Early Biochemical Consequences of Denervation in Fast and Slow Skeletal Muscles and their Relationship to Neural Control over Muscle Differentiation

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1. One week after denervation several biochemical characteristics of the fast extensor digitorum longus and slow soleus muscles from adult rats were investigated and compared with the characteristics of the corresponding unoperated contralateral muscles. 2. After these short periods of denervation-induced atrophy, the isolated myosins showed unchanged ATPase (adenosine triphosphatase) activities, but there was the expected difference between fast and slow muscle. 3. The specific activities of several soluble enzymes and their characteristic patterns were found to be only slightly modified in both the extensor and soleus muscles after denervation, as were most of the activities measured in the isolated mitochondria. 4. The most significant modifications were in the isolated sarcoplasmic reticulum, and appeared to be specific to either slow or fast muscle. 5. Denervation of slow muscle led to a marked increase of Ca^{2+} -transport rates, and of the specific activity of the Mg^{2+} -activated K^{+} -modulated Ca^{2+} -stimulated ATPase, together with changes in the polyacrylamide-electrophoretic profiles of the microsomal membrane protein. Transformation of these several properties of slow muscle sarcoplasmic reticulum to those of fast muscle sarcoplasmic reticulum was further substantiated by electron-microscopic analysis after negative staining. Control experiments with tenotomized soleus muscle gave negative results. 6. The isolated sarcoplasmic reticulum from fast muscle showed a slight diminution of ATPase-linked Ca^{2+} -transport activity and a selective increase of rotenone-insensitive NADH-cytochrome *c* reductase activity, in addition to a greater emphasis on slow-type electrophoretic components of the structural membrane protein. 7. The significance of these results in relation to specific differentiating influences from motor nerves is discussed.

Mammalian skeletal muscles are physiologically distinguishable into fast and slow muscles. At first neural control over the speed of muscle contraction, akin to a continuously acting differentiating factor (see Eccles, 1963), was envisaged as accounting for ontogenically determined changes in muscle contractile characteristics, as well as changes after cross-innervation between fast and slow muscles (Buller *et al.*, 1960a,b).

However, structural and kinetic studies on isolated myosins provided a molecular basis satisfying a more rigorous definition of muscle differentiation according to two fundamental types (Seidel *et al.*, 1964; Bárány *et al.*, 1965; Maddox & Perry, 1966; Trayer *et al.*, 1968; Johnson & Perry, 1970). A general correlation between rate of ATP hydrolysis by myosin and the

intrinsic speed of muscle shortening is derived mainly from developmental (Trayer & Perry, 1966) and comparative studies (Bárány, 1967) in several mammalian species. A complete switch from synthesis of fast-type myosin to slow-type myosin has been demonstrated in a cat fast muscle after re-innervation with a slow motor nerve (Buller *et al.*, 1969).

Experimental evidence is being accumulated to show that neurogenic control extends to additional biochemical characteristics of fast and slow muscles, and that either slow or fast motor innervation has a predominant differentiating influence when individual molecular properties are considered. For example, the preponderance of the H-isoenzyme of lactate dehydrogenase in slow as compared with fast muscles (Dawson *et al.*, 1964) was accounted for by a directing influence from slow motor nerves on the synthesis of the H-form subunits in slow muscle, coincident with the beginning of motile activity (Margreth *et al.*, 1970b).

After cross-innervation of fast and slow muscles in the cat, Buller *et al.* (1969) have reported that the

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transformation of slow to fast myosin ATPase* was small in the re-innervated slow muscle, compared with the marked changes in lactate dehydrogenase isoenzymes, and with the co-ordinated changes of the ATPase and Ca^{2+} -uptake activities in the isolated sarcoplasmic reticulum (Mommaerts *et al.*, 1969).

The present experiments were undertaken with the aim of investigating the effect of denervation after a short time (1 week) on the catalytic properties of myosin, the biochemical characteristics of the isolated sarcoplasmic reticulum, and the enzymic composition of the mitochondria and soluble sarcoplasm, in the fast extensor digitorum longus and the slow soleus muscles of adult rats.

Experimental

Materials

Animals and muscles. Young adult (150–250g) male albino rats from an inbred Wistar strain were used. Right unilateral sciactomy was done the stated number of days (usually 7) before the muscles were removed under light anaesthesia with Nembutal. The sciatic nerve was transected up in the thigh, and about 1cm was removed. Tenotomy was performed by severing the Achilles tendon. The animals were stunned with a blow on the head and killed by exsanguination, and the muscles were removed from the hind legs immediately. The muscles were usually the extensor digitorum longus and soleus and, in some experiments, the gastrocnemius muscle. The muscles from the unoperated leg served as controls.

Chemicals and enzymes. EGTA was obtained from Fluka A.G., Buchs SG, Switzerland. Sucrose (AnalaR grade) and Nonidet P40 were products of British Drug Houses Ltd., Poole, Dorset, U.K. The $^{45}\text{CaCl}_2$ (2–10mCi/mg of Ca) came from The Radiochemical Centre, Amersham, Bucks., U.K. Pre-mixed scintillation fluors ('Omnifluor') were purchased from NEN Chemicals G.m.b.H., Frankfurt/Main, Germany. Acrylamide and *NNN'*-methylenebisacrylamide were obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., and were recrystallized before use (Loening, 1967). Amido Black 10B came from E. Merck, Darmstadt, Germany. Crystalline enzymes were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, through Biochemia, Milano, Italy. Bovine serum albumin, 2-mercaptoethanol (type I), Triton X-100, rotenone, cytochrome *c* (type III), NADH, succinate, DL- α -glycerophosphate, oxaloacetate, α -oxoglutarate, pyruvate, fructose 6-phosphate, fructose 1,6-diphosphate and ATP (disodium salt) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. ATP was converted into the imidazole or tris salt shortly

before use, after treatment with Amberlite IR-120 resin. L-Aspartic acid was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. All other products were analytical-grade reagents.

General methods

Preparation of tissue homogenates. After removal, the individual extensor and soleus muscles from seven to ten rats were each rapidly chilled in a small beaker kept on ice, then they were weighed on a torsion balance, and pooled in ice-cold 0.3M-sucrose. In each experiment, unless otherwise stated, the operated muscles and the corresponding muscles from the opposite side of the body were used for paired comparisons. All subsequent operations were done in the cold-room without further delay. The muscles were freed from excessive blood, then were trimmed of any obvious connective tissue and vessels before being finely minced with scissors on a pre-cooled watch-glass. About 1g of tissue mince was homogenized with 9vol. of 0.3M-sucrose in a flat-bottomed Perspex homogenizer equipped with a loosely fitting motor-driven pestle operated at 1500rev./min. In the experiments where phosphofructokinase activity was to be determined, the extracting medium was 0.3M-sucrose containing 5mM-disodium EDTA, pH8.0, to avoid inactivation by acid pH and Ca^{2+} (Margreth *et al.*, 1967). The coarse tissue homogenates were centrifuged at 650g for 10min (International PR-6 refrigerated centrifuge) to yield an unpurified myofibrillar–nuclear sediment, which served as the starting material for isolation of myosin. The supernatant solution was immediately subjected to differential centrifugation for isolation of the mitochondria, microsomal and soluble-protein fractions. In some experiments with the gastrocnemius muscle (see legend to Fig. 1), the tissue homogenates were made in 20mM-potassium phosphate, pH8.0, as described by Margreth *et al.* (1970b). The crude extract obtained by centrifuging the homogenates at 10000g for 30min was used for assay of glycolytic enzymes.

Isolation of myosin. The method used was adapted from the KI extraction method of Szent-Györgyi (1951), with certain modifications derived in part from the purification procedure of Bárány (1967). For this purpose, the crude myofibrillar sediment was washed three times by resuspension in 9vol. of 0.05M-KCl and re-centrifugation at the original speed to remove entrapped soluble proteins. Myosin was extracted from the washed sediment with 9vol. of 0.6M-KI–6mM- $\text{Na}_2\text{S}_2\text{O}_3$ for 10min in the cold, with occasional stirring with a small glass rod. After centrifugation at 13000g for 20min (Sorvall RC2-B centrifuge), the sediment was discarded. The supernatant solution was filtered through filter paper and diluted with water to lower the final concentration of

* Abbreviations: ATPase, adenosine triphosphatase; EGTA, ethanedioxybis(ethylamine)tetra-acetate.

KI to 0.025 M (Szent-Györgyi, 1951). The precipitated myosin was collected by centrifugation at 2500g for 10 min and was resuspended in a small volume of 0.5 M-KCl. Contaminating actomyosin was separated by centrifugation at 150000g for 60 min in the SW-50 rotor of a Spinco L2 50 centrifuge. The supernatant solution containing purified myosin was used for ATPase assays. The yield of purified protein varied in the several experiments between 10 and 16 mg/g fresh tissue wt., without any consistent difference between extensor and soleus, as well as between denervated and control muscles.

Preparation of subcellular fractions. Mitochondria were sedimented from the myofibrillar supernatant fraction by centrifugation at 6500g for 20 min, before being washed once by resuspension in 0.3 M-sucrose medium and re-centrifuged. The mitochondria were stored frozen at -20°C overnight and were thawed and frozen three times immediately before use. For assay of malate dehydrogenase and aspartate aminotransferase the mitochondria were further disrupted by treatment with 0.1 % Triton X-100 at 0°C for 30 min (Haslam & Krebs, 1968).

'Heavy' and 'light' microsomal fractions were isolated from the mitochondria-free supernatant by centrifugation for 30 min at 30000g and 80000g respectively. These fractions were suspended in a small volume of 0.3 M-sucrose and a sample of the suspension was immediately used for determination of Ca^{2+} -uptake and ATPase activities, the residual portion being stored at -20°C and used within 1 day. The soluble sarcoplasm protein fraction was separated by further centrifugation of the 80000g supernatant at 150000g for 60 min. The centrifugations were done in a Sorvall RC2-B centrifuge and, for the higher speeds, in the 50 Ti rotor of a Spinco L2 50 centrifuge.

Polyacrylamide-gel electrophoresis of microsomal protein. Microsomal fractions were isolated by differential centrifugation between 6500g for 20 min and 30000g for 30 min, and were washed once in 0.3 M-sucrose and once in 0.6 M-KCl-0.005 M-imidazole, pH 7.4. The washed microsomal protein was dissolved in a solution containing phenol-acetic acid-water (4:2:1, w/v/v), 0.1 % Nonidet P40 and 0.5 % mercaptoethanol. Polyacrylamide-gel electrophoresis was done in a Shandon disc-electrophoresis apparatus under the conditions described by Panet & Selinger (1970), on samples of about 100 μg of protein, by applying a constant current of 3 mA/gel column for 3.5 h. The gels were stained with 1 % (w/v) Amido Black in 7 % (v/v) acetic acid. Densitometric tracings of the stained gels were obtained by scanning at 620 nm in a Gilford recording spectrophotometer.

Determination of protein. This was determined by the method of Lowry *et al.* (1951).

Electron microscopy. Microsomal preparations were examined in a Siemens Elmiskop I A electron

microscope, after negative staining with 1 % ammonium molybdate solution, pH 7.1 (Margreth *et al.*, 1970a).

Enzyme assays

Assay of myosin ATPase. The conditions for assay of EDTA-activated and Ca^{2+} -activated ATPase activities were those described by Seidel (1967). After preincubation of about 100 μg of purified myosin in 1 ml of tris-buffered medium, pH 7.5, containing either 0.5 M-KCl and 5 mM-EDTA, or 25 mM-KCl and 10 mM- CaCl_2 , for 2 min at 37°C , the reaction was started by adding ATP-tris (5 mM, final concentration). The P_i liberated during 3 min incubation at 37°C was determined as described by Lindberg & Ernster (1956).

Assays of mitochondrial enzymes. All enzyme activities were determined at 30°C and at pH 7.5 by spectrophotometric methods in a Unicam SP.800 recording spectrophotometer.

Succinate-cytochrome *c* reductase (EC 1.3.99.1) and glycerol phosphate-cytochrome *c* reductase (EC 1.1.99.5) activities were measured from the reduction of cytochrome *c* at 550 nm ($\epsilon_{\text{mM red-ox}} = 19.1$; Minakami *et al.*, 1962) by the methods of Sottocasa *et al.* (1967) and Ringler & Singer (1959) respectively. Malate dehydrogenase activity (EC 1.1.1.37) in disrupted mitochondria was measured by following the oxidation of NADH at 340 nm in 3 ml of medium containing 50 mM-tris-HCl, pH 7.5, 0.1 mM-NADH, 50 μM -oxaloacetate and 1–2 μg of mitochondrial protein. It had been established in preliminary experiments that these concentrations of oxaloacetate were optimum for activity of mitochondrial malate dehydrogenase under the conditions of assay. Progressive inhibition of the activity was obtained, by increasing the concentration of oxaloacetate in the medium above approx. 0.1 mM. Aspartate aminotransferase (EC 2.6.1.1) was measured from the oxidation of NADH, in a phosphate-buffered medium, containing 33 mM-aspartate and 6.6 mM- α -oxoglutarate as described bySizer & Jenkins (1962).

Assays of soluble enzymes. Assays of enzyme activities were all based on spectrophotometric methods. Product formation was measured at 340 nm in the Unicam spectrophotometer by the rate of oxidation of NADH, at 25°C , with the exception of malate dehydrogenase and of aspartate aminotransferase, which were both measured at 30°C . The conditions for assay of these two enzymes were similar to those used for the respective mitochondrial activities. However, with soluble malate dehydrogenase, the final concentration of oxaloacetate in the reaction medium was 0.1 mM.

Lactate dehydrogenase (EC 1.1.1.27) was assayed at pH 7.4, as described by Margreth *et al.* (1970b). The pyruvate-inhibition method of Wilson *et al.*

(1963) was used to determine the isoenzyme ratios. Aldolase (EC 4.1.2.13) was assayed as described by Wu & Racker (1959), and glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) as described by Brosemer (1965). Phosphofructokinase (EC 2.7.1.11) was assayed at pH 8.0 essentially as described by Margreth *et al.* (1967). The activity was usually measured in the myofibrillar supernatant fraction from homogenates prepared in 0.3 M-sucrose–5 mM-EDTA solution, pH 8.0 (see details in the text). All the other activities were assayed on the 150000g soluble supernatant fraction of the muscle homogenates prepared in 0.3 M-sucrose without additions, or in crude muscle extracts in 20 mM-potassium phosphate, pH 8.0, as described above.

Assays of activities in microsomal fractions. Rotenone-insensitive NADH-cytochrome *c* reductase activity was assayed as described by Margreth *et al.* (1971), in the presence of 1.5 μ M-rotenone (Sottocasa *et al.*, 1967). The rate of reduction of cytochrome *c* at 550 nm was measured at 30°C in the Unicam spectrophotometer. ATPase activity was assayed by incubating 40–50 μ g of microsomal protein at 30°C for 10 min in 1 ml of medium of the following composition: 0.05 M-imidazole-HCl buffer, pH 7.1, 5 mM-MgCl₂, 5 mM-ATP-imidazole salt, 5 mM-sodium azide. The P_i liberated was determined as described by Lindberg & Ernster (1956).

Ca²⁺-uptake activity was measured by incubation of 40–50 μ g of microsomal protein at 24°C in 1 ml of medium of the following composition: 0.02 M-histidine-HCl buffer, pH 7.2, 5 mM-MgCl₂, 4 mM-ATP-imidazole salt, 5 mM-sodium azide, 100 mM-KCl, 5 mM-oxalate-imidazole, and 0.1 mM-⁴⁵CaCl₂. The Ca²⁺-uptake was determined by a Millipore filtration method (Martonosi & Feretos, 1964) on Millipore type GS filters (0.22 μ m mean pore size) and liquid-scintillation spectrometry. Both the filter and filtrates were counted for radioactivity in 5 ml of scintillation fluid (4 g of Omnifluor in 1 litre of toluene).

Results

Effect of denervation on muscle weight

Table 1 shows that, after a 1-week denervation period, the average decrease in muscle weight did not exceed about one-third of the original weight. The greater percentage loss of weight in soleus as compared with extensor muscle (Table 1) is in agreement with the observation that the slow muscles undergo atrophy after denervation more rapidly than the fast muscles (Hník, 1962). Tenotomy of soleus muscle led to a decrease in muscle weight similar to the decrease caused by denervation at corresponding periods (Table 1).

Catalytic properties of myosins and metabolic enzyme patterns of the extensor and soleus muscles at early stages of denervation-induced atrophy

The results in Table 2 show that the EDTA-activated and Ca²⁺-activated ATPase activities of isolated myosins from both fast extensor and from slow soleus muscle are essentially unaffected by denervation. The myosins of the extensor muscles invariably exhibit ATPase activities at least twice that of the myosins of the soleus muscles (Bárány, 1967; Margreth & Salviati, 1971). Bárány *et al.* (1965) were also unable to find significant modifications of the catalytic properties of the myosins of cat fast muscles after long-term denervation.

Fig. 1 shows the results of preliminary experiments with rat gastrocnemius, at various periods after denervation. Soluble extracts prepared from this muscle by using a low-ionic-strength phosphate buffer had a relatively unchanged content of glycolytic enzymes during the first week after denervation. Thereafter, the specific activities of aldolase, total lactate dehydrogenase and phosphofructokinase decreased somewhat abruptly, at times coincident with the onset of irreversible ultrastructural damage

Table 1. *Changes in whole muscle weight after denervation or tenotomy*

The symmetrical muscles from the two hind legs of rats, in a range of body weights from approx. 150 to 250 g, were used. The average fresh weight of the extensor and soleus muscles, and the means $\pm$ S.E.M. of percentage changes, are reported. Sciactomy of the muscles of the right hind leg or tenotomy of the right soleus muscle was done 1 week before the muscles were used. The contralateral muscles served as controls.

Muscle	Operation	Total no. of muscle pairs	Av. muscle wt. (mg)		Decrease (%)
			Control	Operated	
Extensor	Denervation	88	76.6	63.7	16.8 $\pm$ 0.7
Soleus	Denervation	96	69.4	45.7	34.2 $\pm$ 1.0
Soleus	Tenotomy	30	71.2	47.9	32.6 $\pm$ 1.9

Table 2. *ATPase activities of myosin of control and denervated extensor digitorum longus and soleus muscles from rats*

The values in each experiment represent the analysis of the myosin isolated from the pooled muscles of several rats. The contralateral muscles from the unoperated leg were used as controls. The experimental conditions for isolation of myosin and for ATPase assays are given in the Experimental section.

			Myosin ATPase ($\mu\text{mol of P}_i/\text{min per mg of protein}$)	
Muscle			EDTA-activated	Ca ²⁺ -activated
Expt. 1	Extensor	Control	2.52	0.70
		Denervated	2.49	0.71
	Soleus	Control	1.14	0.32
		Denervated	0.94	0.33
Expt. 2	Extensor	Denervated	2.00	0.86
	Soleus	Control	0.93	0.41
		Denervated	1.14	0.39

in denervated rat skeletal muscle (Pellegrino & Franzini, 1963).

Enzyme assays in the soluble-protein fraction of tissue homogenates prepared in 0.3M-sucrose gave results in substantial agreement in both extensor and soleus muscles at 1 week after nerve section (Table 3). The changes induced by denervation in the specific activity of several soluble enzymes were relatively small and indiscriminate. In particular, similar decreases were found for two enzymes which are characteristically present in different amounts in fast and slow muscles, namely lactate dehydrogenase and NAD-linked glycerol phosphate dehydrogenase (see, e.g., Pette, 1966; Opie & Newsholme, 1967) and for malate dehydrogenase and aspartate aminotransferase, two enzymes that are present in more similar amounts in extensor and soleus muscles (Table 3). These results, as well as the unchanged isoenzyme ratio of lactate dehydrogenase in extensor muscle and its slightly decreased value in soleus after denervation (Table 3), are different from the evidence obtained from chronic denervation experiments of fast and slow muscles by Dawson & Kaplan (1965) and by Brody (1965).

Phosphofructokinase activity was measured separately in crude muscle extracts prepared in 0.3M-sucrose–5 mM-EDTA (see the Experimental section). The respective mean values $\pm$ S.E.M. for the denervated and control muscles (in μmol of fructose 1,6-diphosphate formed/min per mg of protein) were respectively 0.59 ± 0.04 and 0.93 ± 0.12 (extensor muscle, 5 experiments), and 0.21 ± 0.02 and 0.30 ± 0.02 (soleus muscle, 7 experiments). Some differential-centrifugation experiments indicated that about 15–20% of the activity co-sedimented with the microsomal fractions (Margreth *et al.*, 1967) in both extensor and soleus muscles, and that the percentage

distribution was similar after denervation. However, there was a varying degree of inactivation of phosphofructokinase under these conditions, which affected mainly the unsedimentable (150000g at 60 min) form of the enzyme, and which caused somewhat erratic results that could not be considered with enough confidence.

The results obtained from measurement of several enzyme activities in the mitochondria of the extensor and soleus muscles are presented in Table 4. In agreement with previous observations (Margreth *et al.*, 1970a; Alvarado Rigault & Blanchaer, 1970; Margreth & Salviati, 1971) there was a striking difference between control preparations from fast muscle and slow muscle, in the activity of flavin-dependent glycerol phosphate dehydrogenase, which, on a protein basis, was several times higher in the mitochondria from extensor muscle. The results show that denervation of the extensor muscle caused a 40% decrease in activity, which was statistically significant ($P < 0.01$). All the other changes in fast-muscle mitochondria were found to be non-significant by paired analysis of the denervated and control mean values. The enzymic activities of the mitochondria from soleus muscles (Table 4) were not changed significantly after denervation, neither was there a significant change in the yield of mitochondrial protein.

Effects of denervation on biochemical function and the enzymic and protein composition of isolated sarcoplasmic reticulum

ATPase-linked Ca²⁺-transport activity. In substantial agreement with earlier observations (Sreter & Gergely, 1964; Mommaerts *et al.*, 1969), preliminary experiments with isolated sarcoplasmic

Fig. 1. Changes in activities of soluble enzymes in the rat gastrocnemius muscle after denervation

The enzyme activities were assayed on crude extracts prepared in 20mM-potassium phosphate, pH8.0, from muscles that had been denervated at the times indicated, and from the contralateral (control) muscles. $\circ$, Phosphofructokinase; $\bullet$, aldolase; $\square$, lactate dehydrogenase. The conditions for enzymic assays were as reported in the Experimental section. Lactate dehydrogenase activity was measured with 0.33mM-pyruvate. Each point is the mean value of four to 11 determinations on denervated muscles. The arrows on the ordinates show the mean values of the total number of analyses in the control muscles (21, 13 and 14 determinations for phosphofructokinase, aldolase and lactate dehydrogenase activity respectively). The vertical bars indicate twice the S.E.M.

reticulum from normal muscles had shown that the initial rates of Ca^{2+} uptake varied about fivefold between the extensor and soleus muscles (Margreth & Salviati, 1971). There was also a definite correlation between rates of Ca^{2+} uptake and the extent to which the Mg^{2+} -dependent ATPase activities in the two kinds of microsomal preparations were inhibited by added EGTA (cf. Mommaerts *et al.*,

1969). The EGTA-sensitive fraction of the ATPase activity in either kind of preparation was largely stimulated by the addition of 0.1M-KCl, as was the initial rate of Ca^{2+} uptake (cf. Duggan, 1967; Margreth *et al.*, 1970a). Based on these results, the difference between the total ATPase activity, which is probably an admixture of several ATPase enzymes (Boegman *et al.*, 1970), and the activity determined

Table 3. *Effect of denervation on the enzymic composition of the soluble sarcoplasmic fraction of the extensor and soleus muscles*

Results are reported of paired analyses on the 150000g × 60 min supernatant fraction from homogenates of the denervated (7 days) and contralateral muscles, for the number of experiments reported in parentheses. Activities are expressed as μmol of NADH oxidized/min per mg of protein. Mean values are reported $\pm$ S.E.M., when more than three experiments were done. 'Isoenzyme ratio' is the ratio between the lactate dehydrogenase activities measured at low and at high pyruvate concentrations.

	Extensor muscle			Soleus muscle		
	Control		Denervated	Control		Denervated
Yield of soluble protein (mg/g of muscle)	23.7 $\pm$ 1.3	(13)	23.5 $\pm$ 1.6	19.8 $\pm$ 1.3	(12)	22.7 $\pm$ 1.7
Malate dehydrogenase	9.50	(3)	7.12	12.23	(3)	11.63
Aspartate aminotransferase	1.41	(3)	1.22	1.29	(3)	1.05
Glycerol phosphate dehydrogenase	0.39 $\pm$ 0.03	(10)	0.32 $\pm$ 0.03	0.086 $\pm$ 0.005	(11)	0.083 $\pm$ 0.008
Lactate dehydrogenase						
Pyruvate (0.33 mM)	10.55 $\pm$ 0.94	(8)	9.56 $\pm$ 0.92	4.39 $\pm$ 0.38	(11)	3.59 $\pm$ 0.33
Pyruvate (10 mM)	9.22 $\pm$ 3.69	(4)	8.54 $\pm$ 1.44	1.67 $\pm$ 0.42	(6)	1.54 $\pm$ 0.32
Isoenzyme ratio	1.1		1.1	2.6		2.3

Table 4. *Enzymic composition of the mitochondria of control and denervated extensor and soleus muscles*

Mitochondria were isolated by differential centrifugation from homogenates of the denervated and contralateral muscles (see the Experimental section). The values represent the means $\pm$ S.E.M. of the analyses of several mitochondrial preparations obtained for the number of experiments given in parentheses. Activities are expressed as μmol of cytochrome *c* reduced/min per mg of protein (*); or μmol of NADH oxidized/min per mg of protein (†).

	Extensor muscle			Soleus muscle		
	Control		Denervated	Control		Denervated
Yield of mitochondrial protein (mg/g of muscle)	1.49 $\pm$ 0.13	(8)	1.20 $\pm$ 0.16	1.48 $\pm$ 0.13	(8)	1.94 $\pm$ 0.27
Succinate-cytochrome <i>c</i> reductase*	0.206 $\pm$ 0.019	(10)	0.170 $\pm$ 0.017	0.176 $\pm$ 0.022	(9)	0.171 $\pm$ 0.018
Glycerol phosphate-cytochrome <i>c</i> reductase*	0.059 $\pm$ 0.010	(10)	0.035 $\pm$ 0.005	0.007	(12)	0.007
Malate dehydrogenase†	8.04 $\pm$ 1.00	(6)	5.59 $\pm$ 0.55	5.11 $\pm$ 0.31	(5)	4.39 $\pm$ 0.54
Aspartate aminotransferase†	1.58 $\pm$ 0.37	(6)	1.38 $\pm$ 0.25	1.32 $\pm$ 0.20	(4)	1.48 $\pm$ 0.44

in the presence of 1 mM-EGTA, was used as a measure of the ATPase component of the Ca^{2+} -transport system of reticular membranes. The results in Table 5 show that the EGTA-sensitive fraction of Mg^{2+} -activated ATPase in sarcoplasmic reticulum fragments from control soleus was about one-third that of the respective fraction from control extensor, in both the absence and presence of K^+ .

After denervation, the yield of the microsomal fraction per unit of fresh tissue weight was significantly increased in soleus muscle as compared with the control muscle ($P < 0.01$), and it was also slightly above the control values when calculated per whole muscle, from the results in Table 1. However, the

yield of microsomal protein was virtually unmodified in extensor after an identical denervation period (Table 5). A differential biochemical response to denervation of the isolated sarcoplasmic reticulum from fast extensor and from slow soleus muscle is clearly indicated by the results on ATPase activities also given in Table 5. Whereas the specific activities of both Mg^{2+} - and $\text{Mg}^{2+} + \text{K}^+$ -dependent ATPases decreased slightly in isolated sarcoplasmic reticulum from extensor muscle, they were considerably increased in the respective fraction from soleus, after a 1-week denervation period. It also appears from the results in Table 5 that the enhanced rate of ATP hydrolysis in the microsomal fraction from

Table 5. Comparison between fast extensor and slow soleus muscles with respect to denervation-induced changes of the ATPase activity of isolated sarcoplasmic reticulum

Results are reported of paired analyses of the microsomal fraction isolated by differential centrifugation from homogenates of denervated muscles (7 days) and of the contralateral muscles. The microsomal fractions were isolated by centrifugation of the tissue homogenates between 6500g and 30000g, as described in the Experimental section. Protein yields are the mean values $\pm$ S.E.M. of several experiments with the pooled muscles of seven to nine rats. The total number of experiments is reported in parentheses. Activities are expressed as μ mol of P_i /min per mg of protein and are the mean values of five (extensor) or four (soleus) experiments $\pm$ S.E.M. The ATPase activities were assayed in a medium of the basic composition reported in the Experimental section, in the absence and presence of 100mM-KCl, and with and without 1mM-EGTA. The EGTA-sensitive fraction of the ATPase activity in either condition is the difference between the total activity and the activity measured in the presence of 1 mM-EGTA. This was about zero in two experiments with control soleus.

	Extensor muscle			Soleus muscle		
	Control	Denervated	Change (%)	Control	Denervated	Change (%)
Yield of microsomal protein (mg/g of muscle)	1.07 $\pm$ 0.09 (8)	1.03 $\pm$ 0.11	-4	0.91 $\pm$ 0.07 (9)	1.42 $\pm$ 0.13	+56
Mg ²⁺ -activated ATPase						
Total	1.48 $\pm$ 0.23	1.43 $\pm$ 0.21	-3	1.04 $\pm$ 0.12	1.65 $\pm$ 0.19	+59
EGTA-sensitive fraction	0.33 $\pm$ 0.05	0.24 $\pm$ 0.03		0.11*	0.19 $\pm$ 0.06	
Mg ²⁺ +K ⁺ -activated ATPase						
Total	2.06 $\pm$ 0.38	1.86 $\pm$ 0.29	-10	1.36 $\pm$ 0.14	1.85 $\pm$ 0.19	+36
EGTA-sensitive fraction	0.66 $\pm$ 0.16	0.42 $\pm$ 0.18		0.19 $\pm$ 0.14	0.23 $\pm$ 0.04	

* Average value of two experiments.

denervated soleus was accounted for mainly by an increased activity of the EGTA-sensitive component of the ATPase (Table 5).

Fig. 2 shows the time-course of ATP-dependent Ca²⁺ uptake, measured in the presence of oxalate, in sarcoplasmic reticulum isolated from denervated and control extensor and soleus muscles. The reticular membrane fragments from denervated extensor accumulated calcium oxalate at slightly diminished rates compared with the corresponding fraction from the control muscle. However, the initial rate of Ca²⁺-accumulation was markedly accelerated in sarcoplasmic reticulum isolated from the soleus muscle, with more than a twofold increase 1 week after denervation. These effects of denervation on Ca²⁺-uptake activity are therefore consistent with the concurrent changes in the ATPase activities of the microsomal fraction from fast and slow muscle (Table 5). The correlation appeared to be less complete with respect to the total Ca²⁺-uptake values, recorded after a 10-min incubation period (Fig. 2). The finding that in denervated soleus the maximum calcium oxalate loading capacity of the fragmented sarcoplasmic reticulum increased more than the Ca²⁺-transport rates can probably be explained by additional consequences of denervation-induced atrophy, such as swelling of the isolated microsomal vesicles (Sreter, 1970).

Further experiments were designed to test the specificity of denervation-induced increases in the effectiveness of the Ca²⁺-transport mechanism in reticular membranes from soleus muscle. For this purpose, we investigated the effects of muscle disuse caused by tenotomy which produced a degree of muscle atrophy similar to that found after denervation (see Table 1). The initial rate of Ca²⁺ uptake, and Ca²⁺ accumulation in 10 min, were respectively on average (three experiments) 0.07 and 0.46 μ mol of Ca²⁺/mg of protein in the microsomal fraction from tenotomized soleus muscle, and 0.11 and 0.52 μ mol of Ca²⁺/mg of protein, in the corresponding fraction from the control muscle. Brody (1966) reported that in the gastrocnemius muscle of the guinea pig, tenotomy did not affect the maximum Ca²⁺-accumulating capacity of the sarcoplasmic reticulum, in contrast with denervation.

NAD-linked electron-transport activity. Rotenone-insensitive NADH-cytochrome *c* reductase activity of reticular membranes is consistently found to be more active in the slow muscles than in the fast muscles of the same species (Margreth *et al.*, 1971). This enzyme system is fairly evenly distributed between 'heavy' and 'light' reticular-membrane fragments (Margreth *et al.*, 1971).

The results obtained from investigation of rotenone-insensitive NADH-cytochrome *c* reductase

Fig. 2. Comparison of sarcoplasmic reticulum fragments from denervated and control extensor and soleus muscles, with respect to the time-course of Ca^{2+} uptake *in vitro*

Values are means of seven experiments with paired muscles. The vertical bars indicate twice the S.E.M. Denervated muscles and the contralateral, unoperated muscles, were removed 7 days after nerve section. The microsomal fractions were isolated from 10% (w/v) homogenates prepared in 0.3 M-sucrose, by differential centrifugation between 6500g and 30000g. Ca^{2+} uptake was determined as reported in the Experimental section. $\circ-\circ$, Control extensor muscle; $\circ---\circ$, denervated extensor muscle; $\bullet-\bullet$, control soleus muscle; $\bullet---\bullet$, denervated soleus muscle.

activity in reticular membrane fragments isolated from the extensor and soleus muscles after denervation were almost the opposite of the changes in the ATPase and Ca^{2+} -uptake activities (Table 6). The rate of reduction of cytochrome *c* was considerably enhanced in microsomal fractions from denervated extensor muscle, as compared with the contralateral muscle. The difference was significant ($P < 0.01$) in both 'heavy' and 'light' microsomal fractions. Denervation led to a slight increase of the reductase activity in these fractions from soleus muscle, but the change was found to be non-significant by paired statistical analysis.

Electrophoretic patterns of sarcoplasmic-reticulum protein. Martonosi (1969) and Panet & Selinger (1970) have reported that the structural protein of reticular membranes derived from rabbit skeletal muscle is electrophoretically heterogeneous. By polyacrylamide-gel electrophoresis, the reticular membrane protein was resolved into a major component that moved slightly from the origin and was identified with the Ca^{2+} -transport ATPase (Martonosi, 1969; Panet & Selinger, 1970; MacLennan *et al.*, 1971) and a number of additional small bands.

When sarcoplasmic reticulum fragments from rat extensor were analysed by the procedure of Panet & Selinger (1970), the electrophoretic pattern of the solubilized structural protein (Plate 1a) was similar to that reported by these authors. However, the protein electrophoretic pattern of microsomal fractions from rat soleus muscle differed considerably. The main distinguishing features were several major bands with intermediate and fast mobilities, as well as a decreased amount of material at or near the origin (Plate 1b). The electrophoretic patterns from

Table 6. Effect of denervation on the NADH-cytochrome *c* reductase activity (rotenone-insensitive) of sarcoplasmic-reticulum fragments from the extensor and soleus muscles

Activities are expressed as nmol of cytochrome *c* reduced/min per mg of protein. Mean values of several experiments $\pm$ S.E.M. are given when more than three determinations were done. Each determination represents the analysis of microsomal preparations isolated from the pooled muscles of several rats. The percentage changes of microsomal reductase activity in denervated as compared with the contralateral muscles are reported in parentheses. 'Heavy' microsomal fractions: fractions isolated by centrifugation of the tissue homogenates between 6500g and 30000g. 'Light' microsomal fractions: fractions sedimented between 30000g for 30min and 80000g for 30min (see the Experimental section). NADH-cytochrome *c* reductase activity was measured as reported in the Experimental section, in the presence of 1.5 μM -rotenone.

Muscle		No. of expts.	NADH-cytochrome <i>c</i> reductase activity	
			'Heavy' microsomal fractions	'Light' microsomal fractions
Extensor	Control	5	21.4 $\pm$ 3.0	27.6 $\pm$ 2.9
	Denervated		48.8 $\pm$ 6.0 (+128%)	53.8 $\pm$ 7.7 (+95%)
Soleus	Control	3	62.7	89.1
	Denervated		78.7 (+25%)	111.2 (+25%)

the respective microsomal fractions of the denervated extensor and soleus muscles (Plate 1c and 1d) were found to be appreciably modified, as the result of differential changes. After denervation, there was a greater emphasis on the minor protein components of reticular membranes from extensor (cf. Plate 1c and 1a). However, the electrophoretic pattern of isolated reticular membrane protein from denervated soleus muscle was characterized by an increased prominence of the material immediately below the origin (cf. Plate 1d and 1b).

Discussion

From biochemical and ultrastructural investigations on denervation of skeletal muscles, distinctive patterns of changes have been outlined for myofibrils and several subcellular components of the sarcoplasm. Denervation enhances the catabolism and decreases the rate of synthesis of the contractile proteins (Goldberg, 1969; Margreth *et al.*, 1966), and causes selective damage of the myofibrils *in situ* (Pellegrino & Franzini, 1963; Muscatello *et al.*, 1965).

The major biochemical changes observed in the present investigation of the extensor and soleus muscles after 1 week of denervation are concerned essentially with the sarcoplasmic reticulum since the proliferative response to denervation is almost unique for this membrane system (Pellegrino & Franzini, 1963; Muscatello *et al.*, 1965; Schiaffino & Settembrini, 1970). Denervation-induced hypertrophy of the sarcoplasmic reticulum of frog skeletal muscle, where it has been described in most detail, is apparently accounted for by an enhanced synthesis of reticular membrane protein (Muscatello *et al.*, 1965; Margreth *et al.*, 1966). The wide occurrence of proliferative changes of reticular membranes in denervated skeletal muscles from adult rats (Pellegrino & Franzini, 1963; Hájek *et al.*, 1967) extends, even more strikingly, to the developing gastrocnemius muscle of young animals denervated at birth (Schiaffino & Settembrini, 1970).

There are several published biochemical studies of the changes of the Ca^{2+} -uptake activity of the isolated sarcoplasmic reticulum from denervated skeletal muscles. Brody (1966) reported a steady increase of the maximum Ca^{2+} -uptake activity in microsomal fractions from the gastrocnemius muscle of the guinea pig, starting from about 2–4 days after denervation. Similar results were obtained independently by Howell *et al.* (1966) in the denervated gastrocnemius and diaphragm muscles of rats. Sreter (1970), in reinvestigating these effects in the rat gastrocnemius, attributed them mainly to the passive swelling of the sarcoplasmic reticulum after denervation, which would result in an increased calcium oxalate loading capacity of the isolated vesicles. He found, however, that active Ca^{2+} binding was progressively lower in

fragmented sarcoplasmic reticulum from the gastrocnemius muscle with increasing time after denervation, but was unchanged in the respective fraction from soleus, as measured at 2 weeks (Sreter, 1970).

However, the present evidence delineates some specific modifications induced by denervation on reticular membranes, at the molecular level of organization and therefore distinguishable from volume changes or other kinds of morphological damage of the microsomal vesicles. A critical factor, which may be important for the results obtained, and which would also explain, in part at least, certain definite discrepancies between the present results and those of Sreter (1970), is concerned with the time after denervation, and the distinctive features of the early and late stages of denervation-induced atrophy of muscle (Muscatello *et al.*, 1965). A mixed fibre population can be an additional source of interpretative error in denervation studies, owing to the uneven distribution of atrophic changes between fast and slow skeletal-muscle fibres. It is relevant that the constituent fibres of the extensor digitorum longus and soleus muscles of the rat have considerable homogeneity with respect to both contractile characteristics (Close, 1964) and morphological organization of the sarcoplasmic reticulum (Schiaffino *et al.*, 1970).

Our results demonstrate that denervation causes a marked increase of the Ca^{2+} -uptake activity in fragmented sarcoplasmic reticulum from the slow soleus muscle, and to only a slight decrease of this activity in the corresponding fraction from the fast extensor muscle. The following lines of evidence support the interpretation that these specific effects of denervation are directly linked to the modified molecular composition of reticular membranes, with respect to the concentration of the ATPase component, and possibly other component(s) of the Ca^{2+} -transport system. (i) The observed changes in Ca^{2+} -uptake activity mainly reflect the acceleration or slowing, respectively, of transmembrane Ca^{2+} -transport, rather than simply being caused by variations in the maximum capacity for intravesicular calcium oxalate accumulation. (ii) The greater effectiveness of Ca^{2+} transport in fragmented sarcoplasmic reticulum from denervated slow muscle is associated with an increase in the specific activity of the membrane-bound Mg^{2+} -activated and K^{+} -modulated Ca^{2+} -stimulated ATPase. (iii) The electrophoretic pattern of this fraction from slow muscle indicates a concurrent increase in the relative amount of molecular protein species, which are supposedly related to part at least of the ATPase enzyme.

Electron-microscopic observations of negatively stained reticular membranes are consistent with the biochemical changes in slow-muscle sarcoplasmic reticulum after denervation. Microsomal preparations from the normal extensor and soleus muscles

EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis patterns and densitometric tracings of microsomal protein from denervated and control extensor and soleus muscle. (a) Control extensor muscle; (b) control soleus muscle; (c) denervated extensor muscle; (d) denervated soleus muscle. Electrophoresis was done as described in the Experimental section. Migration was towards the cathode. The tracing of the stained gels was done on a Gilford scanner.

had shown differences in composition, with respect to the proportion of typical reticular membranes with projecting 3.0 nm (30 Å) particles, from that of smooth-surfaced membranes. The subunit particles, though not identical with the Ca^{2+} -stimulated ATPase (MacLennan *et al.*, 1971), are still believed to be a structural requirement for Ca^{2+} transport (Inesi & Asai, 1968; Martonosi, 1969; Baskin & Deamer, 1969; Margreth *et al.*, 1970a). Accordingly, we found that the sarcoplasmic reticulum from isolated fast extensor muscle consisted mainly of granular-surfaced vesicles, whereas the corresponding fraction from slow soleus was largely admixed with smooth membranes, similar to microsomal preparations from heart muscle (Baskin & Deamer, 1969). When sarcoplasmic reticulum fragments from denervated soleus muscle were compared in one experiment with the control preparation, there appeared to be a marked increase in the number of granular-surfaced vesicles. Therefore the composition of this fraction from the denervated muscle was intermediate between that of the microsomal fractions of fast and slow muscles. Therefore, these lines of evidence combine to indicate that denervation effects a partial transformation of some correlated biochemical and ultrastructural properties of slow-muscle sarcoplasmic reticulum to those of fast-muscle sarcoplasmic reticulum.

Denervation leads to a marked increase in the rate of reduction of cytochrome *c* from NADH in sarcoplasmic reticulum fragments from fast extensor muscle. Further, the enhanced electron-transport activity is associated with the gain of new, slow-type electrophoretic components by the membrane protein (see the Results section). Whether these molecular protein species, which are distinct from the Ca^{2+} transport-ATPase, have a direct functional and/or 'structural' relationship to the NAD-linked electron-transport chain of muscle microsomal material (Imai *et al.*, 1966; Margreth *et al.*, 1971) cannot be decided with any confidence at present. It is, however, noteworthy that the increase in microsomal NADH-cytochrome *c* reductase activity in extensor muscle after denervation could be interpreted as a reversed ontogenesis. At birth, the activity is almost as high in fast-muscle sarcoplasmic reticulum as in slow-muscle sarcoplasmic reticulum, but it declines steadily in fast muscle during postnatal development (Margreth *et al.*, 1971).

Both the enhanced activity of the NAD-linked electron-transport chain in fast muscle sarcoplasmic reticulum and of the Ca^{2+} -transport system in slow-muscle sarcoplasmic reticulum after denervation are akin to 'over shooting' reactions from the loss of a negative feedback control. As a result of these dual, differential changes, the biochemical properties of the sarcoplasmic reticulum of the denervated extensor and soleus muscles show some convergence towards

a pattern that encompasses both the fast-type and slow-type characteristics.

The transitory de-repression of Ca^{2+} -uptake activity in the sarcoplasmic reticulum of denervated slow soleus muscle of the rat is specially notable, because of the similar, but permanent effects observed after re-innervation, with fast nerve fibres of previously denervated slow skeletal muscles (Mommaerts *et al.*, 1969). However, the activity of the ATPase-linked Ca^{2+} -transport system of reticular membranes increases steadily in fast skeletal muscles during post-natal development (Fanburg *et al.*, 1968; Holland & Perry, 1969), but remains remarkably stable in slow muscles during the same period (see Perry, 1970), as well as during work-induced hypertrophy (Margreth & Salviati, 1971).

These lines of evidence suggest that the relative deficiency in specialization of slow skeletal muscle with respect to Ca^{2+} -transport mechanisms is the expression of blocked differentiation of this function through active repression by specific neurogenic mechanisms. The fact that mature slow skeletal muscle fibres are competent to develop this biochemical property relatively early after denervation is also consistent with the hypothesis of a continuously acting regulatory influence from motor innervation, and a somewhat special role of slow motor neurones for separation of skeletal muscles into fast and slow muscles (Buller *et al.*, 1960a,b; Eccles, 1963). From this interpretation there emerge some interesting new perspectives with regard to neural mechanisms of homeostatic regulation of the biochemical characteristics of slow muscle (Margreth & Salviati, 1971), as well as to a reappraisal of the previously considered 'undifferentiated' state of this muscle.

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