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# EFFECTS OF DENERVATION ON THE COMPOSITION AND SYNTHESIS OF SARCOLEMMAL PROTEINS

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## Summary

In vitro synthesis of proteins and changes in polypeptide composition of sarcolemma were studied in innervated and denervated extensor digitorum longus muscle of the rat. A technique of evacuating myoplasm from muscle slices was used as a preliminary step in the preparation of three membrane fractions, M, H and S, containing sarcolemma. On the basis of findings from the previous study and the present investigation, it was concluded that the M fraction was most enriched with extrajunctional sarcolemma.

In vitro incorporation of [$^3$H]leucine into membrane proteins of the M fraction showed an apparent linear increase in the rate of protein synthesis from 1—10 days after denervation. The relative increase at 10 days was 137% greater than that of innervated controls. Fractions H and S showed a smaller relative increase.

Polypeptide composition of M, H and S fractions based on SDS gel electrophoresis of innervated and denervated muscle, showed qualitative and quantitative changes. The most striking difference was a nominal 29 000 component in M that constituted a disproportionately large peak. Following 10 days of denervation the M fraction underwent significant compositional changes in its electrophoretic profile, the most dramatic of which was a large reduction in the proportion of the 29 000 component. The denervation-induced compositional change is discussed in light of known alterations in the chloride conductance of the muscle plasmalemma.

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## Introduction

Denervation of skeletal muscle induces striking changes in the electrical properties of the extrajunctional sarcolemma, the latter of which have been well characterized from a functional standpoint (for reviews see Refs. 1—5). It is reasonable to infer that quantitative and qualitative alterations in the composition of sarcolemmal proteins may accompany such changes, and that there may also be changes in the rate of membrane protein synthesis. An obvious example is the increase in extrajunctional acetylcholine sensitivity following denervation [6], which is due to de novo synthesis of extrajunctional acetylcholine receptors [7—10]. However, little attention has been given to alterations in polypeptide composition and membrane protein synthesis of extrajunctional sarcolemma following denervation.

We report the results of a study in which changes in protein composition and rate of in vitro protein synthesis of the sarcolemma, following denervation of the extensor digitorum longus of the rat, were investigated. Analysis was carried out on membrane fractions prepared from evacuated muscle slices [11]. Results indicate that both the rate of membrane protein synthesis and the composition of membrane proteins are altered following 10 days of denervation.

## Methods

*Experimental preparations.* The extensor digitorum longus muscles of male Sprague-Dawley rats were denervated bilaterally by removal of a 1 cm segment of the common peroneal nerve [11] and the animals were allowed to survive for varying periods of time. Innervated muscles were dissected bilaterally from normal, unoperated rats and, in one case, from a bilaterally sham-operated rat.

*Isolation of muscle membrane fractions from evacuated muscle slices.* Evacuated muscle slices, consisting of connective tissue stroma, basal lamina and sarcolemma, were prepared according to Wallis et al. [11]. This procedure eliminates virtually all myoplasmic constituents as a first step in the preparation of membrane fractions. Evacuated muscle slices were homogenized and subjected to a series of differential and discontinuous sucrose gradient centrifugations, which yielded three membrane fractions containing sarcolemma, according to specific $\alpha$-bungarotoxin binding distribution; viz., M, H and S, recovered from 0.5/0.7 M, 0.7/0.85 M and 1.0/1.5 M interfaces of a discontinuous sucrose gradient, respectively [11].

*Preparation for in vitro membrane protein synthesis.* Four muscles of two anesthetized rats were rapidly excised and placed in a petri dish containing ice-cold Eagle's minimum essential medium with glutamine and lacking leucine (Gibco), and which was equilibrated with a 95% $O_2$/5% $CO_2$ mixture. The muscles were secured to two rubber washers mounted on a plexiglass dowel so as to approximate their in situ resting length. Silk thread ties, attached to the muscle tendons, were inserted into the slits of the washers. A plastic, 10 $cm^3$ syringe barrel sealed at the needle end served as a disposable incubation chamber. The syringe contained 7 ml of Eagle's minimum essential medium, supplemented with 0.1 unit/ml insulin (Sigma), and 10 mM glucose. The syringe piston was modified so that humidified 95% $O_2$ and 5% $CO_2$ could be bubbled

through the medium during the period of incubation. The incubation chamber was placed in a water bath maintained at 37°C.

Excised muscles were preincubated for 15 min at which time 200 μCi of [$^3$H]leucine L-[3,4,5-$^3$H(n)]leucine, spec. act. 84 Ci/mmol (New England Nuclear), was injected into the incubation medium. Muscles were incubated for varying time periods up to 4 h. Following a given period of incubation, the muscles were removed in pairs and placed into ice-cold distilled water, and evacuated muscle slices were prepared as described by Wallis et al. [11].

Individual membrane fractions of interest (viz., M, H and S), or an aggregate membrane fraction were prepared. In the latter case, a one-step sucrose gradient, (i.e., 0.32/1.5 M sucrose) was used to combine the individual membrane fractions into a single membrane fraction. Fractions were recovered by aspiration, sedimented and frozen.

Frozen samples were thawed and resedimented by centrifugation at 8000 × *g* for 15 min, and each pellet was solubilized with 1 N NaOH. Aliquots from each sample were taken for scintillation counting and protein determination. Protein was determined by a modification of the Lowry et al. procedure [12], using bovine serum albumin in 1 N NaOH as standard. Specific radioactivity was expressed as cpm/μg protein.

*Gel electrophoresis.* SDS-polyacrylamide gel electrophoresis was used to fractionate major membrane polypeptides. The discontinuous system described by Laemmli [13], as modified by Ames [14], was found to be satisfactory for the analysis of the small samples available in this study.

Polyacrylamide gels were prepared from a stock solution of 30% acrylamide (w/v) (Eastman)/0.8% *N,N'*-bismethylene acrylamide (Eastman). Stacking and separating gels were composed of 5% and 10% polyacrylamide, respectively. All gels and buffers contained a final concentration of 0.1% SDS. Gels were less than 1 mm thick and a Tris-glycine buffer was used for electrophoresis [13].

Samples for electrophoresis were solubilized overnight in a sample buffer, described by Laemmli [13], and then placed in a boiling water bath for 2 min. Molecular weight standards, consisting of bovine serum albumin (monomer and dimer), ovalbumin and cytochrome *c* (Sigma) were solubilized in the same buffer, with the exception of β-mercaptoethanol. Final concentration of each of the standards was 0.1 μg/μl.

Electrophoresis was carried out at room temperature, using a constant voltage of 150 V for about 2 h. The gels were fixed in a solution of glacial acetic acid/95% ethanol/water (1 : 2 : 6, v/v/v), stained in fixing solution containing 0.025% Coomassie blue and destained in 0.1 M acetic acid. Gel tracts, containing individual fractionated samples, were cut out of the slabs and scanned at 620 nm using a modular microdensitometric system. The density profiles were recorded on a strip chart recorder.

## Results

*In vitro synthesis of membrane proteins in innervated and denervated muscles*

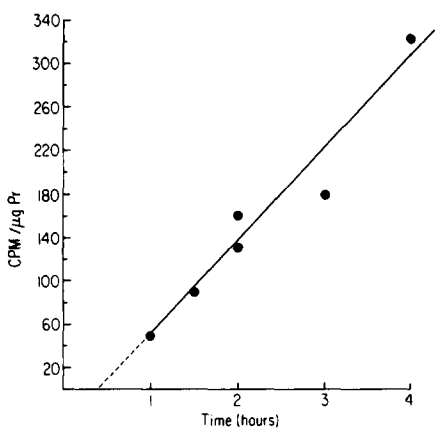
Controls muscles were incubated for varying times to characterize the maximum rate of [$^3$H]leucine incorporation into membrane protein. Following the period of incubation, pairs of muscles were removed and used to prepare an

aggregate membrane fraction from evacuated muscle slices containing a mixture of membrane fractions of interest (i.e., M, H and S; see Ref. 11). Fig. 1 depicts the change in specific radioactivity of the proteins of the aggregate membrane fraction as a function of incubation time in vitro and indicate that the rate of protein synthesis was linear for at least 4 h of the incubation in vitro. Extrapolation of the linear-regression line back to the X-axis revealed that labelled proteins were incorporated into the membrane after a lag of 25 min. The addition of 1 mM cycloheximide to the incubation medium inhibited membrane protein synthesis by 98% (data not shown). Extraction of lipids from the aggregate membrane fraction with a diethylether/ethanol solution (3 : 2, v/v) resulted in the extraction of 3% of the total radioactivity of the fraction.

The foregoing experiments demonstrated that: (1) the in vitro system was capable of supporting membrane protein synthesis at a constant rate for at least 4 h; (2) nascent membrane protein required a minimum of 25 min before incorporation into membranes; (3) the proteins synthesized were dependent on cytoribosomal machinery; and (4) most of [$^3$H]leucine precursor was incorporated into membrane proteins.

Based on morphological and biochemical criteria, membrane fractions M, H and S, prepared by the evacuation technique [11] contained sarcolemma and were selected for study. Each was analyzed with respect to the relative rate of membrane protein synthesis and polypeptide composition of extensor digitorum longus, a fast twitch muscle type, from normal and denervated muscles.

The labelling of membrane proteins in each of the fractions with time after denervation is shown in Table I. All three fractions showed increases in specific radioactivity following denervation. 10 days after denervation, the M fraction



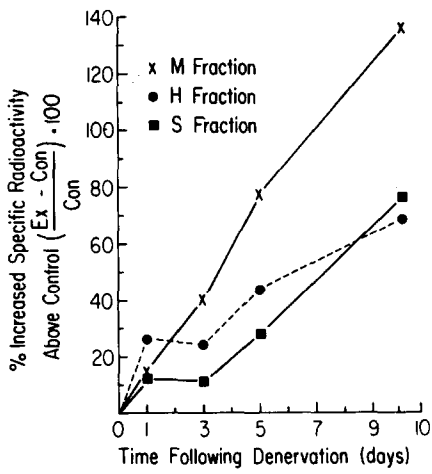




Fig. 1. In vitro synthesis of total membrane protein slices as measured by rate of incorporation of [$^3$H]-leucine into protein (Pr) of the aggregate membrane fraction, composed of membranes making up M, H and S fractions. Extrapolation of the linear regression line to the time axis (dashed line), intercepts at about 25 min.

Fig. 2. Relative changes in the relative change in specific radioactivity of proteins from EMS plasma membrane fractions following denervation by [$^3$H]leucine incorporation into protein of M, H and S membrane fractions with time after denervation of extensor digitorum longus.

TABLE I

INCORPORATION OF [$^3$H]LEUCINE INTO PROTEINS OF M, H AND S MEMBRANE FRACTIONS FOLLOWING DENERVATION OF EXTENSOR DIGITORUM LONGUS

Results are expressed as cpm/μg ± S.E. for three separate experiments.

| Experiment | Membrane fraction | cpm/μg protein |
|---|---|---|
| Control | M | 60 ± 9 |
| 1 day denervated | M | 69 ± 17 |
| 3 day denervated | M | 84 ± 16 |
| 5 day denervated | M | 107 ± 20 |
| 10 day denervated | M | 142 ± 13 ** |
| Control | H | 96 ± 17 |
| 1 day denervated | H | 121 ± 27 |
| 3 day denervated | H | 119 ± 24 |
| 5 day denervated | H | 138 ± 16 |
| 10 day denervated | H | 162 ± 28 |
| Control | S | 74 ± 5 |
| 1 day denervated | S | 83 ± 16 |
| 3 day denervated | S | 82 ± 11 |
| 5 day denervated | S | 95 ± 9 |
| 10 day denervated | S | 131 ± 21 * |

* Difference between experimental and control significant with $P < 0.05$.
** Difference between experimental and control significant with $P < 0.005$.

exhibited the largest increase in specific radioactivity, at which time it was 2.5-times higher than innervated control. Smaller relative increases in specific activity was also apparent in the H and S fractions following 10 days of denervation.

When the relative increase in specific radioactivity of membrane protein with time after denervation was plotted (Fig. 2), the percent increase of the M fraction was almost linear, reaching a maximum after 10 days (the longest time interval studied after denervation) of 137% above control. In contrast to M, both H and S showed no change in the relative rate of labelling between 1 and 3 days after denervation, but exhibited moderate relative increases later.

*SDS electrophoresis of membrane proteins*

A comparison of the densitometric profiles of the three membrane fractions from innervated muscle, designated $M_0$, $H_0$ and $S_0$ (subscript denotes days after denervation) are presented in Figs. 3, 4 and 5. Because the yield of protein from each of the fractions was small the amount of each fraction applied to the gel was not determined. However, in other experiments in which the protein content of individual fractions was determined, [11], the mean values for the three experiments were 14.1 ± 2.2, 12.4 ± 1.6 and 6.9 ± 0.6 mg for $M_0$, $H_0$ and $S_0$, respectively. Nevertheless, comparisons in composition and proportions of components can be made. As is clearly seen in Figs. 3, 4 and 5, the densitometric profiles reveal major differences in polypeptide composition between membrane fractions $M_0$, $H_0$ and $S_0$. One of the most striking of these differences is the presence of a major peak of $M_0$ (Fig. 3, large open arrow), which is just discernible by inspection of the electrophoretograms of $H_0$, but hardly so in $S_0$ (cf. Figs. 3, 4 and 5, large open arrow). This polypeptide in $M_0$ has a

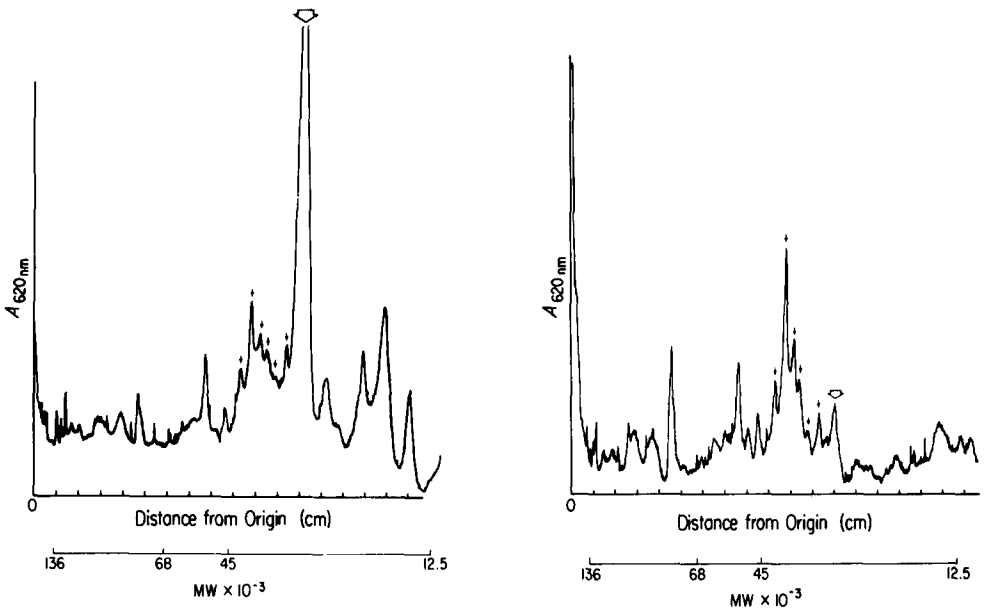



Fig. 3. Densitometric profile of a Coomassie blue stained electrophoretogram of a $M_0$ membrane fraction, prepared from innervated extensor digitorum longus muscles. The nominal $M_r$ of the single large peak is 29 000 (large open arrow). The small arrows designate the 'sextet' of peaks common to M, H and S fractions.

Fig. 4. Densitometric profile of a Coomassie blue stained electrophoretogram of a $H_0$ membrane fraction, prepared from innervated extensor digitorum longus muscles. The 29 000 peak is designated by the large open arrow. The small arrows designate the 'sextet' of peaks common to M, H and S fractions.

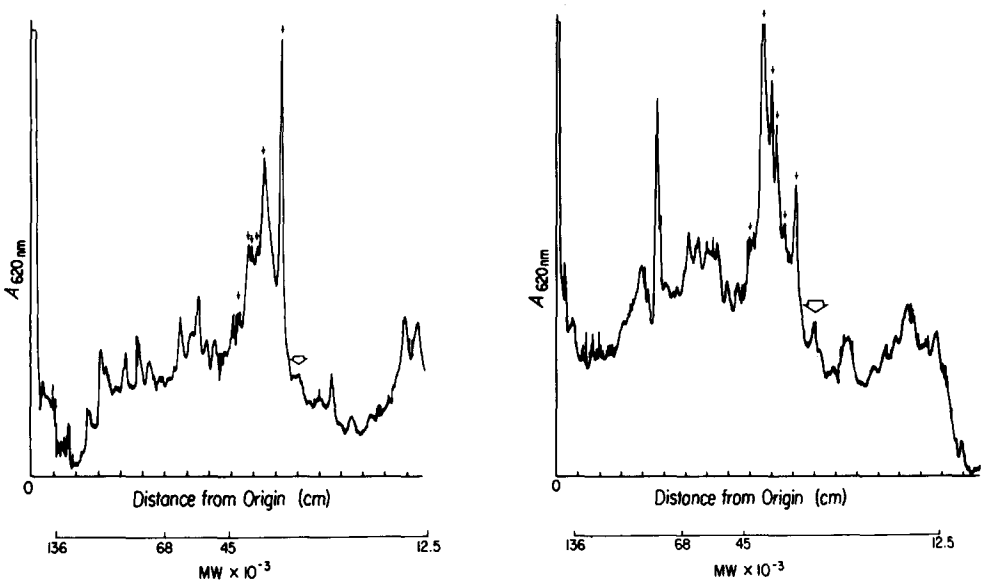



Fig. 5. Densitometric profile of a Coomassie blue stained electrophoretogram of an $S_0$ membrane fraction, prepared from innervated extensor digitorum longus muscles. The apparent 29 000 peak is designated by the large open arrow. The small arrows designate the 'sextet' of peaks common to M, H and S fractions.

Fig. 6. Densitometric profile of a Coomassie blue stained electrophoretogram of an $M_{10}$ membrane fraction, prepared from extensor digitorum longus muscle that had been denervated for 10 days. The 29 000 peak (large open arrow) is markedly diminished (cf. Fig. 3). The 'sextet' of peaks common to M, H and S membrane fractions are very much more prominent.

nominal molecular weight of 29 000 and comprises about 38% of the Coomassie blue stained electrophoretic profile of the $M_0$ fraction. Also of interest is the larger proportion of membrane polypeptides in $M_0$ below 29 000 as compared to $H_0$ and $S_0$.

Above the 30 000 range the densitometric profiles of $M_0$, $H_0$ and $S_0$ are quite similar. Of special note is a region containing six bands that is common to all three membrane fractions (Figs. 3, 4 and 5, small arrows). This 'sextet' of bands ranges in molecular weight from approx. 31 000—42 000 and constitute the major components of the $H_0$ and $S_0$ gels.

Following 10 days of denervation the M fraction (denoted $M_{10}$) undergoes the most dramatic changes in polypeptide composition. The 29 000 component so prominent in the $M_0$ electrophoretogram is not readily visible by inspection but an apparent residual peak can be discerned in the densitometric profile (Fig. 6). The other major peaks below 29 000 have also been greatly reduced in size, and the densitometric profile is now dominated by the 'sextet' of bands (Fig. 6, small arrows) as is typical of $H_0$ and $S_0$. Minor changes in the polypeptide pattern of the $H_0$ and $S_0$ fractions were also seen following 10 days of denervation (data not shown).

*Control experiments*

Following neurotomy, neural degeneration of intramuscular nerve fascicles occurs, the products of which are removed by phagocytic cells within 10—15 days. It could be argued that the change in the composition of the M fraction following 10 days of denervation could be due to a loss of axonal components. Whilst this remains a possibility it is unlikely because the amount of axonal material, specifically nerve membrane, probably constitutes a small fraction of the protein in control M fractions. Nevertheless, an experiment was done in which the standard membrane fractions were prepared from normally innervated muscles from which the endplate regions had been surgically excised before preparation of evacuated muscle slices (endplate-free muscles). If the loss of the major components in the M fraction was due to the removal of axonal membrane protein following denervation, then the preparation of an $M_0$ fraction from muscle in which intramuscular nerve branches and terminals had been excised should yield a similar result to that of denervated muscle.

Endplate-free muscle were prepared by the extirpation of an approx. 8-mm section through the belly of the muscle. The remaining proximal and distal portions were used for the preparation of muscle membrane fractions. To assess the efficacy of endplate removal in these muscles, acetylcholinesterase was assayed in the $P_4$ fraction from the same preparation from which the M fraction was analyzed by electrophoresis. Previous experiments showed that about 93% of the acetylcholinesterase activity of evacuated muscle slices was associated with the $P_4$ pellet [11]. A comparison of acetylcholinesterase activity between $P_4$ from endplate-free muscle and $P_4$ from normal muscle should indicate the extent to which the endplate regions had been eliminated in the former. In one such experiment, acetylcholinesterase activity of the $P_4$ fraction from endplate-free muscles was reduced by 70% relative to that of $P_4$ from normal muscles, indicating that the majority of endplate regions had been removed. The electrophoretic profile of the M fraction from the endplate-free muscles was not dif-

ferent from that of normal muscle (data not shown). Thus, it can be concluded that the major peaks in densitometric profiles of the $M_0$ fraction were not derived from axonal membranes.

Another control experiment was carried out to determine if the major bands in $M_0$ were derived from nonspecific adsorption of cytoplasmic proteins to the plasmalemma during the preparation of evacuated muscle slices. To test this possibility evacuated muscle slices, prepared in the usual manner from innervated muscles, were placed in 500 ml of cold 0.3 M $Na_2SO_4$ to dissociate proteins bound non-specifically [15]. Following a minimum of 30 min in $Na_2SO_4$, the evacuated slices were placed in a flask of cold 0.09% triethanolamine and swirled for approx. 5 min and were than removed, blotted and used for the standard preparation of membrane fractions. The electrophoretic profile of $M_0$ after $Na_2SO_4$ extraction was similar to that of an $M_0$ electrophoretic profile from untreated muscles (data not shown). Thus, the major proteins of the M fraction from innervated muscle were not likely to be derived from sources other than membranes. These findings, moreover, indicate that the major components of $M_0$ were probably integral membrane proteins.

## Discussion

On the basis of previous work [11] and findings of the present study related to changes in polypeptide composition in response to denervation, we conclude that the M fraction is most enriched with respect to sarcolemma. As such, incorporation of labelled precursor into the protein of this fraction should provide information about the effects of denervation on the rate of sarcolemmal protein synthesis.

Denervation for 10 days leads to an almost linear increase in the rate of incorporation into membrane proteins of this fraction, reaching a value 2.5-times higher than that of innervated controls. This would appear to represent an increase in the rate of sarcolemmal protein synthesis following denervation; however, this inference presumes that the rate at which the amino acid pool was labelled was not affected significantly by denervation and no information on this point is available. Nonetheless, increases in rough endoplasmic reticulum and polysomes have been reported in denervated muscle [16,17]. On the other hand, the electrophoretic profiles of the M fraction indicate that the synthesis of a major component, and probably some minor ones may be partially or even completely inhibited following denervation.

Because membrane protein degradation is thought to be a random event [18], another possible explanation for the increase in specific radioactivity of the M fraction membranes is that the rate of sarcolemma protein turnover has decreased following denervation. Although information concerning the effects of denervation on sarcolemma protein degradation was not obtained in the present study, other results indicate that general protein degradation increases after denervation [19,20]. Enhanced activities of glycosidases, acid phosphatases, proteolytic enzymes and lysosomes have also been observed following denervation [21—23]. Indeed, Goldspink [20] found an increase in both synthesis and degradation of muscle protein in 10-day denervated extensor digitorum longus muscles of the rat. In addition, certain glycosyltransferases and

possibly some exoglycosidases have been reported to exhibit increased activities in a plasma membrane fraction prepared from denervated muscle [24]. Thus, protein synthesis and degradation both appear to increase following denervation resulting in an increased rate of protein turnover.

Considering that both electrical and functional properties of the sarcolemma are altered following muscle denervation, it is not surprising that polypeptide composition should also be altered in response to denervation. The most striking change is the virtual loss of a 29 000 component in the M fraction following 10 days of denervation. This component comprises the largest proportion of membrane protein in the M fraction from innervated muscle. A phosphorylated protein with a molecular weight of 28 000 was reported in a low-density membrane fraction derived from a microsomal pellet of rat skeletal muscle [25]. The latter polypeptide also decreased in amount following 10 days of denervation. The similarity in electrophoretic mobility and the effects of denervation in both cases suggest that the two polypeptides could be the same; however, no experimental evidence exists at this time to justify this supposition. In fact, the fractions from which the two polypeptides are derived have quite different characteristics (cf. Refs. 11 and 25).

Is there a physiological correlate that can be ascribed to major reductions of certain membrane polypeptides following denervation? In normally innervated rat muscle, $Cl^-$ accounts for 85—90% of the resting ionic conductances [26, 27]. Following 2.5 weeks of denervation, Camerino and Bryant [26] reported about an 80% decrease in $Cl^-$ conductance in the extensor digitorum longus muscles of rats. The conductance pathway for $Cl^-$ in rat muscle is thought to be mediated by transmembrane aqueous 'channels', passing through the lipid bilayer of the sarcolemma [27]. Such 'channels' are most likely composed of integral membrane proteins according to the fluid mosaic model [28]. If this is true, then a decrease in $Cl^-$ conductance in denervated rat muscle may reflect a reduced density of chloride 'channels'. In this context, it is of interest that a 50—75% reduction in the density of 80 Å intramembranous particles in extrajunctional sarcolemma of denervated rat has been reported by Tipnis and Malhotra [29], using freeze-fracture techniques.

While the functional significance of the loss of the 29 000 component in the M fraction following denervation is not known, it would be of interest to correlate changes in the M fraction polypeptide composition with changes in chloride conductance. In this respect, a comparison of polypeptide composition of M fractions prepared from evacuated muscle slices of normal, denervated and myotonic goats could provide a test of this hypothesis. Myotonia is characterized by an abnormally low $Cl^-$ conductance [30,31].

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