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Mg^{2+}–Ca^{2+}-Activated Adenosine Triphosphatase System Isolated from Mammalian Brain*

Soll Berl and Saul Puszkin

ABSTRACT: A Mg^{2+}- or Ca^{2+}-activated adenosine triphosphatase (ATPase) has been isolated from whole brain of rat or cat. Its properties are similar, in many respects, to those of muscle actomyosin. Additional studies are presented which further characterize this protein. The Mg^{2+}-activated ATPase activity of the brain protein was dependent upon the relative concentrations of adenosine triphosphate (ATP) and Mg^{2+}. When the ATP concentration exceeded that of the Mg^{2+} the enzyme activity was inhibited. Polyethylenesulfonate also inhibited the Mg^{2+}-activated ATPase activity of the brain protein at concentrations effective with muscle actomyosin. Antisera prepared against the brain proteins showed single immunodiffusion bands against the specific antigen. There was no cross-reaction between rat and cat antigen and their respective antiserum. However, antiserum prepared against cat muscle actomyosin did cross-react with the cat brain protein but not with the rat brain protein. Proteins with actin-like and myosin-like properties have been isolated from whole bovine brain. Mixtures with each other or their counterparts from muscle resulted in a marked stimulation of the Mg^{2+}–ATPase activity of the myosin and myosin-like proteins. Such mixtures also showed increased relative viscosities which fell sharply upon the addition of ATP and rose again over periods of 30–60 min. Disc electrophoresis on acrylamide gel with and without urea revealed distinct differences among brain actomyosin-like protein, striated muscle actomyosin, and vascular actomyosin, as well as among brain actin-like protein, striated muscle actin, and vascular actin.

The brain actin-like protein contained bound nucleotide which exchanged with free [^{14}C]ATP in a fashion similar to muscle actin. For the brain actomyosin-like protein we have suggested the name, neurostenin, for the actin-like protein, neurin, and for the myosin-like protein, stenin.

The isolation of a Mg^{2+}- or Ca^{2+}-activated adenosine triphosphatase from whole brains of the rat and cat was recently described (Puszkin *et al.*, 1968). The enzyme presented properties similar to that of muscle actomyosin. This was shown in relation to its method of preparation, solubility, activation, and inhibition of ATPase activity and ability to demonstrate the phenomenon of superprecipitation in the presence of Mg^{2+} and ATP. The present communication compares further the brain protein with the muscle protein.

Perry and Grey (1956) had shown that the ATPase activity of myofibrils was dependent upon the concentration of the ATP and the Mg^{2+}. When the concentration of the former exceeds that of the latter, the enzyme activity of the myofibrils was inhibited. Similar experiments were attempted with the proteins isolated from cat and rat brain. Bárány and Jaisle (1960) reported that upon incubation of actomyosin with polyethylenesulfonate and low concentrations of ATP and Mg^{2+} the ATPase activity of the actomyosin decreased. They were able to demonstrate that this effect was due to splitting of the actomyosin into actin and myosin and they called the polyethylenesulfonate an "interaction inhibitor." The effect of polyethylenesulfonate on rat and cat brain protein was therefore studied. In immunological studies, antisera were prepared against these proteins and their purity and cross-reactivity assayed by immunodiffusion technique.

Furthermore, proteins were isolated from whole bovine brain which combined with each other and with their counterparts isolated from cat striated muscle. These interactions suggested actin-like and myosin-like properties. The actin-like protein contained bound nucleotide and its exchange with free [^{14}C]ATP was compared with that of cat muscle actin. Purity and electrophoretic mobility of the actin-like

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and actomyosin-like protein were assessed by disc electrophoresis.

We would like to point out, however, that we are not equating the brain proteins with that of the muscle but indicating in this paper the similarities and differences between them. At this time, we have no evidence that the proteins in the brain are functioning as a contractile system. To facilitate description and source we suggest the names, neurostenin, for the actomyosin-like protein, neurin, for the actin-like protein, and, stenin, for the myosin-like protein. Brief reports have been published (Puszkin *et al.*, 1969; Berl and Puszkin, 1969).

Materials and Methods

The polyethylenesulfonate was kindly supplied by Dr. M. Bárány, Institute for Muscle Disease, N. Y., and by Dr. Paul W. O'Connell, The Upjohn Company. The $Al(OH)_3$ used was amphojel, Wyeth Lab., Inc., the agar (Noble) was obtained from Difco, and Na_2ATP was obtained from Pabst Laboratories. The [^{14}C]adenosine (U) 5′-triphosphate tetrasodium salt (419 μCi/μmole) was obtained from New England Nuclear. The Ag 1-×4, Cl^- 200–400 mesh (purified Dowex, Bio-Rad Laboratories), was obtained from Calbiochem. The acrylamide and Bis were obtained from Canalco; they were recrystallized from acetone before use.

Preparation of Proteins. Actomyosin was prepared from the fresh back muscles of the cat by the procedure of Szent-Györgyi (1951). The same procedure was used to prepare actomyosin from fresh bovine aorta. Two aortas, each weighing approximately 140 g after removal of loose fat and connective tissue, were minced with scissors and homogenized in a Waring blender with 0.6 M KCl–0.01 M bicarbonate buffer, pH 9.2 (Weber–Edsall solution). The homogenate was kept in the cold room overnight, then treated further as described for the isolation of actomyosin from muscle (Szent-Györgyi, 1951).

Rat and cat brain actomyosin-like protein (neurostenin) was prepared from fresh tissue by extraction with 0.6 M KCl in an 0.01 M bicarbonate buffer, pH 9.2 (Weber–Edsall solution), as previously described (Puszkin *et al.*, 1968). It was purified twice by reprecipitation from 0.05 M Tris-HCl, pH 7.2, containing 0.6 M KCl by dilution of the KCl to 0.1 M with water at 0° at pH 6.3, obtained by addition with constant stirring of 0.125 M sodium acetate buffer, pH 4.9.

Actin was prepared from the long back muscles freshly obtained from the cat and from the fresh bovine aorta. The procedure used was essentially that described by Carsten and Mommaerts (1963). Actin-like brain protein (neurin) was prepared by adaptation of the same method to bovine brain. Muscle and brain proteins were usually prepared simultaneously for purposes of comparison. The brains of freshly killed animals were delivered to the laboratory packed in ice within a few hours after removal from the animals. The brains were carefully cleansed of piaarachnoid, their blood vessels, and choroid plexes. One or two brains were used in each preparation, a brain weighing approximately 400 g. The brains were minced in the cold room and extracted successively with 2 volumes of 0.1 M KCl, 2 volumes of 0.05 M $NaHCO_3$, 5 volumes of 1.0 mM EDTA, and twice with 5 volumes of water. Each extraction was accomplished by gentle stirring with a plastic-coated magnetic stirrer. After each extraction several layers of gauze were used to separate the liquid from the solid tissue. The tissue was then washed twice with 2 volumes of acetone (0°) and extracted once with 5 volumes of acetone in a Waring blender for 15 sec and air dried in a hood overnight. The procedure yielded approximately 90 g of residue/brain and approximately this amount was used immediately and the rest, if any, stored in the deep freeze at −22° for future use. The material was extracted (with magnetic stirrer for 30 min) with 3 volumes and then 1 volume of 0.2 mM ATP–0.2 mM ascorbate, pH 7.5 (4°). The extracts were obtained by filtration through gauze, they were combined, and centrifuged in the Spinco ultracentrifuge (30 rotor) at 105,000*g* for 1 hr. Solid KCl and $MgCl_2$ were added to a final concentration of 0.1 M and 0.1 mM, respectively, for polymerization and the solution was kept at room temperature for 1 hr and centrifuged overnight at 75,000*g* or left overnight in the cold room and then centrifuged at 105,000*g* for 3 hr. The pellet was suspended in 20 ml of 0.2 mM ATP–0.2 mM ascorbate, pH 7.5; the solution was kept in the cold overnight or dialyzed against 20 volumes of the same buffer and centrifuged at 34,000*g* for 20 min. The concentration of protein was approximately 4 mg/ml.

Myosin was prepared from the fresh back muscles of the cat by a method based on the modified procedure described by Richards *et al.* (1967). Myosin-like brain protein (stenin) was prepared by adaptation of the same method to bovine brain. Two fresh brains cleansed of blood vessels and meningeal coverings were homogenized for 1 min in a Waring blender with 3 volumes of 0.37 M KCl–0.05 M histidine-HCl–0.5 mM ATP, pH 6.8 (0°). The homogenate was stirred for 10 min with the aid of a magnetic stirrer and then centrifuged in the Servall centrifuge for 10 min at 9000*g*. The supernatant was diluted with 9 volumes of distilled water, allowed to stand for 20 min (4°), and centrifuged at 30,000*g* in the Servall for 10 min. The precipitate was dissolved in 0.6 M KCl–0.025 M histidine, pH 6.8 (100–125 ml). The solution was diluted with water to 0.3 M KCl and centrifuged in the Spinco at 100,000*g* for 1.5 hr. The supernatant was then diluted with 9 volumes of water, allowed to stand for 1–2 hr (0°), and centrifuged at 30,000*g* for 10 min. The final precipitate was dissolved in 15–20 ml of 0.6 M KCl–0.5 M Tris-HCl, pH 6.8. The concentration of protein was approximately 3 mg/ml. If not used within the next 2 days it was stored in 50% glycerol–0.6 M KCl. It could be recovered from the glycerol by dilution with 20 volumes of water.

Adenosine Triphosphatase Studies. The ATPase activity was assayed by measuring the release of P_i from ATP by the method of Marsh (1959) adapted for detection of 0.1 μg of P_i. The assay medium (1 ml) contained in final concentration 0.03 M imidazole-HCl, 0.06 M KCl, 5×10^{-4} M ATP, 1×10^{-4} M ouabain, 1×10^{-3} M Mg^{2+} (unless otherwise specified), and 0.1 mg of protein (unless otherwise specified). The pH used for the rat neurostenin assay was 6.8 and for the cat, 7.6 (Puszkin *et al.*, 1968). The assays for actin or neurin mixed with myosin or stenin were carried out at pH 6.8. The assay was run at 37° for 30 min and terminated by addition of 0.4 ml of 20% trichloroacetic acid. Protein, inactivated by boiling for 3 min or addition of the trichloroacetic acid prior to the addition of ATP, was used as blanks.

The polyethylenesulfonate studies were performed as described by Bárány and Jaisle (1960).

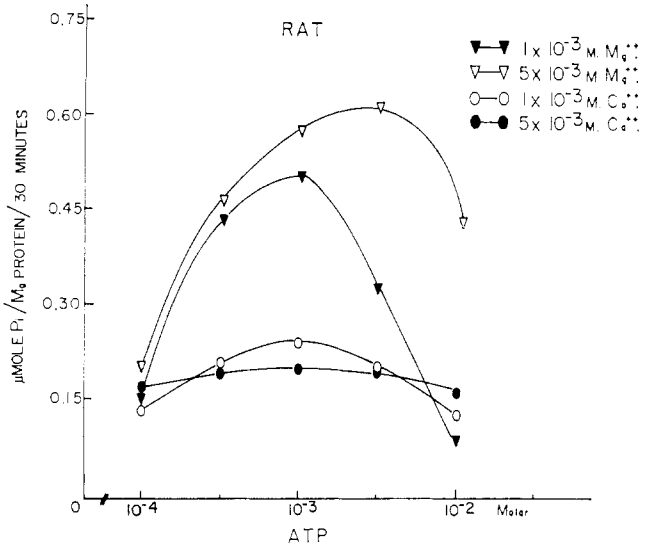

FIGURE 1: Effect of increasing concentrations of ATP with various concentrations of Mg^{2+} and Ca^{2+} on the ATPase activity of the protein isolated from rat brain: imidazole buffer, 0.03 M, pH 6.8, KCl, 0.12 M, final volume 1 ml, 0.1 mg/ml of protein, 37° for 30 min.

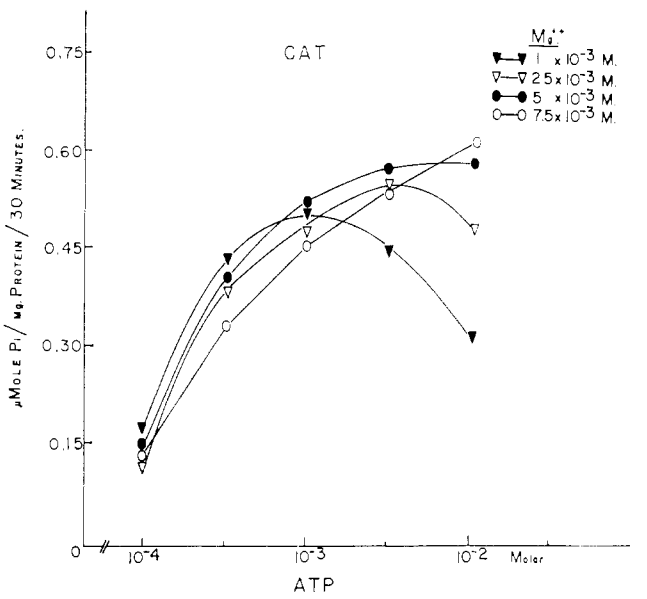

FIGURE 2: Effect of increasing concentrations of ATP with various concentrations of Mg^{2+} on the ATPase activity of cat brain: imidazole buffer, 0.03 M, pH 7.6, KCl, 0.12 M, final volume 1 ml, 0.1 mg/ml of protein, 37° for 30 min.

Antisera. Antibodies to rat and cat protein were raised in individual rabbits by three injections in the footpads and subcutaneously at 3-week intervals. Each injection consisted of 5–8 mg of protein homogenized in 2.5 ml of Freunds adjuvant, complete. The animals were then boosted with weekly intramuscular injections of 2–4 mg of protein in $Al(OH)_3$ for 12–18 weeks.

Immunodiffusion Studies. These were carried out with the Gelman apparatus by the Ouchterlony technique. The plates were prepared with 1% agar, 0.2 M KCl, and 0.4 M sodium pyrophosphate buffer, pH 8.0, and 0.1% sodium azide. The bands were allowed to develop for 2 days at room temperature and then for 5–7 days in the cold room.

Viscosity Studies. Viscometric determinations were performed in a Cannon-Ubbelohde dilution viscometer, size 50. A minimum of 6 ml was used with this instrument. The flow time for this volume of buffer was 254 sec ± 1%. The temperature was 21°. The relative viscosity (η_{rel}) of the protein solutions was determined in 0.05 M Tris, pH 7.2; the KCl concentration was always 0.6 M. The response to ATP ($\eta_{rel, ATP}$) was determined following the addition of less than 1% (v/v) of a 1.5×10^{-2} or 5.0×10^{-2} M solution of ATP in 0.05 M Tris-HCl (pH 7.2) buffer containing 0.6 M KCl. Values for the specific viscosity were calculated according to the formula: $Z_\eta = 2.3 \log \eta_{rel}$/protein concentration (mg/ml). The "sensitivity" toward ATP was calculated according to Portzehl *et al.* (1950). Per cent sensitivity equals $\log \eta_{rel} - \log \eta_{rel, ATP} \times 100/\log \eta_{rel, ATP}$. The actin and neurin were passed through short columns of Sephadex G-25 just prior to the studies to remove the ATP.

Disc Electrophoresis. Gel electrophoresis was performed on a single 7.5% acrylamide gel with and without deionized 8 M urea. The samples were run at 4° on a Canalco electrophoresis apparatus. First a current of 1 mA/gel was applied for 45 min to layer the protein at the surface of the gel, followed by a current of 4–5 mA/gel until the tracking dye passed out of the gel (about 50 min). The buffer was 5 mM Tris-glycine (pH 8.2). The gels were stained for 1 hr in 1% Amido-Schwartz in 7% acetic acid and destained by diffusion overnight in 7% acetic acid; 75–150 μg of protein/gel was used. Recrystallization from acetone of the acrylamide and Bis greatly improved the formation and resolution of the bands.

[^{14}C]*ATP Exchange.* The method is essentially that described by Kuehl and Gergely (1969). The pellets obtained following polymerization of the actin and neurin were gently homogenized in a solution containing 0.2 mM ATP–5.0 mM Tris-HCl, pH 8.0, and depolymerized by dialysis overnight at 4° against 20 volumes of the same solution. They were then centrifuged for 20 min at 30,000*g* and the protein solutions were adjusted to a concentration of 2.4 mg/ml. For each milliliter of protein solution 0.4 ml of a slurry of Ag 1-X4, 200–400 mesh, Cl^- (purified Dowex 1), was added, and the mixture was incubated at 0° for 2 min and centrifuged for 1 min in a clinical centrifuge to remove the free ATP (Hayashi and Tsuboi, 1960; Asakura, 1961). The resin had been previously batch treated several times with 5.0 mM Tris-HCl buffer, pH 8.0; the final resin buffer suspension was 1:1 (v/v) and its pH was 8.0. The protein solutions (4.5 ml containing 2.0 mg/ml) were stirred magnetically at 0° and at zero time 0.5 ml of [^{14}C]ATP (2×10^{-3} M, approximately 130,000 cpm/0.1 ml) was added. At various times (*e.g.*, 2, 5, 10, 15, 30, 45, and 60 min) 0.5-ml samples were removed, added to 0.2 ml of Ag 1-X4 resin slurry, incubated for approximately 30 sec to remove unbound ATP and centrifuged for 1 min in a clinical centrifuge. The radioactivity of the [^{14}C]ATP bound to the protein was determined in 0.2 ml of the supernatant. Blank values were established by treating with resin in a similar manner, [^{14}C]ATP solutions without protein. The complete incubation solution (0.1 ml) was counted prior

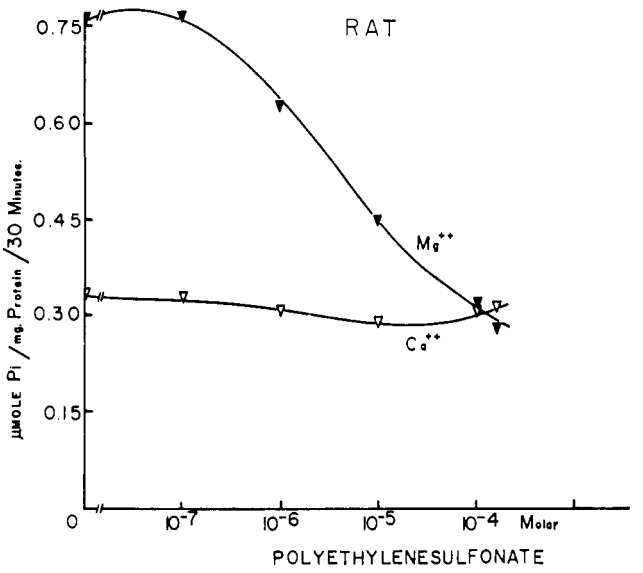

FIGURE 3: Effect of increasing concentrations of polyethylenesulfonate on the ATPase activity of protein isolated from rat brain. Protein preincubated with polyethylenesulfonate for 10 min prior to addition of the ATP: Mg^{2+} or Ca^{2+} 1×10^{-3} M, 0.03 M imidazole buffer, pH 6.8, 0.05 M KCl and 5×10^{-4} M ATP; 0.1 mg of protein, final volume 1 ml, 37°.

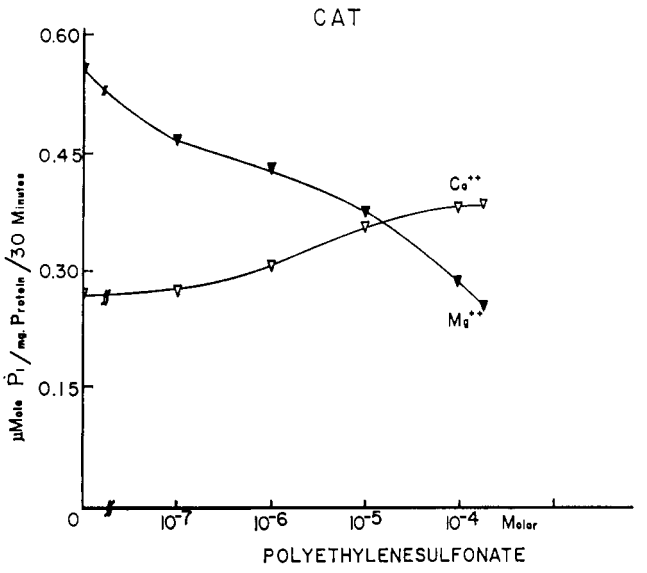

FIGURE 4: Effect of increasing concentrations of polyethylenesulfonate on the ATPase activity of protein isolated from cat brain. See Figure 3 for details, pH 7.6.

to treatment with resin to obtain the initial content of radioactivity. The counting medium was dioxane containing 10% naphthalene, 0.4% 2,5-diphenyloxazole, 0.03% *p*-bis(*O*-methylstyryl)benzene, and 10% water; the instrument was a Packard Tri-Carb scintillation spectrometer.

Protein-bound adenosine nucleotides were determined spectrophotometrically after deproteinization with 7% perchloric acid at 0°. A value of E_{260} of 13.9×10^3 M^{-1} cm^{-1} was used. The values were corrected for absorption at 280 mμ.

Protein was determined by the procedure of Lowry *et al.* (1951). The standard was serum albumin checked against lyophilized actin and neurin and their nitrogen content (Schwartzkopf Analytical Laboratory).

Results

Relative Concentrations of ATP and Cation. Figure 1 shows that as the concentration of the ATP was increased above that of the Mg^{2+} the ATPase activity of the protein isolated from rat brain (neurostenin) was inhibited. This phenomenon was also exhibited by the protein isolated from cat brain (Figure 2). At a Mg^{2+} concentration of 10^{-3} M the optimum ATP concentration was also 10^{-3} M. With both preparations as the molarity of the Mg^{2+} was increased there was a corresponding increase in the optimum ATP concentration. This did not occur with Ca^{2+} as the activating cation and rat brain protein as the enzyme.

Effect of Polyethylenesulfonate. Polyethylenesulfonate in concentrations of 10^{-6} M or greater decreased the Mg^{2+}-activated ATPase activity of the rat brain protein to the level obtained with Ca^{2+} as the activating cation (Figure 3). It had a similar effect on protein isolated from cat brain starting at a concentration of 10^{-7} M (Figure 4). The Ca^{2+}-activated ATPase activity of the brain preparations were either unaffected (Figure 3) or slightly increased (Figure 4) by the polyethylenesulfonate.

ATPase Activity of Myosin and Stenin. In two preparations the Mg^{2+}- and Ca^{2+}-stimulated ATPase activities of stenin were similar to that of myosin and in one preparation the Ca^{2+} activation was lower probably because of denaturation (Table I). In general, in the myosin preparations the Ca^{2+}-ATPase activity was approximately 10 or more times greater than that of the Mg^{2+}. In two preparations of stenin this ratio was approximately 5. However, in all cases both proteins responded with enhanced ATPase activity upon addition of either actin or neurin. In all four mixtures, the effect on the Mg^{2+}-stimulated ATPase activity was considerably greater than on the Ca^{2+}-stimulated enzyme activity. The addition of neurin to myosin or stenin resulted in a 9–60-fold increase in Mg^{2+}–ATPase activity whereas the Ca^{2+}–ATPase activity was unchanged or showed no more than a 2-fold increase. The Mg^{2+}-stimulated enzyme activity usually became greater than that for Ca^{2+} stimulation.

Viscosity Studies. The neurostenin isolated from cat brain (Figure 5) and that isolated from rat brain (Figure 6) upon the addition of low concentrations of ATP responded with a rapid fall in relative viscosity. The viscosity then rose again over a period of 30–50 min. The rate of rise was dependent upon the amount of ATP added, being slower in the presence of greater concentrations of the nucleotide. This could be done repeatedly with the same preparation. The addition of Tris-KCl buffer had no effect on the relative viscosity of the solutions.

The sensitivity to ATP, a measure of the decrease in viscosity in the presence of ATP, was 38.4–45.9% for the cat preparation and 18.9–25% for the rat preparation.

The interaction of stenin with neurin and of these compounds with actin and myosin, respectively, are described in Figure 7. Stenin alone displayed a small decrease in viscosity upon addition of ATP. Neurin and myosin each showed little or no response to ATP. The relative viscosity of the

TABLE I: Effect of Actin and Neurin on the ATPase Activity of Myosin and Stenin.[a]

μmoles of P_i/mg of Protein per min[b]											
Myosin		Stenin		Myosin + Actin		Myosin + Neurin		Stenin + Actin		Stenin + Neurin	
Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
0.055	0.69			0.28	0.65	0.49	1.01				
0.046	0.33					1.17	0.69				
0.026	0.28					0.32	0.26				
	0.87			1.59	1.49	1.26	1.17				
0.042	0.43			1.40	0.56	1.02	0.75				
0.013	0.36			0.43	0.36	0.71	0.52				
		0.071	0.34							0.55	0.39
		0.025	0.13							0.23	0.17
		0.055	0.058					0.15	0.14	0.10	0.091

[a] The myosin and actin were obtained from fresh cat striated muscle. The stenin and neurin, myosin-like and actin-like proteins, respectively, were obtained from fresh whole bovine brain. [b] Calculated on the basis of the amount of myosin or stenin in the assay medium. The actin and neurin had little or no enzyme activity. The incubation medium contained 0.03 M imidazole-HCl buffer, pH 6.8, 0.06 M KCl, 5×10^{-4} ATP, 10^{-3} M Mg^{2+}, 10^{-4} M ouabain, and 0.01–0.05 mg of each protein, final volume 1 ml. Incubation time was 30 min at 37°.

actin was too low to show any response to ATP. The mixture of stenin with neurin or actin and the mixture of myosin with neurin or actin demonstrated an increased relative viscosity. The mixed solutions then responded to the addition of ATP with a rapid fall in viscosity which rose again over a period of 30–60 min. Calculated as per cent sensitivity to ATP, the mixtures demonstrated a marked increase over that of the individual solutions (Table II). The specific viscosity which takes into account the protein concentration, also tended to be higher in the mixed solutions (Table II). Except for the mixtures of actin with myosin and of neurin with myosin the sensitivity to ATP values were lower than has been reported for striated muscle (Portzehl *et al.*, 1950) or for some smooth muscles (Bárány *et al.*, 1966; Filo *et al.*, 1963; Needham and Williams, 1963). They were similar to those reported for slime mold actomyosin-like protein (Adelman and Taylor, 1969).

Immunodiffusion Studies. Protein isolated from rat brain (neurostenin) stimulated antibodies which formed a single band with the antigen (Figure 8). It did not react with the homologous protein isolated from cat brain nor with muscle actomyosin isolated from the cat. Cat brain antigen also raised antibodies against itself which formed a single band (Figure 9); and which did not react with the rat brain protein. On the other hand, cat muscle actomyosin reacted against

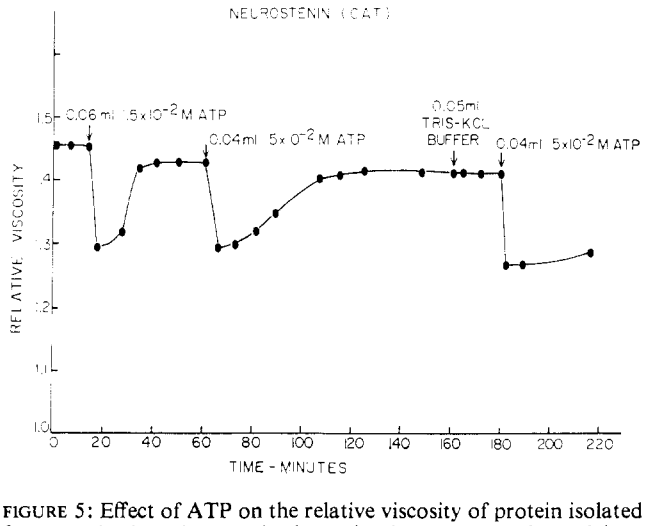

FIGURE 5: Effect of ATP on the relative viscosity of protein isolated from cat brain. Viscometric determinations were performed in a Cannon–Ubbelohde dilution viscometer, size 50, at 21°; 6 ml, 2.38 mg/ml of protein in 0.6 M KCl–0.05 M Tris, pH 7.2, was used. Neutralized ATP solution was added at indicated points.

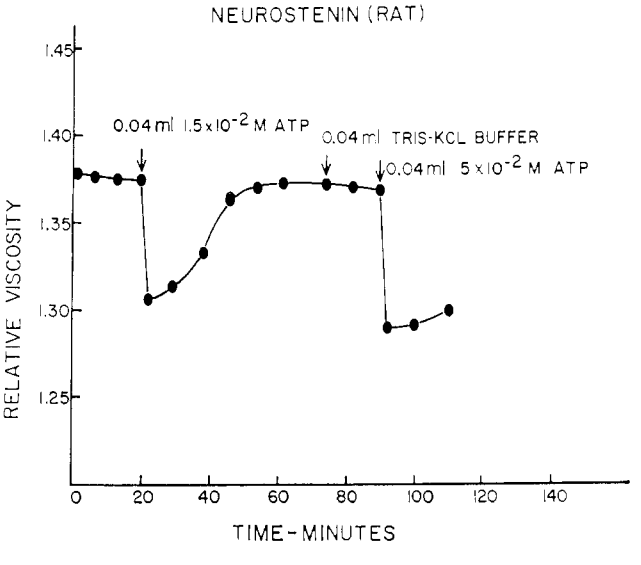

FIGURE 6: Effect of ATP on the relative viscosity of protein isolated from rat brain. See Figure 5 for details. Protein concentration was 2.74 mg/ml.

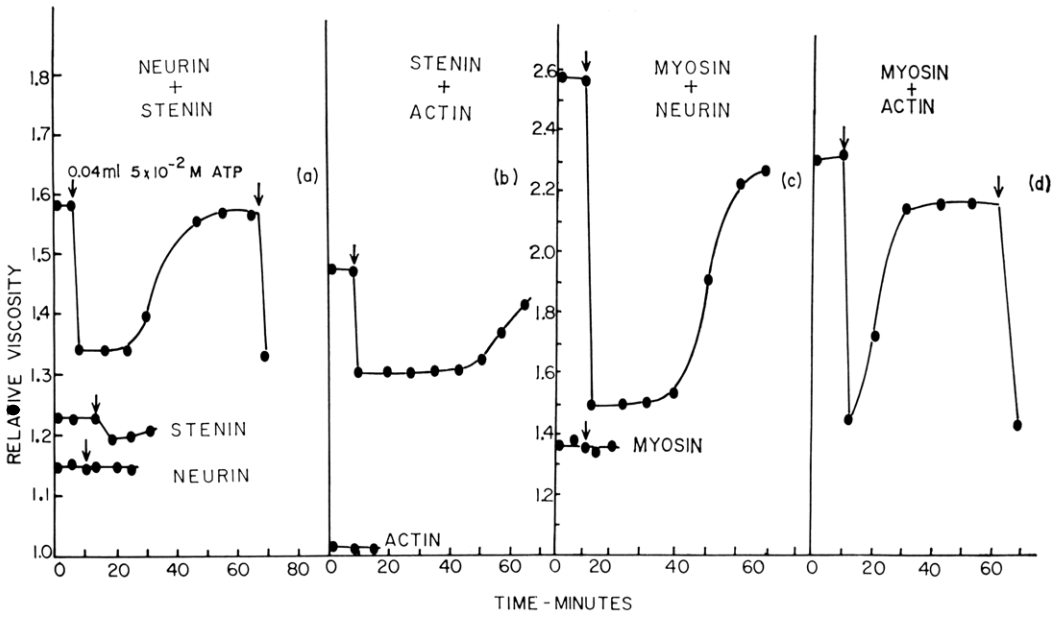

FIGURE 7: Effect of ATP on the relative viscosities of brain actin-like protein (neurin), brain myosin-like protein (stenin), striated muscle actin, and myosin, and their mixtures. Neuron and stenin were isolated from bovine brain and actin and myosin from the long back muscles of the cat. The protein concentrations were, neurin 1.62 mg/ml, stenin, 2.0 mg/ml, actin 1.62 mg/ml, and myosin 2.1 mg/ml. See Figure 5 for additional details.

its antiserum with the formation of at least two, perhaps three, bands (Figure 10). The antiserum also reacted with the protein isolated from cat brain with the formation of at least two bands but did not react with the protein isolated from rat brain.

Disc Electrophoresis. Electrophoresis in acrylamide gel of neurostenin isolated from cat brain revealed a single band which migrated at a faster rate than either actomyosin isolated from striated muscle of the cat or actomyosin isolated from the smooth muscle of bovine aorta (Figure 11). The presence of urea during electrophoresis did not disturb the integrity of the neurostenin band but did cause the formation of several additional bands from both muscle actomyosins which differed from each other in their electrophoretic pattern. Neurin isolated from bovine brain migrated as a single band at a faster rate than did actin isolated from cat muscle or bovine aorta (Figure 12). The latter two proteins also showed predominantly one band. Urea did not alter the single band formation of the neurin but did resolve the cat muscle actin into several additional bands and appeared to intensify a broad band present in the electrophoretic pattern of the actin obtained from aorta. The bands were sharper and there was less tailing in the presence of urea.

TABLE II: Effect of Actin and Neurin on the Specific Viscosity and Sensitivity to ATP of Myosin and Stenin.[a]

	Specific Viscosity		Sensitivity to ATP (%)	
Actin	0.010		0	
Neurin	0.085	0.097	0	0
Myosin	0.15	0.17	3.4	0
Stenin	0.10	0.12	16.8	0
Actin + myosin	0.22		113.6	
Actin + stenin	0.11		49.9	
Neurin + myosin	0.26	0.23	133.6	59.5
Neurin + stenin	0.12	0.18	52.4	73.0

$$\text{Specific viscosity } (Z_n) = \frac{2.3 \log \eta_{rel}}{\text{mg/ml of protein}}$$

$$\% \text{ sensitivity to ATP} = \frac{\log \eta_{rel} - \log \eta_{rel,\,ATP} \times 100}{\log \eta_{rel,\,ATP}}$$

[a] Measurements were made at 21° in a Cannon–Ubbelohde viscometer, size 50. Data from 2 separate sets of preparations are given. See Figure 7 for experimental details.

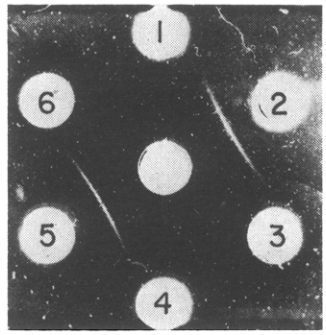

FIGURE 8: Immunodiffusion reactions of rat and cat neurostenin and cat muscle actomyosin with antiserum to the rat brain protein. Center well contained rabbit antiserum to rat neurostenin. Wells 1 and 4 contained cat neurostenin, wells 2 and 5 contained rat neurostenin, and wells 3 and 6 contained cat muscle actomyosin. Wells 4, 5, and 6 contained twice the protein concentration of wells 1, 2, and 3, respectively.

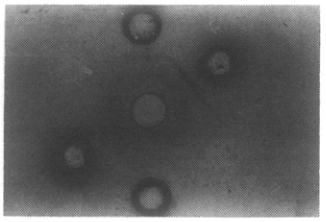

FIGURE 9: Immunodiffusion reactions of rat and cat neurostenin (antigen) with the antiserum to the cat protein. Center well contained rabbit antiserum to cat neurostenin. Upper two wells contained cat neurostenin and the lower two wells rat neurostenin. The darker band had twice the concentration of antigen.

Nucleotide Content and [^{14}C]*ATP Exchange.* The nucleotide content of the protein varied considerably from one preparation to another. Our best results yielded 0.86 mole of nucleotide for muscle actin and 0.68 mole of nucleotide for brain neurin per 60,000 g of protein. Although the ratio of nucleotide to G-actin on a mole to mole basis is very probably 1 (Gergely, 1964), considerable variation in this ratio has been obtained in different laboratories as well as in different preparations in the same laboratory (Martonosi *et al.*, 1960; Martonosi and Gouvea, 1961; Bárány *et al.*, 1961; Strohman and Samorodin, 1962; Kuehl and Gergely, 1969).

The amount of [^{14}C]ATP exchanged with either protein also varied from preparation to preparation and was proportional to the amount of bound nucleotide present in the protein. In Figure 13 it can be seen that under our conditions of preparation and testing maximum exchange had occurred in both proteins in approximately 5 min and both proteins had exchanged approximately an equal amount of ATP. The amount of ATP exchanged by each protein was approximately 0.5 mole/60,000 g. Since the bound nucleotide of G-actin should be completely exchangeable (Martonosi *et al.*, 1960; Kuehl and Gergely, 1969) the results suggest that the preparations contained denatured protein, protein which did not bind nucleotide or protein which contained nonexchangeable nucleotide. The use of 4.7×10^4 daltons as the molecular weight of actin (Rees and Young, 1967) would further decrease the nucleotide:actin ratio. This value, however, was reported for rabbit striated muscle actin. Whether this is true for cat muscle actin or brain actin-like protein remains to be established.

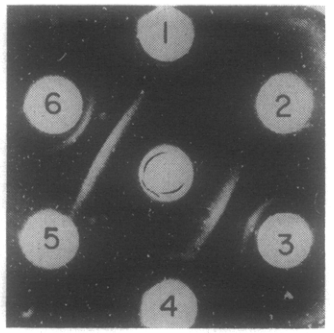

FIGURE 10: Immunodiffusion reaction of cat muscle actomyosin and rat and cat neurostenin with antiserum to cat muscle actomyosin. Center well contained rabbit antiserum to cat muscle actomyosin. Wells 1 and 4 contained cat neurostenin, wells 2 and 5 contained rat neurostenin, and wells 3 and 6 contained actomyosin. Wells 4, 5, and 6 contained twice the protein concentration of wells 1, 2, and 3, respectively.

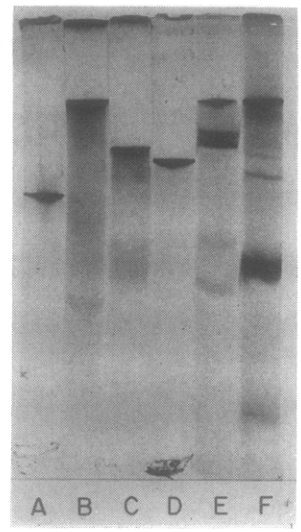

FIGURE 11: Acrylamide gel electrophoresis: A, B, and C did not contain urea; D, E, and F contained 8 M urea in the gel and sample. A and D are neurostenin isolated from cat brain. B and E are actomyosin isolated from cat striated muscle. C and F are actomyosin isolated from bovine aorta.

Discussion

A number of investigators have raised the question as to whether there may be present in nervous tissue a contractile actomyosin-like protein. Thus, Libet (1948), studying Ca^{2+}-activated ATPase activity in squid giant axon, suggested that proteins similar to the myosin system in muscle may be associated with the conduction of nerve impulses. Bowler and Duncan (1967, 1968) in their studies of crayfish nerve and frog brain also voiced the idea that an actomyosin-like protein may be responsible for passive permeability of cells. Mg^{2+}- or Ca^{2+}-stimulated ATPase activity, different from that of the Na^+–K^+–ATPase, has been described as present in the isolated synaptic vesicles of guinea pig brain (Hosie, 1965; Kadota *et al.*, 1967) and rat brain (Germain and Proulx, 1965).

Histochemical techniques have also been applied to the demonstration of Mg^{2+}- or Ca^{2+}-activated ATPase activity in nervous tissue. Naidoo and Pratt (1956) described its distribution in rat brain. The intracortical pattern of distribution of Mg^{2+}-stimulated ATPase activity in rat brain was more intimately studied by Hess and Pope (1959) and Lewin and Hess (1964) and in human frontal cortex by Hess and Pope (1961). Novikoff (1967) has described the distribution of Ca^{2+}-stimulated ATPase activity in dorsal root ganglia and peripheral nerve and has reviewed the findings of others.

Movement of glia and neuronal endings have also been reported. Thus, Benitez *et al.* (1955) described pulsating movements of glia in response to serotonin and Speidel (1935) spoke of movement of nerve endings. Phase contrast, time-lapse cinematography of brain cultures revealed changes in shape and pulsatile activity of glia as well as movement of nerve endings, neuronal nuclear rotation, and bidirectional

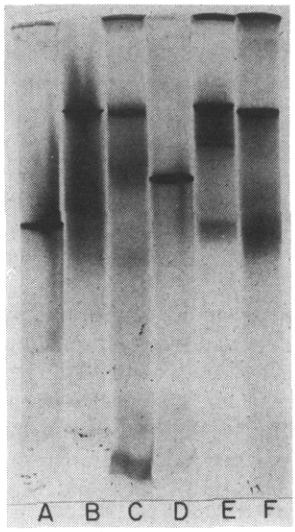

FIGURE 12: Acrylamide gel electrophoresis: A, B, and C did not contain urea; D, E, and F contained 8 M urea in the gel and sample. A and D are neurin isolated from bovine brain. B and E are actin isolated from cat striated muscle. C and F are actin isolated from bovine aorta.

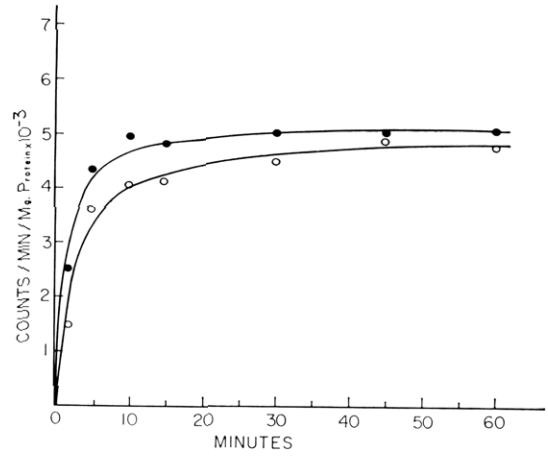

FIGURE 13: [^{14}C]ATP exchange of G-actin and neurin. The reaction mixture contained 1.8 mg of protein, 2×10^{-4} M [^{14}C]ATP (~130,000 cpm/ml) and 5 mM Tris-HCl, pH 8, 0°: (● — ●) G-actin isolated from cat striated muscle; (o — o) neurin isolated from bovine brain.

flow of particles and vacuoles in the axons (Pomerat *et al.*, 1967).

The isolation of actomyosin-like protein from tissues other than striated or smooth muscle gave additional credence to the possibility that such protein might also be present in brain. Thus, actomyosin-like protein (thrombosthenin) has been isolated from blood platelets (Bettex-Galland and Luscher, 1960), from ascites sarcoma cells (Hoffman-Berling, 1956), from liver mitochondria and cell membranes (Ohnishi and Ohnishi, 1962; Neifakh *et al.*, 1965), from erythrocyte membranes (Ohnishi, 1962), and from slime mold (Adelman and Taylor, 1969).

To establish the presence of such a protein in brain would require its isolation so that its chemical and physical properties could be compared with that of muscle protein. Its isolation has been described (Puszkin *et al.*, 1968) and additional studies were undertaken to further establish the nature of the protein. Inhibition of enzyme activity when the ATP concentrations exceeded the Mg^{2+} concentrations (and *vice versa*) and the effect of polyethylenesulfonate on the ATPase activity are additional criteria but not necessarily specific for actomyosin. A more valid characteristic of actomyosin is that at high ionic strength (about 0.6) it responds to low levels of ATP with a fall in viscosity due to dissociation into actin and myosin. The viscosity rises again as the ATP is hydrolyzed. This phenomenon was demonstrated by the brain proteins (Figures 5 and 6) and could be repeated several times with the same sample. The brain protein differed from that of striated muscle in that its sensitivity to ATP was considerably less (Portzehl *et al.*, 1950).

The single sharp bands obtained by immunodiffusion suggest that the brain antigens (neurostenin) contain but a single antigenic site. They also suggest little, if any, contamination by other proteins. The antigenic properties appear to be species and organic specific since the proteins from rat and cat brain did not produce cross-reacting antisera nor did the antisera react with the muscle antigen (actomyosin). However, the muscle antigen did produce antisera which reacted with brain antigen from the same species. Muscle actomyosin from the cat did produce antibodies which reacted with brain protein isolated from the cat but not with that isolated from the rat. The single band obtained with acrylamide gel electrophoresis also supports the purity of the brain preparation.

To finally establish that we were indeed concerned with an actomyosin-like protein it was essential to prepare from brain actin-like and myosin-like protein which would interact with each other as well as with their counterparts isolated from muscle. Such interaction should perhaps result in a stimulation of the Mg^{2+}–ATPase activity and should convert solutions of protein with viscosities insensitive to ATP into solutions of protein with viscosities of increased sensitivity to ATP. We were able to isolate from bovine brain, proteins which were, in these respects, actin like and myosin like in their behavior. The actin-like protein did stimulate the Mg^{2+}–ATPase activity of myosin-like protein and of myosin. Actin did stimulate the Mg^{2+} activity of myosin-like protein as well as that of the myosin. These combinations also demonstrated changes in viscosity and sensitivity to ATP which would be anticipated from actin-like and myosin-like proteins, but to a lesser degree than the muscle proteins. The actin-like protein did contain bound nucleotide and did exchange with free [^{14}C]ATP under conditions which would be characteristic of such a protein. The amount of exchange, however, was much less than reported for muscle actin.

The ATPase activities of the individual preparations show considerable variation. This is probably true in large part because of the instability of the enzymes during preparation. Their activity also is rapidly diminished upon freezing or storage at 4° beyond several days. In addition, varying amounts of actin or neurin may be associated with the myosin or stenin, respectively, in the different preparations. The myosin-like protein isolated from brain exhibited a higher ATPase activity alone and when stimulated by the addition of actin-like protein as compared with the brain actomyosin-like protein isolated as the complex. Similarly,

the myosin-like protein purified from slime mold also exhibited considerably greater ATPase activity than did the isolated actomyosin-like complex (Adelman and Taylor, 1969). This may be related to degree of purity, state of inhibition or inactivation, or perhaps to the ratio of actin-like protein bound to myosin-like protein in the isolated complex. In several preparations the actomyosin-like protein isolated from cat brain, for reasons which we cannot explain, had an approximately five- to tenfold greater enzymatic activity stimulated by either Mg^{2+} or Ca^{2+} than usually encountered.

The possibility that the proteins are being extracted from the smooth muscle of the vasculature has to be considered. The small arteries within the cerebral tissue require consideration since the surface vessels and choroid plexus are readily removed and the intracerebral veins and capillaries do not contain smooth muscle. Approached from two different aspects, (1) the number of arteries entering into the intracerebral tissue of the cat from the surface and the diameter of these vessels (Blinkov and Glezer, 1968) and (2) the volume of the intracerebral vascular bed, it has been calculated that the arterial tissue may comprise approximately 2% of the brain, wet weight. This is not likely to yield the 1–2% of total brain protein which is extracted from brain as neurostenin (Puszkin *et al.*, 1968). The yield of purified neurin was approximately 0.2–0.4% of the total brain protein. Furthermore, partial recovery is to be expected since exhaustive extraction of the tissue is not attempted.

Smooth muscle actomyosins appear to have a different cationic activation than that of skeletal muscle or neurostenin. Actomyosin isolated from both hog (Bohr *et al.*, 1962; Filo *et al.*, 1963) and bovine (Ruegg and Strasser, 1963) carotid arteries were activated to a greater extent by Ca^{2+} than by Mg^{2+}. In the present study this was also true for actomyosin isolated from bovine aorta. The Mg^{2+}- and Ca^{2+}-stimulated ATPase activity, respectively, were 0.014 and 0.028 μmole of P_i min per mg of protein. These values are similar to those reported for hog carotid artery (Filo *et al.*, 1963) and the reverse of that found for neurostenin. The Mg^{2+}- and Ca^{2+}-stimulated activities, respectively (μmole of P_i/min per mg of protein), for the rat brain protein were 0.027 ± 0.011 and 0.013 ± 0.007; $N = 10$ and for the cat brain protein were 0.023 ± 0.009 and 0.015 ± 0.006; $N = 14$ (av $\pm$ std dev) It had been reported previously that cerebral vessels (rat) have a greater Ca^{2+}- than Mg^{2+}-stimulated ATPase activity and that the reverse is true for the nervous tissue (Naidoo and Pratt, 1956). Similarly low Mg^{2+} activities have been described for uterus actomyosin (Needham and Cawkwell, 1956; Needham and Williams, 1963) and for chicken gizzard actomyosin (Bárány *et al.*, 1966). The amount of actomyosin extracted from hog carotid arteries was 2–3 mg/g of tissue (Bohr *et al.*, 1962; Filo *et al.*, 1963), an amount approximately equal to that obtained from whole brain. The yield from bovine aorta in the present study was 510 mg of purified protein from 280 g of cleansed vessel.

The Ca^{2+}–ATPase activities of smooth muscle myosin are much lower than those of striated muscle. This has been reported for myosin isolated from chicken gizzard (Bárány *et al.*, 1966), from uterus (Needham and Williams, 1963), and from arteries (Gaspar-Godfroid, 1964). On the other hand, purified myosin-like protein isolated from the slime mold has approximately three times the activity of rabbit striated muscle myosin (Adelman and Taylor, 1969). However, smooth muscle myosin and slime mold myosin do not show Mg^{2+}–ATPase activation upon addition of the actin-like protein isolated from the same tissue. The myosin-like protein from brain differs from these proteins in that it does show marked Mg^{2+} activation upon addition of either striated muscle actin or the actin-like protein isolated from brain and is in this respect more like striated muscle myosin.

The electrophoretic studies also reveal distinct differences between neurostenin and vascular actomyosin as well as between neurin and vascular actin. Differences between the striated muscle proteins and their vascular analogs are evident as well. The resolution of actin into several bands in the presence of urea during starch gel electrophoresis was noted previously by Carsten and Mommaerts (1963). The changes observed in the presence of 8 M urea probably reflect changes in degrees of polymerization and unfolding of protein molecules (Kielley, 1965) rather than subunit dissociation. They also indicate that the brain proteins are more tightly held together than the muscle proteins.

The antigenic properties of actomyosin and neurostenin also appear to be different, the former producing several bands and the latter only one. In addition, fluorescent antibody binding studies in progress reveal a predominantly nonvascular distribution of the protein antigen. Puchtler, using special staining techniques (Puchtler *et al.*, 1968), had obtained staining of glia and neurons which she believes demonstrates the presence of α-helical myoid fibrils in these cells (personal communication).

In a previous study, we have demonstrated (Puszkin and Berl, 1970) that the colchicine-binding protein associated with neutral microtubules (Weisenberg *et al.*, 1968) has actin-like properties. Whether neurin and colchicine-binding protein are one and the same remains to be established. Electrophoretic studies (unpublished observations) and nucleotide- binding studies (Weisenberg *et al.*, 1968) indicate that they are different and raises the question of whether more than one actin-like protein is present in brain.

The evidence does appear to warrant the conclusion that proteins with properties similar to but not identical with actomyosin, actin, and myosin are present in brain tissue. One may raise the speculation that they function in movement of glia and their extensions as they associate themselves with neurons and blood vessels in that they function in orientation of synaptic junctions in response to transmitter or other chemotropic substances. This latter process, should it occur, would be of particular significance in the establishment of "learned" neuronal pathways. Since the colchicine-binding microtubular protein comprises part but probably not all of the actin-like protein, an important function may be rapid transport of materials and vesicles down the axon from the perikaryon to the synaptic endings (Schmitt, 1968).

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