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In Vitro Studies of Skeletal Muscle Membranes

Effects of Denervation on the Macromolecular Components of Cation Transport in Red and White Skeletal Muscle

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Summary. The effects of denervation on the macromolecular components of active monovalent cation transport in skeletal muscle have been studied using purified sarcolemma membranes. A comparison of membrane activities of fast-twitch, slow-twitch, and mixed-fiber muscles was made to determine what role, if any, the motor nerve has in regulating this important aspect of muscle metabolism. A dramatic increase in the basal sarcolemmal Mg^{++} ATPase activity (three- to fourfold) was found for both major muscle types. An increase in the ouabain-inhibitable ($Na^+ + K^+$)-stimulated enzyme was also found, but the effect was substantially less (1.5- to twofold). [^{3}H]-ouabain binding, as an index of glycoside receptor sites, also increased (two- to threefold) midway in the course of denervation. On the other hand, the phosphorylated intermediate activity, a functional component of the transport system, clearly decreased over the same time course and remained below control values for the remainder of the course. This resulted in a two- to threefold increase in the turnover number, suggesting that active transport of cations should increase dramatically with denervation. The membrane protein patterns on SDS gels were less obvious than the changes observed in the functional components. The major effects appeared after only one week and seemed to be restricted to high molecular weight membrane proteins, especially in the 100,000 to 250,000 daltons range. This effect was more prominent in slow-twitch membranes with an apparent semiquantitative decrease in stain at 240,000 daltons. In gels of membranes from fast-twitch muscles a decreased stain in the range of 100,000 to 110,000 daltons occurred, and this became more obvious with longer periods of denervation. The results suggest that considerable influence on the macromolecular components of active cation transport in skeletal muscle is exerted by the motor nerve. No appreciable difference was found in this effect when the two major types of skeletal muscle, fast-twitch and slow-twitch, were compared, suggesting that motor nerve regulation of this membrane property is qualitatively the same.

Purified surface membranes of mammalian skeletal muscle are a convenient system to evaluate *in vitro* the trophic role of the nervous system.

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In previous reports we (Festoff & Engel, 1974; Reddy, Engel & Festoff, 1976) and others (Andrew & Appel, 1973; Andrew, Almon & Appel, 1974, 1975) have utilized these membranes in a number of studies of junctional and non junctional activities of isolated sarcolemma. We (Festoff, Oliver & Reddy, 1977) have evaluated the catecholamine-sensitive adenylate cyclase system of sarcolemmal membranes (SL) of fast and slow skeletal muscle and found dramatic differences in sensitivity to β-adrenergic stimulation and response to denervation.

The alkali cation transport system is present in and vital to the overall effective functioning of all living cells. The multinucleated skeletal muscle fiber, because of its role in biologic excitation, is especially dependent on efficient and rapid cation transport. Following denervation, gross disturbance in this efficient membrane transport system develops (Drahota, 1962). The most rapidly developing membrane change following denervation was a 10–12 mV depolarization of the resting membrane potential (r.m.p.) (Albuquerque, Schuh & Kaufman, 1971) which was interpreted as an effect on the electrogenic sodium pump. This has been shown to occur in as little as two hours following denervation and to precede all other changes, including the changes in miniature end-plate potentials (mepps), ACh sensitivity, and membrane resistance. It has also been reported that this drop in r.m.p. can be prevented by epinephrine and by dibutyryl cyclic AMP (Bray, Hawken & Hubbard, 1974).

It is generally accepted that the molecular basis for $Na^+ + K^+$ transport across the membranes of living cells involves the ouabain-inhibitable $(Na^+ + K^+)$-stimulated ATPase in isolated membranes. Several authors have suggested an inverse relationship between membrane-bound $(Na^+ + K^+)$-ATPase and hormone-sensitive adenylate cyclase found in the same membrane fractions (*see* Schwartz, Lindenmayer & Allen, 1975).

In the present report, we analyzed the macromolecular components of the monovalent cation transport system of skeletal muscle in normal and denervated rats. We used relatively pure sarcolemmal membranes (SL) of fast (extensor digitorum longus, EDL) and slow (soleus) muscles and assayed $(Na^+ + K^+)$-ATPase, ^{32}P incorporation, and ^3H-ouabain binding under normal conditions and at varying times post-denervation. The patterns of membrane proteins on SDS-polyacrylamide electrophoresis (SDS-PAGE) were also used to evaluate possible macromolecular membrane changes which might have resulted. An increase in the basal Mg^{++}-ATPase occurs during the course (fourfold above the control), while a lesser, but significant, increase in the $(Na^+ + K^+)$-ATPase develops (1.5- to twofold). A two- to threefold increase in ^3H-ouabain

receptor sites also develops, but the level of the phosphorylated intermediate drops, resulting in a marked increase in the turnover number. SDS-PAGE patterns show less consistent changes with some differences observed in the high molecular weight proteins of fast *vs.* slow muscle membranes. The results suggest that the components underlying this active transport system in muscle are under neural control. Specifically which particular component, and how, is the subject of current research.

Materials and Methods

Muscles were isolated from hind limbs of male Sprague-Dawley rats weighing 150–200 g, and denervation was performed as described by Festoff *et al.* (1977). Sarcolemmal membranes were prepared as reported previously (Festoff & Engel, 1974) with a recent homogenization modification (Reddy *et al.* 1976). ATPase assays were performed, using γ-^{32}P-ATP according to a modification (Bais, 1975) of the method of Dunham and Hoffman (1970). The typical assay medium, in a final volume of 0.5 ml, contained 50 μg SL protein, 0.5 mM EGTA, 0.5 mM EDTA, 3 mM Tris-Cl, 4 mM ^{32}P-ATP ($1–2 \times 10^5$ cpm by Cerenkov counting), 30 mM glycylglycine-imidiazole, pH 7.5, and other agents and ions as indicated. Incubation was performed in a shaking water bath at 37°C for 15 min, and the reaction was terminated by adding 0.8 ml of activated charcoal in 1 M HCl. The tubes were vortexed, 0.2 ml 95% ethanol added, and then centrifuged at $1000 \times g$ for 5 min. Aliquots of the supernatant were counted in scintillation vials containing buffer (5 ml) in a Picker Liquimat using Cerenkov radiation (efficiency 35–40%). Boiled and zero membrane controls were similar and were subtracted from the experimental values. Specific activities are expressed as μmoles Pi liberated per hr per mg protein.

The steady-state level of [^{32}P]-incorporation into SL membranes was as described by Reddy *et al.* (1976). [^{3}H]-ouabain binding was performed using a millipore filter assay essentially as described previously (Albers, Koval & Siegel, 1968) with several modifications. SL membranes, 50–100 μg protein, were incubated at 25°C, in a final volume of 0.1 ml, with Tris-Cl 50 mM, pH 7.5; Tris-ATP, 5 mM; $MgCl_2$, 5 mM; NaCl, 100 mM; EDTA, 1 mM; and [^{3}H]-ouabain (10^{-8}–10^{-5} M, 2×10^5 cpm). Incubation began with the addition of labeled glycoside and was terminated by rapidly adding ice-cold glass-distilled water and filtering through millipore filters (HAMK, 0.45 μ, Millipore Corp., Boston, Mass.). The filters were further washed with 10 ml cold water, air-dried, and counted in vials containing 10 ml Instabray (Yorktown Research, S. Hackensack, N.J.) in a Packard 2650 system (Packard Instruments, Downers Grove, Illinois) at an efficiency of 56%.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was performed as previously described (Fairbanks, Steck and Wallach, 1971; Festoff & Engel, 1974). 5.6% gels were loaded with 30 μg of SL protein and electrophoresis stopped when the pyronin Y had migrated 8.5 cm (approximately 90 min). Gels were fixed overnight in 25% isopropanol-10% acetic acid then stained and destained (Fairbanks *et al.*, 1971) prior to photographing.

Protein was estimated by a modification of the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as standard. [^{32}P]-ATP (10–50 Ci/mmol) and [^{3}H]-ouabain (12 Ci/mmol) were obtained from New England Nuclear (Boston). Unlabeled nucleotides and ouabain were obtained from Sigma (St. Louis, Mo.) or Calbiochem (La Jolla, Calif.). All other chemicals were reagent grade.

Results

Effects of Denervation on Sarcolemma ATPases

Our previous studies (Festoff & Engel, 1974; Reddy *et al.*, 1976) were performed with crude SL and sucrose-density gradient purified fractions (SLF) of membranes obtained from gastrocnemius-plantaris (so-called "mixed fiber") muscles. It has been demonstrated that denervation induces a shift of density purified muscle membrane fractions so that plasma membrane markers appear in lighter regions (Andrew & Appel, 1973; Andrew *et al.*, 1974). However, we found that no apparent shift occurs in the membrane fraction prior to density gradient purification, and ouabain-inhibitable ATPase activity remains essentially absent from the post-mitochondrial supernatant fraction during our purification scheme (*unpublished*). Since the input to sucrose-density gradients, "crude" SL, of normal membranes contains significant ouabain-inhibitable $(Na^+ + K^+)$-ATPase (Festoff & Engel, 1974; Reddy *et al.*, 1976), the current study compares activities of SL ATPase derived from both normal and denervated fast or slow muscles. Use of SL eliminates any possible artifact of density banding which might occur with denervation.

A greater total activity of Mg^{++}-ATPase, as well as $(Na^+ + K^+)$-ATPase, appears in soleus than in EDL (Tables 1, 2). However, it appears that the net ATPase stimulation with $Na^+ + K^+$ above the Mg^{++} base ($Na/K\Delta$) is somewhat greater with EDL. Teleologically, in a muscle where a faster return to resting conditions. (i.e., rapid repolarization) is desirable for the completion of an excitation-contraction cycle, one might expect greater sensitivity to Na^+, and hence, pump activity, to be present. These differences, however, are not great.

The effect of denervation on the EDL activities are shown in Fig. 1. These effects are seen in both Mg^{++} basal as well as monovalent cation-stimulated activities. The results are expressed as a percentage of the contralateral controls. A dramatic increase in EDL Mg^{++}-ATPase occurs over the five-week period with a threefold stimulation above control values (Table 1). Less impressive, though significant, is the increase in $(Na^+ + K^+)$-ATPase, increasing more than twofold, over the course of study. Much less of an increase in both enzyme activities is found in soleus membranes although the trend is the same (Fig. 2, Table 2): denervation appears to increase basal (Mg^{++}) as well as $(Na^+ + K^+)$-stimulated sarcolemmal ATPases in both "fast" and "slow" muscles, with the major effect apparent in the Mg^{++} enzyme activity.

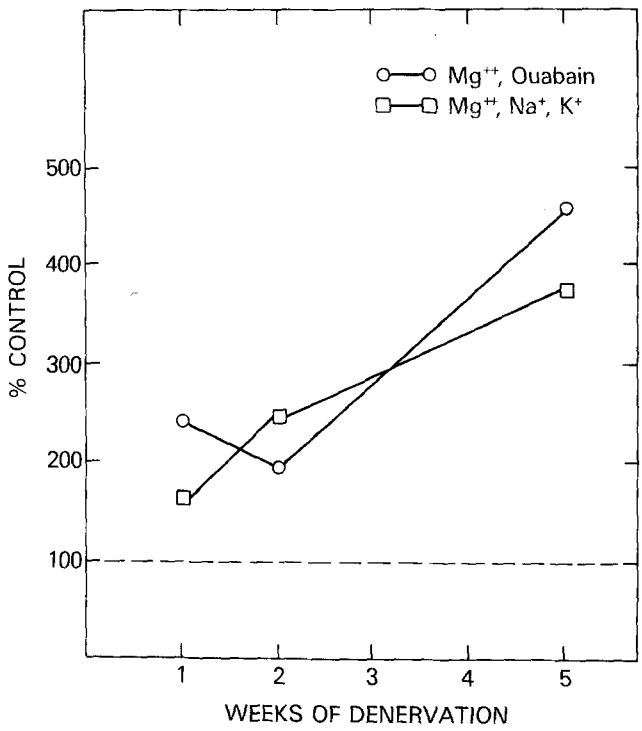

Fig. 1. Effects of denervation on sarcolemmal Mg^{++} and $(Na^+ + K^+)$-ATPases of "fast" (EDL) muscles. Conditions were as described in Materials and Methods. Values are expressed as percentage of control (unoperated). Points represent duplicates (within 5%) of three assays

Table 1.

Muscle[a] (EDL)	Time after denervation (days)	SL Protein (mg/g wet weight)	ATPase Activity (μmoles/hr/mg protein)	
			(Mg^{++})-ATPase	$(Na^+ + K^+)$-ATPase
1. Normal		2.92	4.27 ± 1.09	12.85 ± 3.78
Denervated	7	2.25	9.32 ± 0.55[b]	18.01 ± 0.70[c]
2. Normal		2.73	3.78 ± 0.71	6.96 ± 0.27
Denervated	14	2.83	6.95 ± 0.48[d]	16.55 ± 0.63[b]
3. Normal		1.84	2.63 ± 0.04	7.80 ± 0.27
Denervated	35	1.03	17.54 ± 0.01[b]	27.73 ± 0.80[c]

[a] SL membranes prepared as in Materials and Methods from 20 male S-D rats. Values are means $\pm$ SEM of duplicate determinations from at least three separate assays.
[b] ($p < 0.001$).
[c] ($p < 0.005$).
[d] ($p < 0.01$).

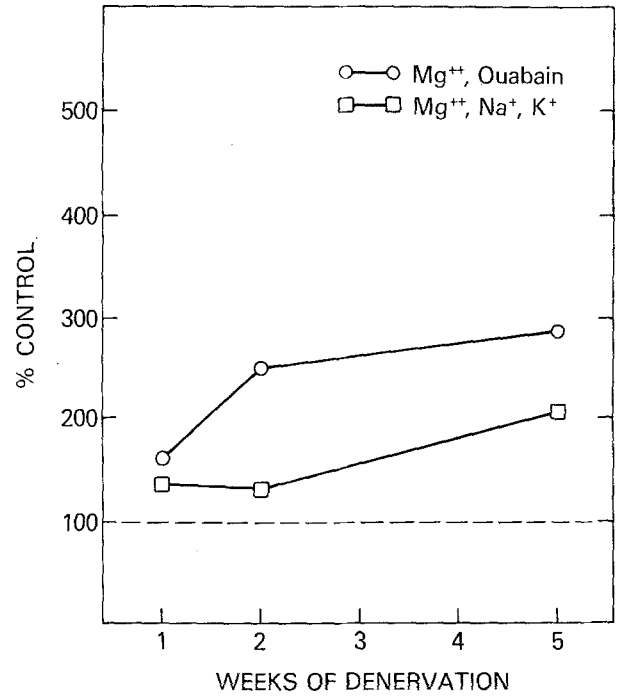

Fig. 2. Effects of denervation on sarcolemmal Mg^+ and $(Na^{++}+K^+)$-ATPases of "slow" (soleus) muscle. Conditions were as described in Materials and Methods. Values are expressed as percentage of control (unoperated). Points are duplicates of three assays and are within 5%

Effect of Denervation of [^{32}P]-Intermediate of the ($Na^+ + K^+$)-ATPase

In our study of the surface membranes of skeletal muscle, we characterized and purified the phosphorylated intermediate of the cation transport ATPase (Reddy *et al.*, 1976) to determine the overall relationship of this enzyme system to Na^+ and K^+ movements in muscle prior to investigating whether this system is under neurotrophic control. The large subunit of the ATPase intermediate can be readily identified by SDS-PAGE (Schwartz *et al.*, 1975), and the relative amounts of this polypeptide analyzed under a variety of conditions, such as denervation, making its estimation a simple and reliable method of analysis for the current study.

In contrast to the above-mentioned increase in ATP hydrolytic activity, the overall effect of denervation on the intermediate is to reduce its levels as shown in Fig. 3. A 25 to 30% reduction in Na-stimulated [^{32}P]-incorporation from γ-labeled ATP into SL membranes of gastrocnemius occurred over the course of denervation. "Mixed" fiber membranes

Table 2.

Muscle[a] (soleus)	Time after denervation (days)	SL Protein (mg/g wet weight)	ATPase Activity (μmoles Pi/hr/Mg protein)	
			(Mg^{++})-ATPase	$(Na^{+}+K^{+})$-ATPase
1. Normal		3.4	7.32±1.68	15.23±0.89
Denervated	7	1.8	11.14±2.59[b]	20.96±1.94[c]
2. Normal		3.3	4.34±0.50	12.99±2.67
Denervated	14	1.5	11.54±1.21[c]	21.99±1.31[c]
3. Normal		2.1	8.81±0.25	17.41±1.07
Denervated	35	0.6	21.55±0.55[b]	34.33±0.54[b]

[a] SL membranes prepared as in Materials and Methods from 20 male S-D rats. Values are means ±SEM of duplicate determinations from at least three separate assays.
[b] ($p<0.001$).
[c] ($p<0.025$).

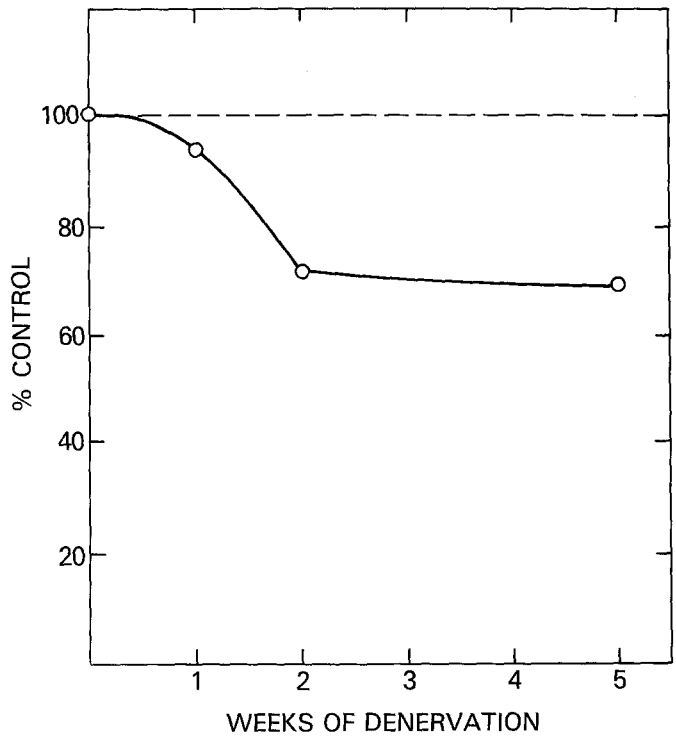

Fig. 3. Effect of denervation on Na^{+}-stimulated phosphorylation of "mixed" fiber (gastrocnemius) sarcolemmal protein. Incorporation of ^{32}P from [γ-^{32}P-ATP], in 10 sec, in tubes containing in final volume of 1 ml: Mg^{++}, 1 μmole; Na^{++}, 100 μmoles; [γ-^{32}P-ATP], 200 nmoles (10 μCi); Tris-Cl, 25 μmoles (pH 7.5); and 200–300 μg of SL protein. Reaction was terminated by injection of an ice-cold solution (6% TCA, 50 mM KH_2PO_4, 1 mM Na_2ATP). Values are percent of control, in duplicate, of three separate assays.

were used in this part of the study because of the absence of qualitative differences between "fast" and "slow" muscles in the ATPase studies (Tables 1 and 2). Though quantitatively reduced, compared to control values, [^{32}P] incorporation into SL membrane proteins was qualitatively similar to that in our previous study (Reddy *et al.*, 1976). The predominant protein phosphorylated was a major protein component of the membrane: a 108,000 dalton peptide, the phosphorylated intermediate of the ($Na^+ + K^+$)-ATPase in these sarcolemmal fragments.

Effects of Denervation on [^{3}H]-Ouabain Binding to Sarcolemmal Membranes

Another marker useful in characterizing plasma membrane components, and more specifically, the transport ATPase complex, is the binding of labeled cardiac glycosides. Ouabain is known to block the Na pump in intact cells and to inhibit the ($Na^+ + K^+$)-ATPase in particulate preparations (Schwartz *et al.*, 1975). Extensive studies of the binding of [^{3}H]-ouabain and other glycosides have been made in red cells (Dunham & Hoffman, 1970, 1971; Gardner & Conlon, 1972), kidney (Lane, Copenhaver, Lindenmayer & Schwartz, 1971), brain and electric eel (Albers *et al.*, 1968) and cardiac tissue (Schwartz *et al.*, 1975). What has emerged from these studies can be summarized as follows: 1) [^{3}H]-ouabain binding is specific for the ($Na^+ + K^+$)-ATPase complex located within the plasma membrane; 2) binding occurs on the outer surface of the membrane to a site which appears closely similar to, but not identical with, the high affinity K^+ site for the enzyme; 3) the K_d for the binding site is usually less than the concentration needed to half-maximally inhibit the enzyme, and a 2:1 ratio has been suggested (Schwartz *et al.*, 1975); and 4) the binding of [^{3}H]-ouabain usually displays first-order kinetics, is saturable, and generally requires Na^+ and ATP. This last observation led to the suggestion that the enzyme is in the phosphorylated form (E_2-P) for binding and, presumably, for enzyme inhibition to occur, although binding can occur in the presence of agents not conducive to phosphorylation (Schwartz *et al.*, 1975). The phosphorylated intermediate in these SL membranes has been shown to be predominantly the E_2-P form (Reddy *et al.*, 1976). Binding of [^{3}H]-ouabain recently has been demonstrated in whole muscle cells of rat soleus (Clausen & Hansen, 1974).

We have utilized relatively purified SL and evaluated the ouabain-binding sites. Specific, saturable binding of [^{3}H]-ouabain to SL of mixed-

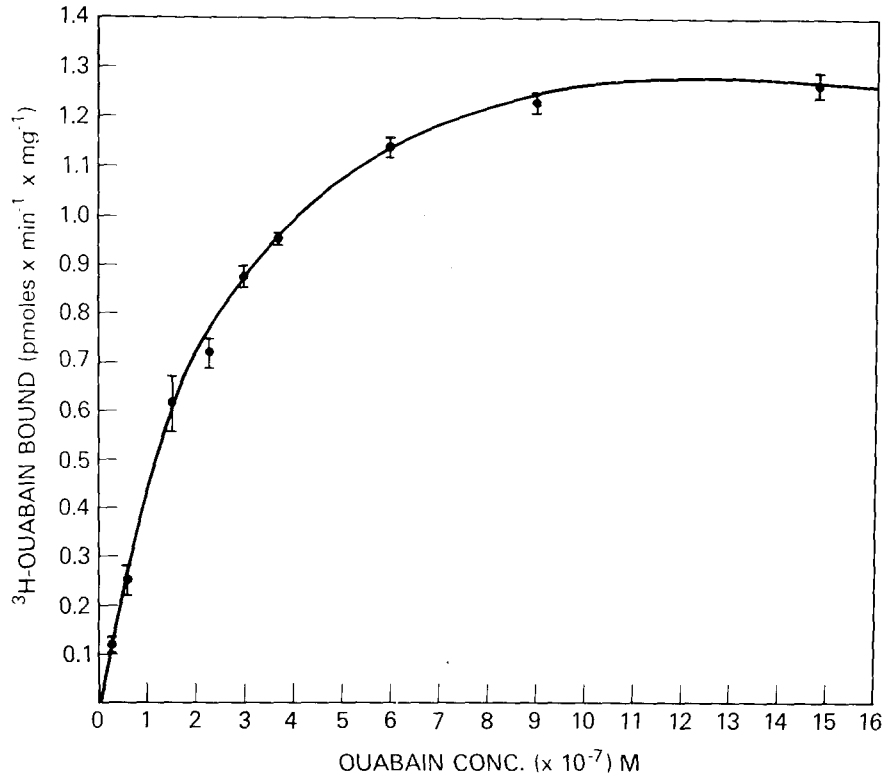

Fig. 4. ^{3}H-ouabain binding to sarcolemmal membranes. Effect of increasing concentration of unlabeled glycoside on binding of ^{3}H-ouabain. Points are means of four different preparations ± SEM

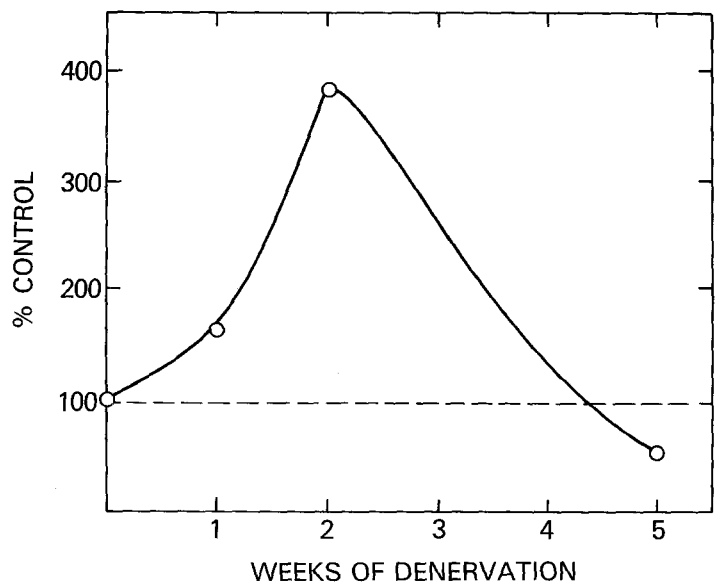

Fig. 5. ^{3}H-ouabain binding to sarcolemmal membranes. Effect of denervation expressed as percent of controls (pmoles per 30 min). Assay performed as given in Materials and Methods. Points are means of either duplicate or triplicate determinations of three separate assays

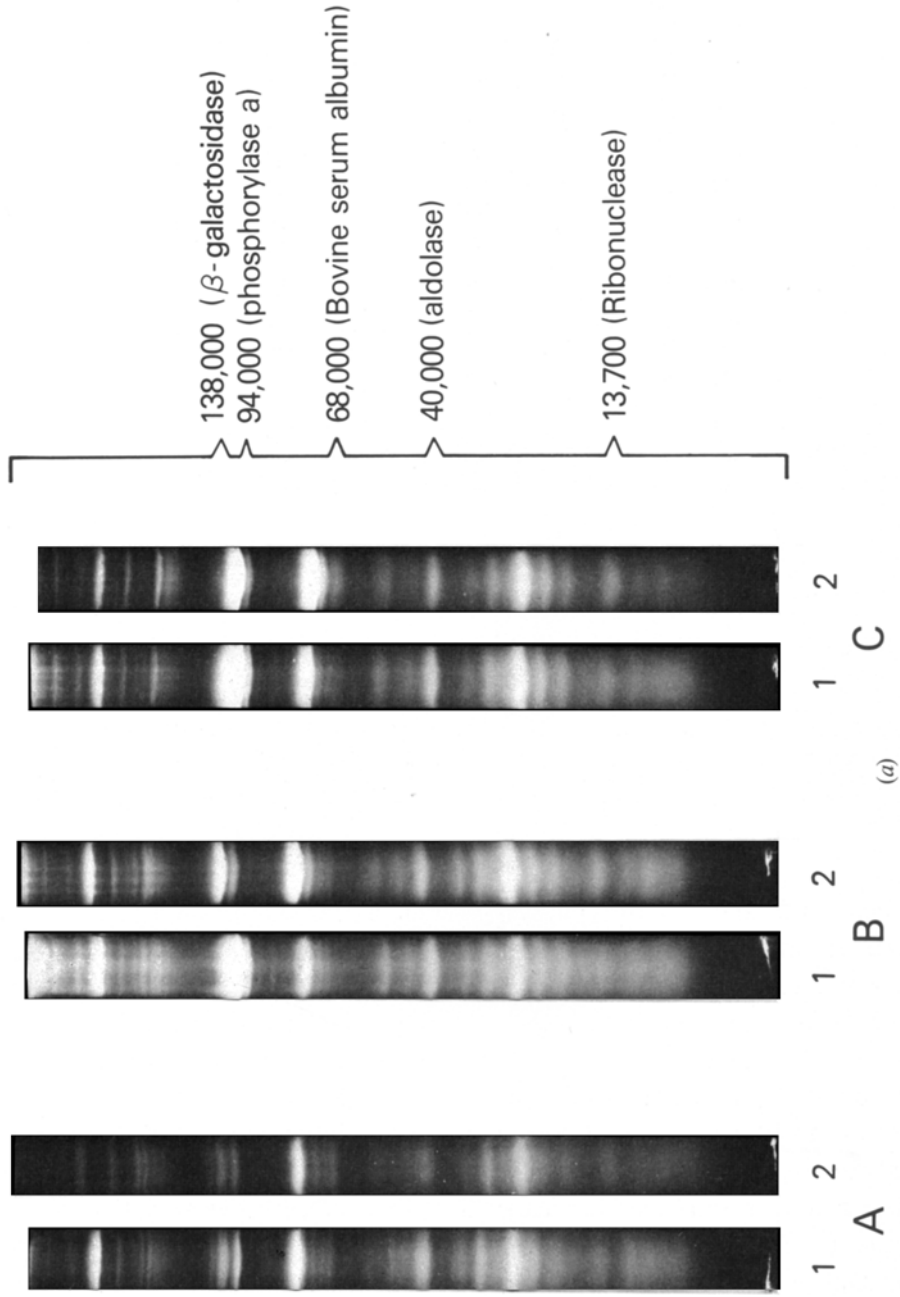

Fig. 6. SDS-PAGE of "fast" and "slow" sarcolemmal membrane proteins. Electrophoresis of 30 μg membrane protein as described previously (Festoff & Engel, 1974). (*a*) One-week denervation: *A* = soleus, *B* = EDL, *C* = gastrocnemius; *1* = normal, *2* = denervated. (*b*) Five-week denervation of EDL sarcolemmal proteins: *A* = normal, *B* = denervated, *C* = standard

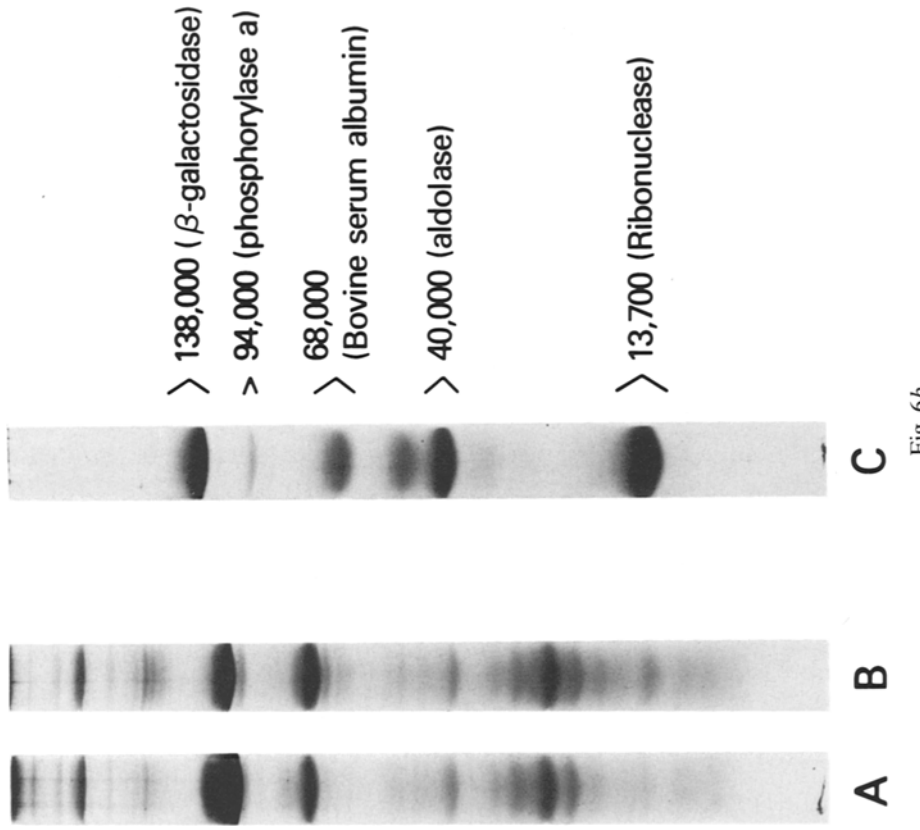

Fig. 6b

fiber membranes is shown in Fig.4. A dissociation constant of 2.25×10^{-7} M can be calculated from these data. This is in good agreement with the dissociation constant of 2.1×10^{-7} M reported by Clausen and Hansen (1974) for the intact muscle and with the K_i of ouabain for $(Na^+ + K^+)$-ATPase of these SL (5×10^{-7} M). The effects of denervation on the binding of [^{3}H]-ouabain to gastrocnemius SL were evaluated and the results are shown in Fig. 5. As can be seen, greater than threefold increase in binding of labeled glycoside occurs after two weeks of denervation. The extent of binding then drops to below control levels by five weeks. It is of interest to note that in the control membranes from the older animals there is also a decrease in the [^{3}H]-ouabain binding, as well as enzyme inhibition. It is known that sensitivity of the rat cardiac enzyme to glycoside, but not the brain enzyme, is less than that in other species (Schwartz *et al.*, 1975), and that older animals, especially the rat, show a decrease in specific activities of a number of enzymes (Williams & Thompson, 1973).

Effects of Denervation on SDS-PAGE Patterns of SL Proteins

Because denervation induced several functional changes in membrane components described above, we anticipated marked alterations in the protein patterns obtained on SDS gels. Using similar membrane preparations, Andrew and Appel (1973) at first found no alteration in major band patterns but subsequently reported a decrease in low molecular weight peptides (Andrew *et al.*, 1975) with denervation. In the present studies, an obvious quantitative change could be demonstrated for soleus (Fig. 6). These changes appeared in the 240,000 mol wt range whereas the EDL shows an apparent decrease in semiquantitative Coomassie blue staining in the range of 100,000 to 110,000 daltons (Fig. 6). Although purely speculative, it is intriguing to consider that some other protein, at a molecular weight similar to or identical with the ^{32}P-intermediate (which is in this same molecular weight range), has been lost from the SL as a consequence of denervation and thereby may account for the observed increase in specific activity. Such a protein might be the anion binding site, recently shown to be in band 3 (100,000 daltons) of red cell membranes (Rothstein, Cabantchik & Knauf, 1976).

Discussion

The molecular components of monovalent cation transport are generally felt to be associated with the plasma membrane of living cells. As a marker enzyme for plasma membrane isolation, the $(Na^+ + K^+)$-ATPase is frequently used. A number of recent reports have shown the association of $(Na^+ + K^+)$-ATPase with surface membrane fractions of skeletal muscle (Boegman, Manery & Pinteric, 1970; Andrew & Appel, 1973; Festoff & Engel, 1974). In addition, we have shown that the phosphorylated intermediate of the $(Na^+ + K^+)$-ATPase is present in isolated sarcolemma and co-purifies with the Na^+- and K^+-stimulated ATP hydrolytic activity (Reddy *et al.*, 1976). Glycoside binding sites, located on the externally disposed face of the surface membrane, have been demonstrated in skeletal muscle (Clausen & Hensen, 1974). We have recently characterized these ouabain-binding sites in sucrose-density purified sarcolemmal membranes (Festoff *et al.*, *in preparation*). These preparations allowed us to evaluate the role of the motor nerve in the regulation of these transport system components in skeletal muscle *in vitro*.

The present results suggest that the increase in sarcolemmal $(Na^+ + K^+)$-ATPase would predict an increase in the number of sites

of active Na^+ and K^+ transport in the muscle membrane. Our data demonstrate that an increase in Mg^{++}-ATPase (fourfold with EDL membranes) and a lesser, but significant, increase (one- to twofold) in $(Na^+ + K^+)$-ATPase occurs with denervation of mixed fiber, red and white skeletal muscle. Though some variability in the degree of stimulation with Na^+ and K^+ occurs even in normal SL, there is a tendency for this degree of activation to diminish with longer periods of denervation. Severin, Boldyrev and Tkachuk (1974) demonstrated an increase in sarcolemmal $(Na^+ + K^+)$-ATPase with denervation. They further showed an inhibition of both normal and denervated membrane $(Na^+ + K^+)$-ATPase to acetylcholine (ACh) and cholinergic analogues. The denervated membranes were 20-fold more sensitive, and they suggested a correlation with the known increase of fast membranes to ACh following denervation.

Since the number of phosphorylation sites and glycoside receptor sites relate to overall sites of Na^+ and K^+ transport, we evaluated these in preparations of gastrocnemius SL. Our ATPase data confirmed the previous authors' findings (Severin *et al.*, 1974), and the apparent glycoside binding sites also increased threefold during the midpoint of the denervation curve. We are presently evaluating whether the K_i for ouabain inhibition of SL $(Na^+ + K^+)$-ATPase (5×10^{-7} in normal SL) changes accordingly in denervated SL. We have demonstrated that [^{3}H]-ouabain binding to SL suggests a single class of sites (Festoff *et al.*, *in preparation*), and it would be of interest if a lower affinity site appears (with denervation), as was demonstrated in or brain $(Na^+ + K^+)$-ATPase (Taniguchi & Iida, 1972).

It has been demonstrated in several systems (Schwartz *et al.*, 1975; Albers, 1976); that the high turnover numbers of ATPase relate to the functional significance of the phosphorylated intermediate in the overall ATPase reaction. Our data (Reddy *et al.*, 1976) have shown turnover numbers (nmoles Pi liberated per mg per min ÷ nmoles Pi incorporated per mg per min) of 11,000 and 12,000 min^{-1} for crude and purified SL. These compare favourably with those reported from other sources. With denervation, this number increases approximately threefold with the increase in $(Na^+ + K^+)$-stimulated ATP hydrolytic activity and the decrease in Na^+-stimulated ^{32}P incorporated. Phosphorylation of SL membrane proteins is not different, qualitatively, from normal membranes, the majority occurring in the 108,000 dalton protein.

The question arises: does the denervation-associated increase in monovalent cation transport sites predicted from the current studies

result from activation of preexisting sites or synthesis of new ones? Both an increase in ouabain binding (at two weeks) and an increase in turnover (at five weeks) were found. It is well known that changes in protein synthesis develop in muscle subsequent to denervation (Guth, 1968). However, as reviewed by Guth (1968), denervation produces dissimilar changes in different muscles: increased amino acid incorporation was observed in diaphragm, while decreased synthesis and increased proteolysis occurred in others. Furthermore, it has been shown that compartmental differences also exist in denervated muscle, with increases in the membranous sarcotubular compartment and decrease in myofibrillar proteins (Gutmann, 1962). Denervation might uncover "latent" pump sites, possibly by effecting other membrane proteins which might serve to inhibit or restrain pump activity in the innervated state. It is known that the usually high muscle chloride conductance diminishes in myotonia (Bryant, 1973) and in denervation (Bryant & Camerino, 1976). The anion binding protein thought to be involved in the obligatory self-exchange of Cl^- across membranes, is present in the 100,000 daltons band 3 of red cell membranes (Rothstein *et al.*, 1976). A decrease in this molecular weight band occurs in the SL of denervated EDL (Fig. 6*a* & *b*) while ATPase activity increases (Table 1). It is possible that neural influences might regulate this important anion channel in skeletal muscle, and that a correlation of increased Na^+ and K^+ transport and decreased Cl^- movement might exist in denervated skeletal muscle. At the present time, however, we are not able to choose between an increase in turnover or an increase in enzyme synthesis. It is possible that the increase in glycoside binding might be due to a lower affinity site only transiently appearing during the course of denervation.

Finally, of what significance, if any, is the marked (fourfold) increase in basal Mg^{++}-ATPase? Histologically, one of the prominent alterations in muscle architecture occurring after denervation is the development of vacuolar degeneration (Gutmann & Zelena, 1962). Numerous membrane-bound vacuoles appear in both "fast" and "slow" skeletal muscle after severing their nerve supply. An increase in permeability to molecules such as glucose (Bass, 1962) and amino acids (Goldberg & Goodman, 1969) also occurs after denervation, possibly reflecting enhanced endocytosis. In red blood cells, correlation of endocytosis and vacuole formation with levels of Mg^{++}-ATPase activity has been made (Penniston & Green, 1968; Ben-Bassat, Bensch & Schrier, 1972; Penniston, 1972). It is possible that the three- to fourfold increase in Mg^{++}-ATPase of SL at five weeks post-denervation corresponds both to the presence of vacuoles in the

muscle at this time and the observed increased permeability (endo- or pinocytosis). Thus, it may be that another of the controls exerted by nerve on muscle is the regulation of its permeability to nonion nutrients and that this is mediated via neural regulation of Mg^{++}-ATPase activity.

Whether this, or any other "neurotrophic" function, represents an influence on *de novo* synthesis (via gene regulation), loss of "endogenous inhibitors," or reorientation of membranes in fractionation, or some other yet to be defined effect is currently under investigation.

The authors express their gratitude to Dr. R.W. Albers for helpful discussions. The present paper is fourth in a series on characterization of muscle membranes *in vitro*. The preceding paper is that of Festoff *et al.* (1977).

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