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Neural Regulation of Mammalian Fast and Slow Muscle Myosins: An Electrophoretic Analysis†

J. F. Y. Hoh*

ABSTRACT: Mammalian nerves to fast and slow muscles have the remarkable property of changing the speed of contraction of muscles following cross-reinnervation. The biochemical basis of speed transformation is the change in myosin in ATPase activity. This paper provides electrophoretic evidence for structural changes in myosin from cross-reinnervated muscles. A method is described for the separation of intact fast and slow muscle myosins by polyacrylamide gel electrophoresis. This method utilizes the fact that ATP and its analogs prevent the formation of myosin polymers in low ionic strength buffers. In this system, normal fast muscle myosin has a higher electrophoretic mobility than slow muscle myosin. Normal rat soleus myosin has a major slow and a minor fast component due to two populations of muscle fibers. The same muscle cross-reinnervated by a fast muscle nerve shows only the fast component. The normal, homogeneous fast extensor digitorum longus muscle has only the electrophoretically fast myosin, but following cross-reinnervation it shows both fast and slow components. These results suggest that mammalian motor nerves can induce or suppress the expression of genes that code for fast and slow skeletal muscle myosins.

Motor nerves exert a trophic effect upon skeletal muscles. A remarkable example of this trophic effect was the discovery by Buller *et al.* (1960) that when nerves to fast- and slow-contracting muscles in a mammalian limb were cut and sutured cross-wise, so that the fast muscle became reinnervated by the nerve to slow muscle and *vice versa* (an operation known as nerve cross-union), the time course of the isometric twitch of these muscles became altered: the fast muscle became slow, while the slow muscle became fast. Detailed physiological analysis of normal fast and slow mammalian muscles by Close (1964) revealed that they differ in the intrinsic speed of shortening of their sarcomeres. This implies that the rate of sliding of thick and thin filaments relative to each other under unloaded conditions is different in fast and slow muscles. Furthermore, Close (1969) showed that the intrinsic speeds of sarcomere shortening in cross-reinnervated muscles were reciprocally altered. These physiological studies suggest that the site of action of the neural influence on speed is the contractile material, *i.e.*, actin or myosin. The work of Bárány (1967) narrowed this to myosin since he showed that the actin-activated ATPase activity of myosins from muscles of different speed was correlated with the speed of muscle shortening, whereas myosin ATPase activity was independent of the source of actin used in the ATPase reaction. This concept of the biochemical basis of speed transformation has been corroborated in studies which showed correlated changes in myosin ATPase activity and muscle speed in cross-reinnervated muscles (Buller *et al.*, 1969; Bárány and Close, 1971).

It has been postulated that mammalian nerves bring about speed changes by regulating the expression of genes that code for myosin (Hoh, 1969; Buller *et al.*, 1969; Guth *et al.*, 1970). A major difficulty in testing this hypothesis is

† From the Department of Pharmacology, University of Illinois at the Medical Center, Chicago, Illinois, 60612, and the Department of Physiology, University of Sydney, N.S.W. 2006, Australia. *Received July 17, 1974.* This work was supported by U. S. Public Health Service N.I.N.D.S. Grant N.B. 05679 to Dr. B. Salafsky and the National Health and Medical Research Council of Australia.

* Present address: Department of Physiology, University of Sydney, N.S.W. 2006, Australia.

the lack of a method for separating myosins of fast and slow muscle fibers. The necessity for separation stems from the fact that most mammalian muscles, including those used for nerve cross-union studies, are composed of mixtures of fast and slow muscle fibers (Close, 1972) and changes in physiological properties and myosin characteristics as a result of nerve cross-union are not necessarily complete (Buller *et al.*, 1969). Myosins from fast and slow muscles are ultracentrifugally indistinguishable (Gergely *et al.*, 1965; Bárány *et al.*, 1965). Polyacrylamide gel electrophoresis, which has been so successfully applied to the separation of many proteins in recent years, has been used extensively on myosin, but only to analyze proteolytic fragments (Nauss *et al.*, 1969; Harrison *et al.*, 1971), or myosin after subunit dissociation (Small *et al.*, 1961; Gaetjens *et al.*, 1968; Perrie and Perry, 1970; Gershman and Dreizen, 1970; Sarkar *et al.*, 1971; Hale and Beecher, 1971) or chemical modification (Locker and Hagyard, 1967; Florini and Brivio, 1969; Paterson and Strohman, 1970; Weeds and Lowey, 1971). This is because intact myosin is insoluble in the low ionic strength buffer systems usually used in polyacrylamide gel electrophoresis. These buffers at the high ionic strength necessary to solubilize myosin are also not suitable for myosin electrophoresis because considerable amount of heat would be produced at the voltage gradient needed to produce a reasonable migration of these high molecular weight, thermolabile molecules. A method of electrophoresis applicable to intact myosin has been developed which overcomes this dilemma. This method makes use of the observation of Brahms and Brezner (1961) that myosin is soluble in low ionic strength solutions containing ATP or other anions which bind strongly to myosin. This method clearly resolves myosin from fast and slow muscle fibers. This paper describes the method and its application to the analysis of the neural regulation of myosin gene expression. Preliminary reports of this work have appeared (Hoh, 1972a,b).

Materials and Methods

Muscles. Experiments were done using the fast extensor digitorum longus (EDL) and the predominantly slow soleus muscles of normal and operated Wistar rats. Nerves to these muscles were surgically crossed when the animals were 3–4 weeks old. Special precautions were taken as previously described (Hoh and Salafsky, 1971) to ensure that each muscle was reinnervated only by the intended nerve. As controls, nerves to these muscles in some rats were cut and allowed to reinnervate their own muscles. This study is based on 16 experimental muscles from 9 operated animals 12–16 months after operation. During the excision of these muscles from the animal, the innervation of each muscle was checked by careful dissection and stimulation of the appropriate nerves. Muscles were stored in 100% glycerol at −20° until used.

Preparation of Myosin. EDL and soleus muscle myosins used during the development of the electrophoretic method were prepared from pooled glycerinated muscles from normal rats by dissociation of actin from three times precipitated actomyosin according to the method described by Bárány (1967) except that the dialysis step to remove Mg^{2+} was omitted. Myosins from experimental muscles were prepared using individual muscles or pairs by direct extraction according to the method of Bárány and Close (1971). Myosins were stored at −20° after mixing with an equal volume of glycerol. The protein concentration in myosin solutions was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Preparation of Electrophoresis Buffer. Electrophoresis was carried out at 4° in a continuous buffer system containing 0.025 M Tris, 0.19 M glycine, 5 mM ATP, and 10% glycerol (v/v) (pH 8.3). A stock solution containing 6.03 g of Tris, 28.8 g of glycine, and 6.16 g of ATP (disodium salt) per 200 ml was prepared; 1 l. of running buffer was prepared using 100 ml of the above Tris-glycine-ATP stock solution, 100 ml of glycerol, and 800 ml of deionized water, the pH being adjusted at 4° with concentratrated HCl to 8.3 before the run. This solution may be reused once or twice with good result within 1 week if stored at 4°.

Preparation of Polyacrylamide Gels. Gels were 3.36% with respect to total monomers, the acrylamide methylenebisacrylamide ratio being 20:1. A stock solution containing 32 g of acrylamide and 1.6 g of methylenebisacrylamide per 200 ml was prepared. To prepare 25 ml of the gel mixture, 5 ml of monomer solution, 2.5 ml of Tris-glycine-ATP stock buffer, 10 ml of 25% glycerol (v/v), and 7.4 ml of deionized water were mixed and deaerated. Polymerization was initiated by adding 40 μl of *N,N,N′,N′*-tetramethylethylenediamine and 0.1 ml of freshly prepared 10% solution of ammonium persulfate. Gel tubes used were 5 mm i.d. × 76 mm long or 6 mm i.d. × 80 mm long. They were filled with the mixture up to 10 mm from the top of the tube and layered with deionized water to ensure a flat horizontal gel surface. Polymerization was allowed to proceed for 1 hr at room temperature.

Conditions for Myosin Electrophoresis. Electrophoresis was done in a cold room or in a refrigerator using either a Canalco apparatus or a Gradipore (Townson and Mercer, Sydney) apparatus specially adapted to hold six tubes in place of a gel plate. Recirculation of buffer between the upper and lower electrophoresis baths was necessary to avoid drastic pH changes which would otherwise occur from the electrolysis of water during the electrophoresis run. The Gradipore apparatus has a built-in pump for this purpose. The buffer was pumped from the lower bath through a cooling coil immersed in ice before returning to the upper bath. In the case of the Canalco apparatus, an external pump in the cold room was used without further provision for cooling. In both cases the buffer was returned to the lower bath by gravity. Gels were prerun at 100 V for 30 min prior to the application of protein. Samples (2–50 μl) containing up to 2 mg/ml of protein in 50% glycerol were applied directly to the top of the gels. During the first 15 min of the electrophoresis, buffer recirculation was stopped to avoid mixing of sample with buffer. With the Canalco apparatus, electrophoresis was carried out at a constant current, usually of 2 mA/tube (about 90 V initially) and in the Gradipore apparatus at a constant voltage ranging between 110 and 180 V (about 2.5–5 mA/tube initially), the duration of each run was usually 4–6 hr. At the end of a run the temperature of the running buffer was usually not more than 8°.

Staining for Myosin ATPase in Gels. A method was developed for localization of calcium-activated myosin ATPase in gels after electrophoresis. Gels were incubated at room temperature or 35° in 10 ml of an ATPase reagent containing 0.025 M Tris, 0.19 M glycine, 0.005 M ATP, 0.035 M $CaCl_2$, and 0.6 M KCl (pH 8.3) (20°) until a white band of calcium phosphate appeared. This took a few minutes to 1 hr at 35° and up to several hours at room temperature. Gels may either be subsequently stained for protein or treated further to produce a more permanent ATPase stain-

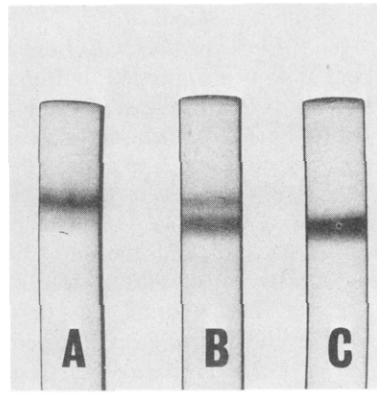

FIGURE 1: Polyacrylamide gel electrophoresis of purified intact myosins from the slow soleus and the fast extensor digitorum longus muscles of the rat. (A) Soleus myosin, 5 μg, (B) soleus myosin + extensor digitorum longus myosin, 5 μg, (C) extensor digitorum longus myosin, 5 μg. Electrophoresis was carried out in a Canalco apparatus in the cold room at 2 mA/gel (92 V initially) for 4.5 hr. Gels were stained with Coomassie Brilliant Blue.

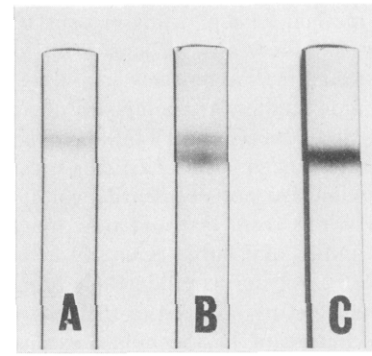

FIGURE 2: Duplicates of gels shown in Figure 1 but stained for calcium-activated myosin ATPase. (A) Soleus myosin, (B) soleus myosin + extensor digitorum longus myosin, (C) extensor digitorum longus myosin. Gels were incubated in the ATPase reagent for 1 hr at 35°, the white bands which appeared were subsequently converted to dark bands by the lead conversion method.

ing in the gel using a modification of the lead conversion method (Allen and Hyncik, 1963): gels were rinsed in deionized water and incubated at room temperature for 30 min in 10 ml of a lead reagent containing 0.1 M Tris maleate and 0.003 M lead nitrate (pH 7.0) to convert the precipitate to lead phosphate. Subsequently gels were washed in running tap water for 3–12 hr to remove excess lead ions. They were finally incubated for 3 min in an ammonium sulfide solution containing 0.5% H_2S (w/v) to give a brownish-black lead sulfide precipitate. Gels were washed several times and stored in deionized water. Gels stained in this way tend to fade over a period of several weeks especially when exposed to strong light.

Staining for Protein in Gels. Gels were stained for protein using 0.05% Coomassie Brilliant Blue in 7% acetic acid (v/v) in a water bath at 70° for 2 hr and destained by diffusion for 24–48 hr in 7% acetic acid in a Hoeffer diffusion destainer.

Results

Electrophoresis of Intact Myosin in Tris-Glycine-ATP Buffer. Attempts to electrophorese myosin using Tris-glycine buffer (without ATP) according to the method of Deyl *et al.* (1969) resulted in precipitation of myosin at the top of the gel. In contrast, using the Tris-glycine-ATP buffer, purified myosin was able to remain in solution and migrated into the gel during electrophoresis. This is shown in Figure 1 in which 5 μg of myosin was loaded per gel and electrophoresis was carried out for 4.5 hr at 2 mA/gel. With loads above 20 μg/gel, some myosin tended to be retained at the top of the gel. Even with 55 μg/gel, the bulk of the protein migrated at about the same rate as with lower myosin loads. However, at such high loads there appeared a minor component of considerably lower mobility, presumably due to an aggregated form of myosin. The presence of calcium-activated ATPase activity in the principal bands (as well as the slower minor band and the tops of the heavily loaded gels) was demonstrable by incubation of gels in the ATPase reagent prior to protein staining thus establishing the intact nature of the myosin. This ATPase activity electrophoresis buffer was replaced by 5 mM sodium pyrophosphate or simply by using 10 mM sodium pyrophosphate in 10% glycerol (v/v) as the buffer. However, these buffers are not satisfactory for the demonstration of calcium-activated myosin ATPase in the gel as pyrophosphate forms a white precipitate with the ATPase reagent as well as inhibiting myosin ATPase.

Other workers have also used pyrophosphate buffers to electrophorese myosin in polyacrylamide gel. Penn *et al.* (1972) used 20 mM sodium pyrophosphate buffer at pH 7.5 in 0.6% agarose gel or 4% polyacrylamide gel. d'Albis and Gratzer (1973) used a buffer of very high ionic strength which has the disadvantage of limiting the permissible voltage gradient. The duration of electrophoresis needed to achieve reasonable migration of myosin into the gel in their study was of the order of 2 days. Neither group of workers have positively identified intact myosin by ATPase staining.

Separation of Fast and Slow Muscle Myosins. Using this method of electrophoresis, myosins from fast and slow skeletal muscles can be clearly resolved into two distinct components. Myosin from the fast EDL muscle (gel C, Figure 1) gave a single fast band while myosin from the slow soleus muscle gave two bands: a major slow band and a faint fast band which is not very clearly resolved in gel A (but see gel D of Figure 3). The difference in mobility of the band for EDL myosin and the major band of soleus myosin is clearly evident in the gel containing a mixture of myosins from these muscles (gel B, Figure 1). No difference in mobility was detected between the fast component of soleus myosin and EDL myosin.

The gels in Figure 2 were duplicates of corresponding gels shown in Figure 1 but had been stained for ATPase by incubating for 1 hr at 35° in the ATPase reagent and subsequently treated with lead reagent and ammonium sulfide. Although this method for staining ATPase has not been shown to be quantitative, it can be seen that the same quantity of EDL myosin incubated for the same duration as for soleus myosin stained more intensely for ATPase than the soleus myosin. The observation is consistent with the higher specific activity of the EDL myosin (Bárány, 1967).

Effects of Reinnervation and Cross-Reinnervation on Soleus Myosin. Reinnervation of the soleus muscle by its original nerve produced no changes in the electrophoretic pattern of soleus myosin (gel D, Figure 3), which is characterized by a major slow component and a minor fast component. Cross-reinnervation by the nerve to EDL, however, resulted in a myosin with a fast component only (gel E, Figure 3). A mixture of these soleus myosins (gel F, Figure 3) showed two approximately equally staining bands; the minor fast component of reinnervated soleus could not be

resolved from the fast component in cross-reinnervated soleus. ATPase staining of companion gels showed that all bands, including the minor fast component of reinnervated soleus, had enzymatic activity. Furthermore, the gel containing the mixture showed that the faster band was more heavily stained.

Effects of Reinnervation and Cross-Reinnervation on EDL Myosin. Reinnervation of the EDL muscle by its original nerve produced no change in the electrophoretic pattern of EDL myosin which is characterized by the fast component only (gel A, Figure 3). Cross-reinnervation by the soleus nerve resulted in the appearance of a slow component in addition to the fast component (gel B, Figure 3). The fast and slow components stained for protein to about the same intensity but the ATPase staining of the slow component is less intense compared with that for the fast component, suggesting that this new slow component has a lower specific activity. A mixture of the two EDL myosins (gel C, Figure 3) showed two components.

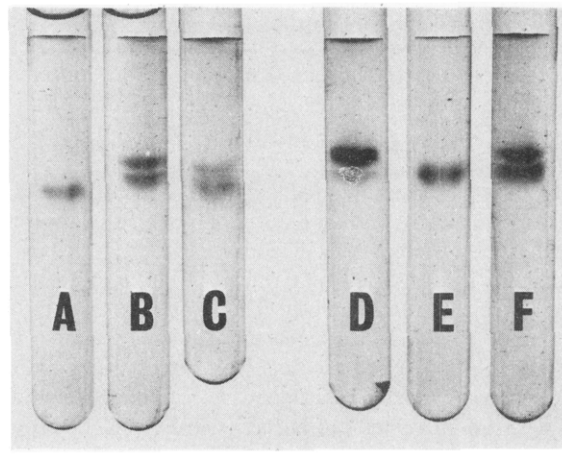

FIGURE 3: Effects of self-reinnervation and cross-reinnervation on the electrophoretic pattern of myosins from the extensor digitorum longus (EDL) and soleus muscles. (A) Control myosin from EDL reinnervated by its own nerve, (B) myosin from EDL cross-reinnervated by nerve to soleus, (C) mixture of myosins in (A) and (B), (D) control myosin from self-reinnervated soleus, (E) myosin from soleus cross-reinnervated by nerve to EDL, (F) mixture of myosins in (D) and (E). All gels were electrophoresed at 180 V for 5 hr and stained by Coomassie Brilliant Blue.

Discussion

The principle which forms the basis of myosin electrophoresis described in this paper is the specific interaction of ATP and its analogs on myosin which prevents its aggregation into filaments in low ionic strength solutions. Noda and Ebashi (1960) first demonstrated that ATP caused the dissociation of myosin polymers. Brahms and Brezner (1961) showed that not only ATP, but also other polyvalent anionic ligands such as pyrophosphate and ADP would keep myosin in solution at millimolar concentrations in the absence of other salts. Josephs and Harrington (1968) using velocity sedimentation studies have shown that in low ionic strength solutions myosin filaments are in a rapidly reversible equilibrium with monomeric myosin. The effect of ATP and other ligands have been shown by Harrington and Himmelfarb (1972) to shift this equilibrium dramatically in favor of filament dissociation at low concentrations of ligand. These authors have produced evidence to suggest that this dissociating effect is due to a specific binding of 1–2 molecules of ligand per molecule of monomer to some site in the rod portion of the myosin molecule.

During the electrophoresis described in this paper, myosin is exposed to a low ionic strength buffer in the presence of a ligand, *i.e.*, conditions very similar to those which ensured virtually complete dissociation of myosin filaments (Harrington and Himmelfarb, 1972). However, myosin under these conditions may not be truly monomeric. It has been shown by Godfrey and Harrington (1970) and Herbert and Carlson (1971), using independent methods, that myosin in high ionic strength solutions is in a rapidly reversible equilibrium between monomeric and dimeric species. A similar monomer $\rightleftharpoons$ dimer equilibrium may exist at low ionic strength in the presence of ligand. The electrophoretic pattern of such an equilibrium system would be indistinguishable from that of a purely monomeric system (Cann, 1972). It is therefore uncertain whether myosin during electrophoresis described above is monomeric or in a rapidly reversible monomer $\rightleftharpoons$ dimer equilibrium.

It is now generally accepted that myosin is a multisubunit protein consisting of two heavy chains of molecular weight about 200,000 (Gershman *et al.*, 1969; Gazith *et al.*, 1970) and several light chains of about 20,000. The electrophoretic separation of fast and slow muscle myosins described in this paper provides support for their overall difference in structure. The uncertainty of whether myosin exists as true monomers or as a rapidly reversible monomer $\rightleftharpoons$ dimer equilibrium is of little consequence in this connection: if such an equilibrium state exists during electrophoresis, independent migration of the two species of myosins would imply that they are sufficiently distinct structurally to prevent the formation of hybrid dimers. Further independent evidence for differences in overall structure is provided by immunological studies (Gröschel-Stewart and Doniach, 1969) and analyses of myosin subunits.

The light chains of myosin extracted from fast and slow muscles are electrophoretically distinct (Locker and Hagyard, 1968; Perrie and Perry, 1970; Sarkar *et al.*, 1971; Lowey and Risby, 1971). Fast muscle myosins show three light chain components. Peptide sequence analyses have confirmed that these are structurally distinct (Weeds, 1969; Weeds and Frank, 1972). Slow muscle myosins show two light chains and peptide analysis shows that they are structurally unrelated to light chains from fast muscle myosin but similar to those from cardiac myosin (Weeds and Pope, 1971). It may therefore be concluded that light chains of fast and slow muscle myosins are products of distinct sets of genes.

Heavy chains from fast muscle myosin contain the unusual amino acid 3-methylhistidine (Trayer *et al.*, 1968; Kuehl and Adelstein, 1969; Huszar and Elzinga, 1971) whereas it is absent in heavy chains of slow and cardiac myosins (Kuehl and Adelstein, 1970). It has been shown that the methyl group is added after the histidine has been incorporated into the polypeptide chain (Hardy *et al.*, 1970), so that this difference alone does not imply a difference in structural genes for the heavy chains of these myosins. However, a comparison of the amino acid sequence of a histidine-containing peptide of cardiac myosin with the homologous 3-methylhistidine-containing peptide of fast muscle myosin revealed differences in structure between the heavy chains of these myosins (Huszar and Elzinga, 1971, 1972). Unfortunately, similar sequence analysis has not been done for skeletal slow muscle myosin. Indirect evidence is available to suggest that the heavy chain of slow muscle myosin is very similar to that of cardiac heavy chain: light meromy-

osin paracrystals prepared from cardiac and slow skeletal muscle myosins are strikingly similar in ultrastructural pattern to each other but differ from that of fast muscle myosin (Nakamura *et al.*, 1971). It is therefore likely that heavy chains of fast and slow muscle myosins are products of different genes.

The above discussion leads to the conclusion that the electrophoretically fast and slow components of myosin are products of different sets of genes. Recent work from this laboratory has revealed that the situation is more complex than this. Fast muscle myosin from a number of species can be further resolved into three distinct components (White and Hoh, 1973), while the fast migrating component of rat soleus myosin, which corresponds to motor units contracting with an intermediate speed (Close, 1967), is distinct from these, and indeed has a mobility intermediate between the three fast components and the slow component corresponding to soleus slow motor units (Hoh and Dunlop, unpublished observations).

Using the method described here, the cross-reinnervated soleus muscle showed only the fast myosin component. Since soleus muscle reinnervated by the original nerve showed no change in myosin compared with normal soleus, it may be concluded that the EDL nerve had induced the synthesis of fast or intermediate myosin in fibers previously synthesizing slow myosin while at the same time the synthesis of slow myosin was suppressed.

Myosin from cross-reinnervated EDL showed both electrophoretically fast and slow components. This heterogeneity is not due to unintended reinnervation by its original nerve, as special precautions had been taken to avoid self-reinnervation and the innervation of muscles were checked (see Materials and Methods). One possible explanation for the observed heterogeneity is that both types of myosin are synthesized in all cross-reinnervated EDL muscle fibers, and this would imply that the nerve to soleus could induce the synthesis of slow myosin but could not suppress the ongoing synthesis of fast myosin. An alternative explanation is that there are two populations of muscle fibers in cross-reinnervated EDL as in the case of normal soleus muscle. In either case, the soleus nerve has been shown to be able to induce the synthesis of slow myosin in the originally fast muscle fibers. In view of evidence cited above that electrophoretically fast and slow myosins are products of different sets of genes, these results on cross-reinnervated muscles clearly imply that the nervous system does alter the expression of these genes.

Several recent studies have provided evidence that the light chains (Samaha *et al.*, 1970; Weeds *et al.*, 1974; Sreter *et al.*, 1974) and heavy chains (Jean *et al.*, 1973) of myosin may be transformed as a result of nerve cross-union. Sreter *et al.* showed that the electrophoretic patterns of light chains from rat cross-reinnervated EDL and soleus muscles were virtually identical, both myosins containing predominantly LC 1_s and LC 1_f, the slowest migrating light chains characteristic of normal slow and fast myosins, respectively. This contrasts with the present findings on intact myosin revealing an absence of electrophoretically slow component in cross-reinnervated soleus myosin while cross-reinnervated EDL myosin showed both fast and slow components. There is no apparent reason to believe that cross-reinnervated soleus myosin used by Sreter *et al.* differed in electrophoretic mobility from that of the present study. Our results may be reconciled by the assumption that the electrophoretic mobility of intact myosin and its ATPase activity are determined principally by the heavy chains. Clearly, studies on subunit composition of electrophoretically homogeneous intact myosins are needed to resolve this problem.

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Neutral and Cationic Sulfonamido Derivatives of the Fluorescent Probe 2-*p*-Toluidinylnaphthalene-6-sulfonate. Properties and Mechanistic Implications†

Frank C. Greene

ABSTRACT: The sensitivity of the fluorescence energy of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) to apparent solvent polarity is considerably greater in methanol–water mixtures and lower primary alcohols than in higher primary alcohols or solvents containing no hydroxyl groups. It is suggested that the behavior of TNS in lower alcohols and their mixtures with water is an anomalous result of the combination of general polarity influences and *changes* in the nature of specific interactions of the probe with hydroxyl groups of such solvents. Upon conversion of the sulfonate group of TNS to sulfonamido, the biphasic behavior observed in alcohols is eliminated, and fluorescent probes are obtained which have more nearly equal sensitivities to polarity in the alcohols and nonhydroxyl solvents. The sulfonamido fluorescent probes are more sensitive to general environmental polarity than TNS, and are superior as probes of environmental polarity.

Fluorescent probes (environmentally sensitive fluorophores) of the *N*-arylaminonaphthalenesulfonate type are widely utilized in contemporary biochemical research, and have proven quite useful, despite some uncertainty concerning the nature of the mechanisms determining their sensitivity (Brand and Gohlke, 1972). Interpretations of the fluorescence characteristics of these probes in pure solvents are based on the premise that the energy and efficiency of emission reflect the degree of unrestrained solvent–excited probe interaction (McClure and Edelman, 1966), and may be taken as indications of microenvironmental polarity (Turner and Brand, 1968). Such interpretations are often extended to studies involving the binding of probes by macromolecules, though additional factors of environmental restraint may be operative (Ainsworth and Flanagan, 1969). In the present investigation, neutral and cationic derivatives of the probe 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS)[1] have been prepared as aids in the study of protein–amphi-

† From the Food Proteins Unit of the Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710. *Received September 16, 1974.*

[1] Abbreviations and symbols used are: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; II, 2-*p*-toluidinylnaphthalene-6-sulfonamide; III, 2-*p*-toluidinylnaphthalene-6-[*N*-β-ethylamine hydrochloride]sulfonamide; 1,8-ANS, 1-anilinonaphthalene-8-sulfonate; 2,6-ANS, 2-anilinonaphthalene-6-sulfonate.