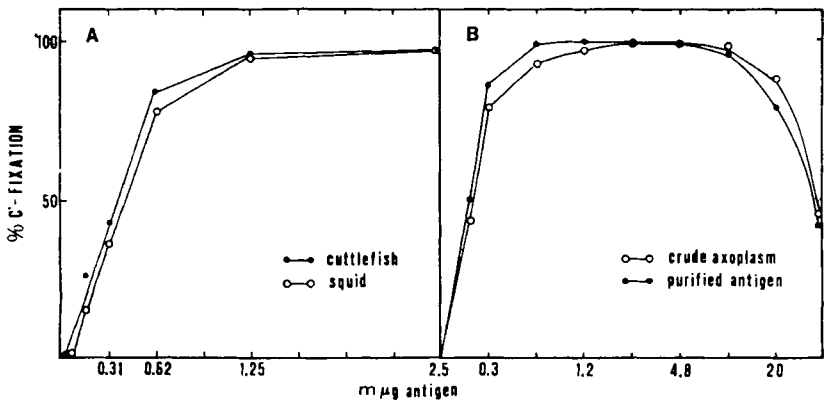

FIG. 5.—*A*: Calibration curves for purified cuttlefish and squid proteins with anti-cuttlefish serum. Fixation was as described in the text. *B*: Complement fixation curves with the purified squid protein and with the crude extract from the axoplasm of squid giant axon. The curve for axoplasm was plotted so that the amount of antigen at maximal fixation corresponded to the amount at maximum for the pure protein. For the axoplasm the point of maximal fixation corresponded to about 25 ng of total soluble proteins.

TABLE 2.—DISTRIBUTION OF THE PROTEIN IN SOME ORGANS AND IN DIFFERENT REGIONS OF THE NERVOUS SYSTEM OF CUTTLEFISH AND SQUID

Species	Organ or N.S. region	μg/mg TSP*	mg/g.w.w.	n†
Sepia off.	Optic lobe	5·1	0·317	(4)
	Hepatopancreas	0·05	0·004	(2)
	Muscle	undetectable		(2)
	Gills	undetectable		(1)
Loligo vulg.	Optic lobe	7·57	0·251	(4)
	Stellate ganglion			
	(a) giant fibre lobe	28·50	0·989	(3)
	(b) neuropil	4·55	0·075	(3)
	(c) cortical layer	3·53	0·072	(3)
	Stellate nerve			
	(a) axoplasm	60·1	2·437	(3)
	(b) sheath	51·7	0·170	(3)
	(c) small fibres	5·27	0·048	(3)
	Kidney	1·6	0·098	(2)
	Hepatopancreas	0·06	0·006	(1)
	Gills	0·13	0·003	(1)
	Muscle	undetectable		(1)
	Systemic heart	undetectable		(1)
	Branchial heart	undetectable		(1)

* TSP = total soluble proteins (see Materials and Methods).
† n refers to the number of specimens examined.

Table 2 summarizes the experimental data on the localization of the protein in different organs and in some structures of the nervous system of squid and cuttlefish. Whether in terms of μg/mg of total soluble proteins or of mg/g.w.w. it is evident that the protein is present in high concentrations in the nervous system, whereas it is absent or nearly absent in non-neural organs. The only apparent exception is represented by the kidney whose content is comparable to that of some nervous structures such as the small fibres in the stellate nerve or the neuropil and the layer containing large neurons in the stellate ganglion. In the nervous tissue the highest amount of protein is present in the axoplasm extruded from the squid giant axon, which contains 100–500 times the quantity detected in non-nervous structures and eight to ten-fold the amount present in the optic lobe. It appears likely that a significant portion of the protein associated with the sheath reflects the presence of contaminating axoplasm in view of the very low content of antigen present in the sheath per wet weight of tissue, as compared to the value referred to total soluble proteins. Soluble proteins constitute in fact a much higher percentage of total proteins in the axoplasm than in the sheath. The presence of this antigen in non-neural organs was also tested by immunodiffusion techniques. A clear precipitin line was observed with extracts from the optic lobes of squid and cuttlefish and from the axoplasm of squid giant axons. Of the non-neural organs of the squid examined, only kidney gave a faint but distinct band. With the complement fixation procedure, no cross-reactivity was observed when beef brain S-100 was used as antigen, even at a concentration of antiserum 20 times higher.

Neither was any cross-reactivity also found in crude extracts from the optic lobes of *Octopus vulgaris* when the concentration of antiserum was the same as that used for the determination on squid and cuttlefish extracts. Values of C′-fixation close to 80–90 per cent were obtained however when the concentration of antiserum was raised 20–25-fold.

DISCUSSION

Several lines of evidence concur in indicating the purity of the protein isolated from the optic lobes of squid and cuttlefish and identified as the most rapidly moving component in acrylamide electrophoresis. A single band was obtained when the purified protein was subjected to electrophoresis in a continuous system containing EDTA and in acrylamide gels in presence of 0·1 % SDS. A single symmetrical peak was also obtained in gel filtration experiments on Sephadex G-100. Additionally, antiserum to the purified antigen gave only one precipitin line when tested with the crude extract from the optic lobes or from the axoplasm of squid giant axons. The protein appears to be very acidic. This was suggested by its late elution from DEAE-cellulose and by its high electrophoretic mobility toward the cathode at alkaline pH, and was confirmed by the elevated content of dicarboxylic amino acids and by the electrofocussing experiments. Molecular weight determinations carried out by gel filtration, SDS electrophoresis and amino acid analysis indicate a value ranging between 13,000 and 15,000. On the basis of experiments of gel filtration carried out in the presence of ^{45}Ca, it has been shown that the protein binds Ca^{2+} ions with a dissociation constant of about $2{\cdot}5 \times 10^{-5}$ M in the presence of physiological concentrations of KCl. In the axoplasm of squid giant axons the total calcium concentration is 0·4–0·5 mM (KEYNES and LEWIS, 1956), of which only 0·005 mM is present as free Ca^{2+} ions (LUXORO and YANEZ, 1968), the rest being bound presumably to axoplasmic proteins. Assuming a molecular weight of 13,500 and two binding sites for Ca^{2+} at nearly physiological concentrations of KCl (300 mM, see Fig. 4), approx. 50 per cent of the total Ca^{2+} content of the axoplasm might be bound to this protein, which is present in this compartment at a concentration close to 2·40 mg/g.w.w.

Complement fixation has proved to be one of the most sensitive techniques in detecting structural differences in proteins (LEVINE, 1962). Since only slight differences in reactivity were found between the proteins from squid and cuttlefish, these antigens must possess similar immunological properties. The close similarity of the two proteins is also suggested by several of their physicochemical characteristics, such as molecular weight, Ca^{2+} binding ability and electrophoretic mobility, which are indistinguishable in the two species. On the other hand, the weak immunologic cross-reactivity observed in *Octopus* probably reflects the presence in such a species of an antigen only distantly related to the squid and cuttlefish protein. This is confirmed by the absence in the optic lobe of *Octopus* of a protein component with the same electrophoretic mobility of the cuttlefish and squid protein. All the immunological assays employed in the present study confirm the absence of this protein in non-neural organs such as muscle and systemic and branchial hearts, and its presence in very small concentrations in hepatopancreas and gills. Only kidney appears to contain appreciable amounts of the antigen. The protein might be present in kidney cells or, alternatively, this organ might have an unusually rich innervation. In the squid nervous system the protein is present both peripherally (stellate nerve and ganglion) and centrally (optic lobe), being most

concentrated in the giant axon and in the giant fibre lobe. The protein is largely intra-axonal, the amount detectable in the sheath probably reflecting the presence of residual axoplasm. It cannot be excluded however that some protein may be present in the periaxonal glial cells. The high concentration of the protein in the giant fibre lobe of the stellate ganglion further indicates its neuronal location and, when compared to the relatively low content of the protein in the small fibres of the stellate nerve and in the ganglion layer containing their neuronal somas, (cortical layer in Table 2) suggests its prevalent association with the giant fibre system.

Two acidic proteins, tubulin and filarin, have been recently isolated and characterized from squid giant axons (HUNNEUS and DAVISON, 1970; DAVISON and HUNNEUS, 1970). These proteins however, appear to be very different from the protein described in this paper on the basis of several properties including molecular weight and amino acid composition. The availability of a pure, brain specific protein, capable of binding calcium and easily measurable at the ng level should stimulate studies aimed at uncovering its role in the invertebrate nervous system. The occurrence of high concentrations of the protein in squid giant axons points to its involvement in axonal functions which, presumably, should be more readily investigated in this system in view of its outstanding simplicity.

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