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THE TRANSFORMATION OF MYOSIN IN CROSS-INNERVATED RAT MUSCLES

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SUMMARY

1. The characteristics of isometric twitch and tetanic contractions have been determined for normal (N-EDL, N-SOL), self-innervated (S-EDL, S-SOL) and cross-innervated (X-EDL, X-SOL) extensor digitorum longus (EDL) and soleus (SOL) muscles of the rat at 35° C. The muscles were then used for biochemical analyses of properties of myosin and actomyosin.

2. The ATPase activities of myosin and actomyosin of X-EDL decreased to the level of those of N-SOL or S-SOL, and the ATPase activities of X-SOL approached those of N-EDL or S-EDL. Of the various ATPase activities, the actin- and Mg^{2+}-activated ATPase activity of myosin and the Mg^{2+}-activated ATPase activity of actomyosin showed the highest degree of correlation with the intrinsic speed of shortening of the muscles.

3. Myosin of normal, self-innervated, and cross-innervated muscles combined with F-actin superprecipitated at rates which were proportional to the speed of muscle contraction.

4. The pH profile curve and the ATP-induced dinitrophenylation reaction revealed that the structure of myosin of X-EDL was altered to that of N-SOL or S-SOL, and the structure of myosin of X-SOL was modified to that of N-EDL or S-EDL.

5. No differences were found in the yield of myosin of normal, self-innervated, and cross-innervated extensor digitorum longus and soleus muscles.

INTRODUCTION

It has been shown that the intrinsic speed of shortening of rat extensor digitorum longus muscle is about two and one half to three times greater than that of rat soleus muscle (Close, 1964, 1965*a*, 1967), and that this difference in the dynamic properties of these two muscles is correlated

with, and probably the result of, differences in the kinetic properties of the myosin adenosinetriphosphatase (Bárány, 1967). Cross-union of the motor nerves of these muscles brings about reciprocal changes in the intrinsic speed of shortening and the force:velocity properties (Close, 1965*b*, 1969). The present work was carried out to determine some of the properties of myosin of cross-innervated fast and slow rat muscles. It will be shown that the reciprocal changes in speed of contraction of cross-innervated rat muscles are directly correlated with changes in the kinetic properties of myosin adenosinetriphosphatase.

METHODS

Physiological methods

Nerve cross-union operations. Operative cross-union of nerves to extensor digitorum longus (EDL) and soleus (SOL) muscles was performed on 3-week-old female Wistar rats; the operative procedure was essentially the same as described previously (Close, 1969) but in this series the cross-union operation was carried out in four different ways as described below. The cross-union was always carried out on the right hind limb.

Group I. Simple cross-union of EDL and SOL nerves was carried out as described previously (Close, 1969) with no precautions taken to prevent inadvertent reinnervation of a muscle by its own nerve. In these animals the right EDL muscle was always innervated partly by SOL nerve and partly by 'fast' nerve fibres from the peroneal nerve; all the EDL muscles from these operated animals were discarded.

Group II. The EDL and SOL nerves were cross-united as in group I. The remainder of the peroneal nerve was transected near the point of entry into the peroneal muscles, and the proximal part was separated from EDL nerve, reflected and tied to fascia over the muscles of the thigh. In these animals the right EDL muscle was always innervated solely by SOL nerve and not one of the muscles which is normally innervated by the peroneal nerve was reinnervated by fibres from that nerve.

Group III. This cross-union operation was the same as that for group II except that only the tibialis anterior and extensor hallucis longus components of the peroneal nerve were reflected and tied back over the thigh. Again the EDL muscle was innervated solely by SOL nerve and the reflected components of the peroneal nerve failed to reinnervate the tibialis anterior and extensor hallucis longus muscles.

In groups I, II and III the right SOL muscle was never reinnervated by fibres from the main SOL nerve.

Group IV. The proximal part of SOL nerve was tied to the distal stump of EDL nerve and the whole of the peroneal nerve was reflected and tied to fascia over the thigh muscles. In these preparations the SOL muscle degenerated completely in the absence of innervation. The EDL muscle was innervated only by SOL nerve and the peroneal nerve failed to reinnervate any of the muscles which it normally innervates.

The EDL and SOL nerves of the left limb were either left intact or transected and re-sutured, thereby producing normal and self-innervated control homologous muscles.

About 30% of normal soleus muscles of Wistar rats are innervated by an accessory nerve in addition to the main soleus nerve; the accessory nerve is of variable size, arises from a branch of the plantaris nerve and innervates muscle fibres which appear to have the same low speed of contraction as the remainder of the SOL muscle. The accessory soleus nerve was not included in the cross-union operations because it is

usually some distance from the main soleus nerve and difficult to detect visually. Consequently about 30 % of the 'cross-innervated' SOL of groups I, II and III contained a variable proportion of normal slow soleus muscle fibres innervated by the unmolested accessory soleus nerve in addition to the cross-innervated fast soleus muscle fibres which were innervated by EDL nerve fibres. These mixed soleus muscles were easily detected at the time of the terminal experiments (see below) and were discarded.

Dissection of muscles for physiological recording. The terminal experiments in which the physiological characteristics of the muscles were determined were carried out between 327 and 454 days after operations. The methods of dissection and preparation of muscles for the experiments were the same as those described for the earlier series (Close, 1969) except that in this series the nerve to plantaris in the right hind limb was left intact in order to test for the presence or absence of the accessory soleus nerve.

TABLE 1. The number of muscles from each type of operation which were preserved for biochemical analysis

Operation	N-EDL	S-EDL	X-EDL	N-SOL	S-SOL	X-SOL
I	0	0	0	6	6	12
II	5	7	12	5	7	12
III	5	5	10	5	6	11
IV	7	0	7	0	0	0

Apparatus and method of determining physiological characteristics. The equipment and method of setting up the preparations for *in situ* recording of isometric contractions have been described (Close, 1969). The usual procedure in these experiments was first to determine the optimal length for twitch contractions at 35° C; normal and self-innervated SOL (N-SOL, S-SOL) and cross-innervated EDL (X-EDL) were stimulated through the sciatic nerve, and normal and self-innervated EDL (N-EDL, S-EDL) and cross-innervated SOL (X-SOL) were stimulated through the common peroneal nerve. Tests were then carried out to determine whether the cross-innervated muscles were pure as regards their innervation. In these tests the gain of the tension transducer amplifier was increased 5–10-fold; the tibial nerve was stimulated to determine whether fibres from the main soleus nerve had inadvertently innervated the X-SOL muscle and whether an accessory soleus nerve was present; the common peroneal nerve was stimulated to determine whether 'fast' nerve fibres had inadvertently innervated the X-EDL. Subsequently, a short series of isometric twitch and tetanic contractions were recorded with the muscle at the optimal length. Stimulation of the muscles was kept to a minimum and all recordings were made with the muscle at about 35° C.

Muscles which had mixed innervation were discarded along with their contralateral control muscles. Pure muscles, innervated solely by one nerve, were excised and the lengths of several superficial muscle fibres were determined microscopically with the muscle held at the optimal length. The tendons of these muscles were cut off near the ends of the muscle fibres, the muscle was blotted, weighed and finally immersed in 500 ml. of glycerol (BDH, analytical reagent) at −9° C to −13° C; several minutes elapsed between excision of the muscle and immersion in glycerol.

The muscles which were preserved in glycerol for subsequent biochemical analysis were all pure as regards their innervation and every cross-innervated muscle was accompanied by its control, normal or self-innervated, contralateral homologue. The number of muscles from each type of operation are listed in Table 1.

All the muscles of one type, e.g. N-EDL, X-SOL, etc., were placed in one glass container fitted with teflon-lined screw-top lids. At the end of the series of physiological measurements the muscles were flown from Canberra to New York for biochemical analysis. The period of storage of the muscles in cold glycerol ranged from 39 to 146 days.

Abbreviations and definitions

P_t The maximum isometric twitch tension.
P_0 The maximum isometric tetanic tension.
F_1 Average length of superficial muscle fibres.
M The wet weight of the muscle. It is assumed that the density of the muscle was unity and M/F_1 is used as a measure of muscle cross-sectional area.
T_c The isometric twitch contraction time, i.e. the time from onset to peak of an isometric twitch.
$T_{\frac{1}{2}r}$ The isometric twitch half-relaxation time, i.e. the time for relaxation from P_t to $0{\cdot}5P_t$.

All the characteristics listed above, except M, were determined with the muscle at the optimal length for twitch contractions at 35° C.

Biochemical methods

Preparation of myosin. The time for shipment of the muscles to New York was 33 hr. The temperature of the glycerol was 19° C when the muscles arrived in the laboratory. Preparation of myosin started immediately. The muscles were washed several times with 0·04 M-KCl, pH 7·0, at 4° C to remove glycerol. They were chopped with scissors and washed again with 0·04 M-KCl until free of visible blood. Then the muscles were blended with 0·01 M-KCl, at 0° C, for 3 min. The blendate was brought to final concentrations of 0·3 M-KCl, 0·15 M potassium phosphate buffer, 10 mM-ATP, and 1·0 mM cysteine, pH 6·6 (25 vol. of this solution per gram of fresh muscle). The extract was stirred at 0° C for 20 min and then centrifuged at 27,000 *g* for 10 min: the residue was saved to prepare actomyosin (see next paragraph), and the supernatant was dialysed at 4° C against 150 vol. of a solution containing 0·01 M-KCl, 1·0 mM Tris-HCl, and 0·5 mM cysteine, pH 7·2, to precipitate the myosin. This dialysis solution was exchanged 4 times during the 36 hr of dialysis. The myosin precipitate was collected, washed with the dialysis solution, and dissolved by homogenization in 0·6 M-KCl. The actin content of these myosin preparations was negligible, as evidenced by their ATP sensitivity (Weber & Portzehl, 1952), which was 5–15 %, and by the slight change in turbidity which was observed in the superprecipitation test upon addition of Mg^{2+}, Ca^{2+}, and ATP (see Fig. 1 in Bárány, Tucci & Conover, 1966). The myosin solutions were further purified by dilution to 0·3 M-KCl, pH 6·5 (Portzehl, Schramm & Weber, 1950), the small amount of precipitate formed (actomyosin) was removed by centrifugation at 37,000 *g*, and the supernatant (pure myosin) was concentrated by dialysis against 150 vol. of the solution containing 0·01 M-KCl, 1·0 mM Tris-HCl, and 0·5 mM cysteine, at 4° C. The myosin precipitate was collected and dissolved in 0·6 M-KCl. The solutions, 10–12 mg myosin/ml., were stored in ice until use.

Preparation of actomyosin. The residue left after extraction of myosin was re-extracted with 20 vol. of a solution containing 0·6 M-KCl, 0·05 M Tris-HCl buffer, pH 7·4 and 0·5 mM cysteine at 4° C for 20 hr. The extract was centrifuged at 37,000 *g*, the residue (connective tissue) was discarded, and the supernatant (actomyosin) was concentrated by dialysis against 0·01 M-KCl, 1·0 mM Tris-HCl, and 0·5 mM cysteine, pH 7·2. After 24 hr of dialysis (with several changes) the actomyosin precipitate was collected and dissolved by homogenization in 0·6 M-KCl (6–8 mg actomyosin/ml.). The ATP sensitivities of these actomyosin preparations were 100–120 %, and

they superprecipitated readily in the presence of Mg^{2+}, Ca^{2+}, and ATP at low ionic strength. The actomyosin solutions were kept in ice until use.

Determination of ATPase activity. To obtain a true correlation between biochemical and physiological analyses, the characteristic ATPase activities of myosin and actomyosin were determined at 35° C. The reaction mixtures, 3·8 ml., were incubated under magnetic stirring in a temperature-controlled water-bath for 10 min, and the reactions were initiated by the addition of 0·2 ml. of enzyme solution kept at 25° C. The reaction time was 1·0 min. The reactions were stopped by the addition of 1·0 ml. of 10 % (w/v) trichloroacetic acid to the assay mixtures. After filtration, 3·0 ml. samples were used to measure inorganic phosphate (P_i) according to the method of Rockstein & Herron (1951). It was determined that under the assay conditions the ATPase activity was a linear function of the enzyme concentration. Furthermore, identical patterns of ATPase activity were obtained in repeated experiments.

The composition of the reaction mixtures was the following: *actin- and Mg^{2+}-activated ATPase activity of myosin:* 30 mM-KCl, 20 mM Tris-HCl buffer, pH 7·4, 6·0 mM-$MgSO_4$, 0·1 mM-$CaCl_2$, 3·0 mM-ATP, 0·5 mg myosin/ml., and 0·25 mg F-actin/ml. *Mg^{2+}-activated ATPase activity of actomyosin:* 30 mM-KCl, 20 mM Tris-HCl buffer, pH 7·4, 6·0 mM-$MgSO_4$, 0·1 mM-$CaCl_2$, 3·0 mM-ATP, and 0·3 mg actomyosin/ml. *K^+-and EDTA-activated ATPase activity:* 0·50 M-KCl, 20 mM Tris-HCl buffer, pH 7·4, 1·0 mM-EDTA, 3·0 mM-ATP, and 0·5 mg myosin/ml., or 0·3 mg actomyosin/ml. *Ca^{2+}-activated ATPase activity:* KCl as specified, 20 mM Tris-HCl buffer, pH 7·4, 10·0 mM-$CaCl_2$, 3·0 mM-ATP, and 0·5 mg myosin/ml., or 0·3 mg actomyosin/ml.

The effect of pH on the Mg^{2+}- and Ca^{2+} activated ATPase activities of actomyosin was determined in the following reaction mixtures: *Mg^{2+}-activated ATPase activity:* 40 mM Tris-acetate buffer at specified pH, 30 mM-KCl, 6·0 mM-$MgSO_4$, 0·1 mM-$CaCl_2$, 3·0 mM-ATP, and 0·3 mg actomyosin/ml. *Ca^{2+}-activated ATPase activity:* 40 mM Tris-acetate buffer at specified pH, 30 mM-KCl, 10·0 mM-$CaCl_2$, 3·0 mM-ATP, and 0·3 mg actomyosin/ml.

In one case (Fig. 2) the ATPase activity was determined at 25° C. In this experiment the alkali lability of various myosins was compared. In contrast to the previously described assay systems, the myosins were incubated in the reaction mixtures, 3·6 ml., under magnetic stirring for 10 min, and the reactions were initiated by the addition of 0·4 ml. ATP. The reaction time was 2·0 min. *To determine the pH profile of the Ca^{2+}-activated ATPase activity* the reaction mixtures contained: 40 mM Tris-acetate buffer at specified pH, 0·6 M-KCl, 10 mM-$CaCl_2$, 0·5 mg myosin/ml., and 3·0 mM-ATP.

Superprecipitation. The ability of myosin to superprecipitate with F-actin was determined by following the decrease in transmittance at 546 mμ (Bárány *et al.* 1966). Myosin (1½ mg) were mixed with 0·75 mg F-actin under magnetic stirring in a final volume of 3·0 ml. containing 0·11 M-KCl, 0·02 M Tris-HCl buffer, pH 7·4, and 0·1 mM-$CaCl_2$, at 25° C. One millilitre of this actomyosin suspension was used for the assay. After the addition of 10 μl. 0·1 M-$MgSO_4$ the reaction was initiated by the addition of 5 μl. 0·02 M-ATP at 25° C. The decrease in percentage of transmittance was recorded with an Eppendorf spectrophotometer and Varian recorder. The rate of superprecipitation was determined from the slope of the linear portion of the curves and it is expressed as Δ % transmittance per sec. Duplicate assays for superprecipitation yielded rates which agreed within 5 %.

Incorporation of tritiated 1-fluoro-2,4-dinitrobenzene ([^3H]FDNB) into myosin. Myosin (5 mg) were used for each determination. To remove the cysteine present in the myosin solutions (about 0·15 μ-mole cysteine), the myosin samples were in-

cubated with twofold molar excess of iodoacetate over cysteine, pH 7·7, 25° C, for 5 min. The myosin samples were diluted to contain 0·5 mg myosin/ml., 0·10 M Tris-HCl buffer, pH 7·7, 24 mM-KCl, and the additions as indicated in Table 10. The reaction, at 25° C, was initiated by the addition of 0·03 μ-mole [^{3}H]FDNB ($4{\cdot}4 \times 10^6$ dpm/μ-mole) and was stopped by the addition of cysteine (400-fold molar excess over FDNB) after $2\frac{1}{2}$ min. After stirring with cysteine for 5 min the myosins were precipitated with 5% (w/v) trichloroacetic acid, washed three times with 2% trichloroacetic acid, once with 0·1% trichloroacetic acid, and dissolved in 2% (v/v) NH_4OH by homogenization. Samples were taken from this homogenate to determine the protein by the biuret method and the radioactivity by liquid scintillation counting (Bárány, Bailin & Bárány, 1969).

Preparation of F-actin. The procedure described previously (Bárány, Conover, Schliselfeld, Gaetjens & Goffart, 1967) was used to prepare F-actin from the skeletal muscles of rabbit.

Determination of proteins. The biuret method of Gornall, Bardawill & David (1949) was used for measuring the optical density at 320 mμ (Bárány & Bárány, 1959).

Preparation of [^{3}H]FDNB solution. This has been described recently (Bárány *et al.* 1969).

RESULTS

Physiological studies

Table 2 shows the properties of the six groups of muscles. The mean values and the pattern of significance of difference between the means for normal and operated muscles are essentially the same as those obtained from an earlier series of measurements on rat muscles (Table 1, Close, 1969). The most important point to note is that for any particular type of muscle (N-EDL, X-SOL, etc.) there is no statistically significant difference between the means for T_c or $T_{\frac{1}{2}r}$ of the present series (Table 2, below) and those from the earlier series of measurements in which the force:velocity properties and isometric characteristics were determined (Table 1, Close, 1969). It has been shown earlier that there is a well defined inverse relation between intrinsic speed of shortening and the isometric twitch contraction time of normal and cross-innervated rat EDL and SOL muscles and it will be assumed that the force:velocity properties of the muscles used in the present work were the same as those of the muscles used in the previous investigation (Table 1, Fig. 4, Close, 1969). The large number of muscles used in the present work has yielded more accurate mean values of isometric twitch and tetanic tensions per unit cross-sectional area of muscle and this has revealed that the small residual changes in twitch:tetanus ratio at long times after nerve cross-union are due principally to changes in tetanic contractions; this effect, hitherto unrecognized, is dealt with more fully in the sections below.

There are several minor differences between the results in Table 2 below and those of the earlier work (Table 1, Close, 1969). The differences

TABLE 2. Mean values ± S.D. of measurements on a number (n) of normal (N-EDL, N-SOL), self-innervated (S-EDL, S-SOL) and cross-innervated (X-EDL, X-SOL) extensor digitorum longus (EDL) and soleus (SOL) muscles. Operations were performed on 3-week-old rats and all records of contraction were made with the muscle at about 35° C.

The abbreviations above the columns are for the muscle weight (M), fibre length (F_1) at optimal length for twitch contractions, the maximum isometric twitch (P_t) and tetanic (P_0) tension, and the isometric twitch contraction (T_c) and half-relaxation ($T_{\frac{1}{2}r}$) times. Probability values for differences between means have been determined from t values and the mean and S.D. are in italic figures where $P < 0{\cdot}01$ for the differences between means for operated and normal homologous muscles

Muscle	Days after operation	Body weight (g)	M (mg)	F_1 (mm)	P_t (g)	P_0 (g)	P_t/P_0	P_tF_1/M (kg/cm²)	P_0F_1/M (kg/cm²)	T_c (msec)	$T_{\frac{1}{2}r}$ (msec)	Temp. ° C
N-EDL	383	247·7	122·2	12·4	50·8	296·6	0·173	0·513	3·001	12·56	8·68	35·01
(n = 17)	± 54	± 33·0	± 7·7	± 0·25	± 7·1	± 40·2	± 0·014	± 0·022	± 0·381	± 0·93	± 0·25	± 0·26
(S-EDL)	382	246·5	129·6	*13·4*	47·1	271·0	0·176	0·473	2·797	12·54	8·77	34·97
(n = 12)	± 18	± 17·3	± 13·7	*± 0·71*	± 9·0	± 42·0	± 0·03	± 0·08	± 0·355	± 0·98	± 0·42	± 0·17
X-EDL	383	247·1	*72·9*	*14·7*	*27·25*	*96·8*	*0·282*	0·539	*1·913*	*21·33*	*25·74*	35·0
(n = 29)	± 37	± 27·0	*± 8·8*	*± 1·27*	*± 6·2*	*± 19·9*	*± 0·036*	± 0·093	*± 0·271*	*± 2·85*	*± 4·2*	± 0·36
N-SOL	421	247·5	116·9	14·0	44·4	174·7	0·255	0·532	2·095	37·8	54·75	34·84
(n = 16)	± 18	± 35·4	± 13·6	± 0·87	± 5·1	± 21·6	± 0·025	± 0·053	± 0·222	± 3·4	± 5·3	± 0·225
S-SOL	381	245·7	103·1	*15·24*	*36·5*	*148·7*	0·246	0·511	2·085	38·0	55·18	*35·04*
(n = 19)	± 8	± 17·5	± 29·4	*± 0·975*	*± 7·04*	*± 25·0*	± 0·02	± 0·077	± 0·292	± 3·04	± 4·83	*± 0·2*
X-SOL	400	246·5	112·5	*19·76*	*24·4*	155·5	*0·156*	*0·415*	*2·673*	*15·6*	*19·31*	35·0
(n = 35)	± 25	± 26·3	± 25·6	*± 0·75*	*± 9·2*	± 48·0	*± 0·022*	*± 0·084*	*± 0·38*	*± 0·83*	*± 2·1*	± 0·1

between the two series in regard to muscle weight and isometric tensions are correlated, in most instances, with differences in age and body weight, but the greater mass and tensions of X-EDL muscles of the present series were probably due to improvement in innervation of this muscle by SOL nerve.

One important difference is the higher P_t/P_0 of N-SOL in the present series compared with that of the earlier series (Table 2, above; Table 1, Close, 1969); the explanation for this difference is not known but, as indicated by values for P_tF_1/M and P_0F_1/M, it is clearly the result of a difference in the twitch response rather than the tetanic response; one possible explanation is that neuromuscular transmission may have been impaired in the older animals used in the earlier series, with the result that some of the muscle fibres may not have been activated during twitch responses of the muscle to indirect stimulation.

EDL muscles. Table 3 shows the correlation matrix for simple linear correlation coefficients between a number of variables representing different properties of the fifty-eight normal, self-innervated and cross-innervated EDL muscles which are described in Table 2. It may be noted that there was no significant correlation between the post-operative period, body weight, or temperature and any of the variables shown in Table 3. The results in Table 3 show that variations in P_tF_1/M and P_0F_1/M are uncorrelated and that of these two, the variation in P_0F_1/M is the more important factor determining variation in P_t/P_0. In this connexion, there is no significant difference between means of P_tF_1/M of normal, self-innervated or cross-innervated EDL muscles (Table 2) and the differences in P_t/P_0 of these muscles are due principally to differences in P_0F_1/M. Table 3 shows that T_c is correlated with P_0F_1/M but not P_tF_1/M, consequently the correlation between T_c and P_t/P_0 involves only the component of P_t/P_0 which is correlated with P_0F_1/M; this is an interesting and unexpected result which indicates the need for caution in any attempt to interpret variations in P_t/P_0 in terms of the concept of the active state.

SOL muscles. Table 4 shows the simple linear correlation coefficients for the relations between variables representing properties of the seventy control and cross-innervated SOL muscles which are described in Table 2. Again there was no significant correlation between post-operative period, body weight or temperature, and any of the variables in Table 4. The results for SOL muscles in Table 4 are virtually the same as those for EDL muscles in Table 3 except that there are significant correlations between P_tF_1/M and T_c and between P_tF_1/M and $T_{\frac{1}{2}r}$. The results for SOL muscles are complicated, however, because P_tF_1/M and P_0F_1/M of the X-SOL were not the same for all the three main types of operations.

Table 5 illustrates the main differences between the six groups of X-SOL

muscles. There is no significant difference between the mean $P_t F_1/M$ of group I X-SOL and the means for N-SOL, S-SOL, N-EDL, S-EDL and X-EDL, and $P_0 F_1/M$ of group I X-SOL is about the same as that for normal EDL muscles. Group II and III X-SOL had lower $P_t F_1/M$, $P_0 F_1/M$ and P_t/P_0 than those of group I X-SOL but these differences were not correlated with variations in T_c; the correlation coefficient was $-0{\cdot}018$ ($P > 0{\cdot}9$) for the relation between T_c and $P_t F_1/M$, and $0{\cdot}244$ ($P > 0{\cdot}1$) for the relation between T_c and P_t/P_0 of the 35 X-SOL muscles.

TABLE 3. Simple linear correlation coefficients for relations between variables representing properties of the fifty-eight normal, self-innervated and cross-innervated EDL muscles described in Table 2. Probability values for the significance of the correlation coefficients were determined from t values, and the coefficients are given in italic figures where $P < 0{\cdot}001$

	P_t/P_0	$P_t F_1/M$	$P_0 F_1/M$	T_c
$P_t F_1/M$	*0·4916*	—	—	—
$P_0 F_1/M$	*−0·8013*	0·0321	—	—
T_c	*0·7997*	0·1712	*−0·7652*	—
$T_{\frac{1}{2}r}$	*0·8109*	0·1550	*−0·8258*	*0·9583*

TABLE 4. Simple linear correlation coefficients for relations between variables representing properties of the seventy normal, self-innervated and cross-innervated SOL muscles described in Table 2. Probability values for the significance of the correlation coefficients were determined from t values, and the coefficients are given in italic figures where $P < 0{\cdot}001$

	P_t/P_0	$P_t F_1/M$	$P_0 F_1/M$	T_c
$P_t F_1/M$	*0·6977*	—	—	—
$P_0 F_1/M$	*−0·6694*	0·0513	—	—
T_c	*0·9207*	*0·5969*	*−0·6695*	—
$T_{\frac{1}{2}r}$	*0·9108*	*0·5530*	*−0·6987*	*0·9870*

It is clear from the results in Table 5 that minor differences in the properties of cross-innervated soleus muscles can arise through differences in operations and the condition of neighbouring muscles but they do not invalidate the general conclusion that the net changes in P_t/P_0 following simple nerve cross-union are due principally to changes in $P_0 F_1/M$ and that there is little or no change in $P_t F_1/M$. In support of this conclusion, it was found that there was no significant difference between the mean $P_t F_1/M$ of any pair from the series N-EDL, S-EDL, X-EDL, N-SOL, S-SOL and group I X-SOL.

The observation that normal EDL and SOL muscles differ in regard to the maximum tetanic tension per unit cross-sectional area of muscle (Table 2) is the only new physiological finding which has emerged from the present work. It is of interest to note that this difference in dynamic pro-

perties is probably not due to differences in the number of filament cross-bridges per half sarcomere because, as indicated in Table 6 (see below), the myosin yield, and presumably the myosin content per gram of fresh muscle, is approximately the same in normal EDL and SOL muscles. More work will have to be carried out to determine whether there is a real difference in the intrinsic strength of the contractile materials of fast and slow muscles or whether the tension developed in tetanic contraction is limited by extrinsic factors.

TABLE 5. Mean values ± S.D. for isometric twitch contraction (T_c) and half-relaxation ($T_{\frac{1}{2}r}$) times and the maximum isometric twitch (P_tF_1/M) and tetanic (P_0F_1/M) tensions per unit cross-sectional area of muscle, of cross-innervated soleus muscles obtained from different types of operations. The second column merely shows whether the contralateral control soleus muscle was normal or self-innervated. Temperature = 35° C

Operation	Contralateral muscle	T_c (msec)	$T_{\frac{1}{2}r}$ (msec)	P_tF_1/M (kg/cm²)	P_0F_1/M (kg/cm²)
I	Normal	15·75	18·7	0·505	3·02
	(n = 6)	± 0·25	± 1·55	± 0·055	± 0·149
	Self-innervated	15·8	19·1	0·505	2·813
	(n = 6)	± 0·46	± 1·23	± 0·055	± 0·195
II	Normal	15·6	19·5	0·344	2·368
	(n = 5)	± 1·52	± 3·8	± 0·087	± 0·69
	Self-innervated	15·3	19·64	0·356	2·7
	(n = 7)	± 0·64	± 2·86	± 0·036	± 0·238
III	Normal	15·7	19·7	0·404	2·624
	(n = 5)	± 0·51	± 1·43	± 0·01	± 0·246
	Self-innervated	15·5	19·25	0·37	2·455
	(n = 6)	± 0·65	± 1·45	± 0·048	± 0·17

Biochemical studies

Table 6 shows the muscle weights and the amount of myosin isolated from the muscles. As much as 39–50 mg pure myosin was isolated per gram of fresh muscle, owing to a selective extraction of myosin from the exhaustively washed and blended muscles and the essentially complete recovery of the extracted myosin by precipitation through dialysis. There is no systematic variation in the yield of myosin among normal, self-innervated, and cross-innervated extensor digitorum longus and soleus muscles. Bárány, Bárány, Reckard & Volpe (1965) have determined the myosin content of rabbit extensor digitorum longus and soleus muscles and have found 67–74 mg myosin per gram of fresh muscle. On the assumption of a similar amount of myosin in rat and rabbit muscles, it appears from the data of Table 6 that about 60 % of the total myosin of the rat muscle cells was made available for the biochemical studies.

Most of the remaining myosin was also isolated in the form of acto-

myosin, since the residue obtained after extraction of myosin was re-extracted to prepare actomyosin as described in Methods. The yield of this actomyosin was 16–20 mg per gram of fresh muscle. This means 12–15 mg myosin per gram muscle, when 75 % of the weight of actomyosin is taken as myosin. Thus, the total yield of myosin was 51–65 mg per gram of fresh muscle.

TABLE 6. Yields of myosin of normal (N-EDL, N-SOL), self-innervated (S = EDL, S-SOL), and cross-innervated (X-EDL, X-SOL) extensor digitorum longus (EDL) and soleus (SOL) muscles from rats.

The muscle weight refers to the fresh weight

Muscle	Muscle weight (g)	Yields of myosin (mg/g muscle)
N-EDL	2·08	39
S-EDL	1·56	50
X-EDL	2·12	44
N-SOL	1·87	44
S-SOL	1·96	39
X-SOL	3·94	46

TABLE 7. ATPase activities of myosin of normal (N-EDL, N-SOL), self-innervated (S-EDL, S-SOL), and cross-innervated (X-EDL, X-SOL) extensor digitorum longus (EDL) and soleus (SOL) muscles from rats.

The ATPase activities are expressed as μ-mole P_i/min.mg myosin in the presence of the indicated activators. Temperature = 35° C

Muscle	Actin + Mg^{2+}	K^+ + EDTA	Ca^{2+} + 0·05 M-KCl	Ca^{2+} + 0·3 M-KCl	Ca^{2+} + 0·6 M-KCl
N-EDL	1·17	1·19	1·04	1·05	0·94
S-EDL	1·20	1·11	1·05	0·97	0·89
X-EDL	0·55	0·54	0·59	0·64	0·57
N-SOL	0·50	0·57	0·46	0·40	0·43
S-SOL	0·52	0·51	0·47	0·44	0·41
X-SOL	0·90	1·00	0·68	0·59	0·56

Changes in the ATPase activity of myosin. Table 7 summarizes the characteristic ATPase activities of myosin of normal, self-innervated, and cross-innervated rat muscles. There is virtually no difference between the ATPase activities of myosin of N-EDL and S-EDL. In contrast, the ATPase activities of myosin of X-EDL are much lower than those of myosin of N-EDL or S-EDL. Thus, the actin- and Mg^{2+}-activated, and the K^+- and EDTA-activated ATPase activities of myosin of X-EDL are about one-half of those of myosin of N-EDL or S-EDL. The Ca^{2+}-activated ATPase activity of myosin of X-EDL, in the presence of 0·05 M-KCl, shows a similar 50 % reduction, whereas its Ca^{2+}-ATPase activity, in the

presence of 0·3 M-KCl or 0·6 M-KCl, is 63 %. The ATPase activities of myosin of N-SOL and S-SOL are also equal within the limits of experimental error. However, myosin of X-SOL shows an increased activity; the actin- and Mg^{2+}-activated, and the K^+- and EDTA-activated ATPase activities of myosin of X-SOL are about 1·8 times higher than those of myosin of N-SOL, or S-SOL. With respect to the Ca^{2+}-activated ATPase the increases are 1·5-, 1·4-, and 1·3-fold in the presence of 0·05, 0·3, and 0·6 M-KCl, respectively. Finally, a comparison of myosin of N-EDL and S-EDL with that of N-SOL and S-SOL shows that the myosin of the extensor digitorum longus muscles has 2·1–2·4 times higher ATPase

TABLE 8. ATPase activities of actomyosin of normal (N-EDL, N-SOL), self-innervated (S-EDL, S-SOL), and cross-innervated (X-EDL, X-SOL) extensor digitorum longus (EDL) and soleus (SOL) muscles from rats.

The ATPase activities are expressed as μ-mole P_i/min.mg actomyosin in the presence of the indicated activators. Temperature = 35° C

Muscle	Mg^{2+}	K^+ + EDTA	Ca^{2+} + 0·05 M-KCl	Ca^{2+} + 0·3 M-KCl
N-EDL	0·89	0·28	0·73	0·58
S-EDL	0·91	0·32	0·72	0·45
X-EDL	0·44	0·18	0·41	0·28
N-SOL	0·42	0·19	0·42	0·24
S-SOL	0·47	0·18	0·36	0·25
X-SOL	0·70	0·25	0·57	0·36

activities than the myosin of the soleus muscles. It should be mentioned that these differences were obtained at 35° C, i.e. when the ATPase activities are maximal.

Changes in the ATPase activities of actomyosin. Results similar to those described in Table 7 were obtained when the characteristic ATPase activities of actomyosin of normal, self-innervated, and cross-innervated extensor digitorum longus and soleus muscles were compared (Table 8). The ATPase activities of actomyosin of X-EDL muscle, in the presence of Mg^{2+}, K^+ and EDTA, Ca^{2+} and 0·05 M-KCl, or Ca^{2+} and 0·3 M-KCl, are 50–60 % of the same ATPase activities of actomyosin of N-EDL or S-EDL muscles. These ATPase activities of actomyosin of X-SOL are 1·4–1·6 times higher than those of actomyosin of N-SOL or S-SOL. There is no difference in the ATPase activities between either actomyosin of N-EDL and S-EDL, or actomyosin of N-SOL and S-SOL, although the ATPase activities of actomyosin of the EDL muscles are 1·6–2·1 times higher than those of the SOL muscles.

The Mg^{2+}- and Ca^{2+}-activated (0·05 M-KCl) ATPase activities of actomyosins, given in Table 8, are 75–80 % of these ATPase activities of

myosins given in Table 7 and this is consistent with the 75–80 % myosin content of actomyosin. However, in the presence of Ca^{2+} and 0·3 M-KCl, the ATPase activity of actomyosin is only 54 % of that of myosin, whereas in the presence of K^+ and EDTA the activity is reduced to 30 %. This latter decrease is explained by the known inhibitory effect of actin on the K^+ and EDTA-activated ATPase activity of myosin.

The differences in the ATPase activities of actomyosin that result from the cross-innervation of the muscles hold true over a wide pH range. This is demonstrated in Table 9 by comparing the Mg^{2+}- and Ca^{2+}-activated

TABLE 9. The effect of pH on the Mg^{2+}- and Ca^{2+}-activated ATPase activities of actomyosin of normal (N-EDL, N-SOL), self-innervated (S-EDL, S-SOL), and cross-innervated (X-EDL, X-SOL) extensor digitorum longus (EDL) and soleus (SOL) muscles from rats.

The ATPase activities are expressed as μ-mole P_i/min.mg actomyosin at the indicated pH values. Temperature = 35° C

Muscle	Mg^{2+} pH 6·0	Mg^{2+} pH 7·5	Mg^{2+} pH 9·0	Ca^{2+} pH 6·0	Ca^{2+} pH 7·5	Ca^{2+} pH 9·0
N-EDL	0·36	0·87	1·21	0·61	0·75	1·18
S-EDL	0·40	0·94	1·15	0·65	0·72	1·01
X-EDL	0·19	0·44	0·67	0·32	0·34	0·43
N-SOL	0·15	0·45	0·58	0·35	0·30	0·46
S-SOL	0·16	0·42	0·58	0·32	0·32	0·48
X-SOL	0·30	0·78	1·02	0·46	0·49	0·65

ATPase activities of actomyosins, at pH 6·0, 7·5, and 9·0, and at low ionic strength. At all pH values the ATPase activities of actomyosin of the X-EDL muscle are decreased uniformly to half of the activities of actomyosin of the N-EDL or S-EDL muscles. The Mg^{2+}- activated ATPase activity of actomyosin of the X-SOL muscle exhibits a 1·7–1·9-fold increase over that of actomyosin of N-SOL or S-SOL, independent of the pH; furthermore, its Ca^{2+}-activated ATPase is increased 1·4–1·6-fold, irrespective of pH. At the three pH values studied, the Mg^{2+}- and Ca^{2+}-activated ATPase activities are 2·2-fold higher in actomyosin of N-EDL and S-EDL than in that of N-SOL and S-SOL. Apparently the low ionic strength favours the difference in the enzymic activities of actomyosins from fast and slow muscles.

Changes in the superprecipitation. Cross-innervation changes the speed of contraction of rat muscles (Close, 1969). Therefore, it was of interest to examine whether a similar change in the contractile properties of myosin takes place. The pure myosin of normal, self-innervated, and cross-innervated muscles was combined with F-actin, in a weight ratio of myosin to actin of 2·5:1 to saturate the myosin with F-actin. The ionic strength and pH of the superprecipitation reaction were near physiological. Because

of the rapidity of the superprecipitation reaction these studies were made at 25° C to obtain measurable rates.

Fig. 1 shows typical superprecipitation curves. The speed of superprecipitation is greatest for myosin of N-EDL closely followed by that of myosin of X-SOL. However, the speed is much lower for myosins of X-EDL and N-SOL muscles. There was no difference in the rate of superprecipitation between myosins of either N-EDL and S-EDL, or N-SOL and S-SOL muscles (this is not shown in Fig. 1).

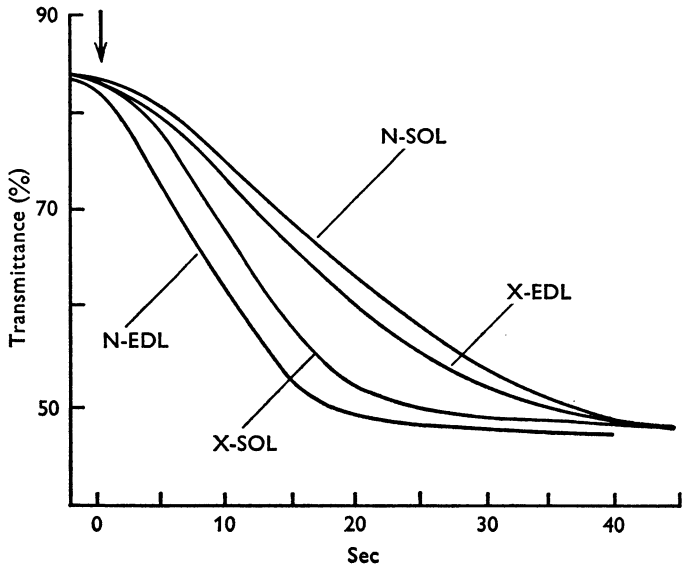

Fig. 1. Superprecipitation of myosin of normal and cross-innervated rat muscles combined with F-actin. The arrow indicates the addition of ATP. Abbreviations: N-EDL, myosin of normal extensor digitorum longus muscle; X-EDL, myosin of cross-innervated extensor digitorum longus muscle; N-SOL, myosin of normal soleus muscle; X-SOL, myosin of cross-innervated soleus muscle. Temperature = 25° C.

A comparison of the speed of superprecipitation (Δ % transmittance per sec) for the various myosins in duplicate determinations gave the following mean values: $-2{\cdot}30$ for N-EDL, $-2{\cdot}33$ for S-EDL, $-1{\cdot}26$ for X-EDL, $-1{\cdot}08$ for N-SOL, $-1{\cdot}03$ for S-SOL, and $-2{\cdot}14$ for X-SOL. Thus, in the model contraction system myosin of cross-innervated soleus resembles myosin of normal extensor digitorum longus, and myosin of cross-innervated extensor digitorum longus behaves like myosin of normal soleus.

Changes in the structure of myosin. Fast and slow muscle myosins show several differences in their molecular structure (Sreter, Seidel & Gergely,

1966; Bárány & Bárány, 1969; Samaha, Guth & Albers, 1970*a*). Two of these differences were utilized in the present work to detect possible changes in the structure of myosin that may result from the cross- innervation of rat muscles: (i) the alkali lability of slow muscle myosin ATPase; (ii) the ATP-induced changes in the dinitrophenylation of the myosins of fast and slow muscles.

1. Sreter *et al.* (1966) were the first to show that incubation of rabbit slow muscle myosin at pH 9·0, 25° C, for 10 min, leads to a rapid loss of ATPase activity, while the fast muscle myosin of rabbit remains unaffected. Samaha *et al.* (1970*a*) extended these observations to the slow and fast muscle myosins of the cat, by exposing the myosins to pH 10·5, 25° C, for 10 min. These authors also showed that the slow muscle myosin is relatively acid-stable, in contrast to the fast muscle myosin which is acid-labile. In view of these findings, we incubated the rat muscle myosins in the pH range from 4·8 to 10·0, at 25° C, for 10 min, and subsequently determined their Ca^{2+}-activated ATPase activities. Fig. 2 shows the results. Since the specific ATPase activities of these myosins differ greatly, the data in Fig. 2 are expressed on a percentage basis, taking the Ca^{2+}-ATPase activity at pH 7·4 as 100 %. (In the legend of Fig. 2 the 100 % values for the Ca^{2+}-ATPase activity, expressed as μ-mole P_i per min per mg myosin, are given.)

The ATPase activity of myosin of N-EDL and S-EDL muscles is increased 2·7–3·0 times when the pH is raised from 7·4 to 9·5 and 10·0 (Fig. 2). In contrast, the ATPase activity of myosin of X-EDL muscle shows no increase in the alkaline pH range but rather a slight decrease. The myosin from N-SOL and S-SOL show only a decrease in their ATPase activity at pH 9·5 and 10·0 whereas the ATPase activity of myosin of X-SOL is increased 2·2–2·3 times at pH 9·5 and 10·0, as compared to that at pH 7·4. These data classify the myosin of X-EDL as alkaline-sensitive, slow-muscle type of myosin, and the myosin of the X-SOL as alkaline-stable, fast-muscle type of myosin.

The acid parts of the pH profile curves in Fig. 2 show a tendency to separate the myosins of N-EDL, S-EDL, and X-SOL into one group, and the myosins of X-EDL, N-SOL, and S-SOL into another. This is based on the small difference in the ATPase activity that is observed at pH 5·3. Because of the limited amount of myosin we were unable to perform more experiments in the acid pH range; therefore, it remains an open question whether the difference at pH 5·3 reflects an acid stability of myosins from X-EDL, N-SOL, and S-SOL muscles, or a relatively higher Ca^{2+}-ATPase activity at pH 5·3.

2. Bárány & Bárány (1969) have shown that the ATP-mediated changes in the dinitrophenylation of slow muscle myosins are less pronounced

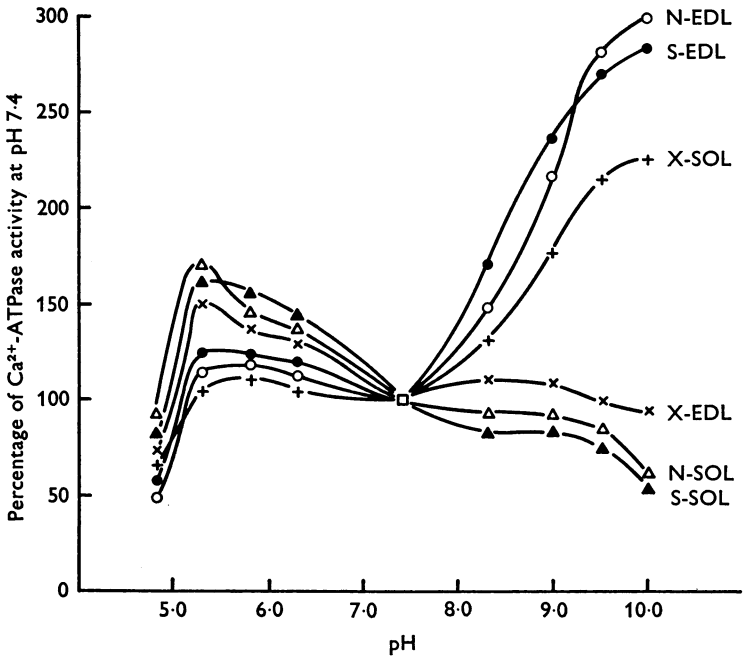

Fig. 2. The pH-profile of the Ca^{2+}-activated ATPase activity of myosin of normal (N-EDL, N-SOL), self-innervated (S-EDL, S-SOL), and cross-innervated (X-EDL, X-SOL) extensor digitorum longus (EDL) and soleus (SOL) muscles from rats. The 100% Ca^{2+}-ATPase activities at pH 7·4, expressed as μ-mole P_i per min per mg myosin, were the following: N-EDL, 0·35; S-EDL, 0·32; X-EDL, 0·22; N-SOL, 0·18; S-SOL, 0·20; X-SOL, 0·27. Temperature = 25° C.

TABLE 10. Incorporation of tritiated 1-fluoro-2,4-dinitrobenzene ([^{3}H]FDNB) into myosin of normal (N-EDL, N-SOL), self-innervated (S-EDL, S-SOL), and cross-innervated (X-EDL, X-SOL) extensor digitorum longus (EDL) and soleus (SOL) muscles from rats.

The incorporation is expressed as μ-mole of tritiated 2,4-dinitrophenyl group bound per 500 mg of myosin per 2½ min under conditions as indicated. Temperature = 25° C

Muscle	No additions	3·0 mM-$MgCl_2$ + 1·0 mM-ATP	1·0 mM-EDTA + 1·0 mM-ATP
N-EDL	0·183	0·135	0·336
S-EDL	0·196	0·145	0·349
X-EDL	0·202	0·191	0·257
N-SOL	0·203	0·196	0·245
S-SOL	0·215	0·194	0·263
X-SOL	0·225	0·152	0·338

than those of fast muscle myosins. Table 10 shows the incorporation of [^{3}H]FDNB into the myosins of normal, self-innervated, and cross-innervated rat muscles. In the presence of 3·0 mM-$MgCl_2$ + 1·0 mM-ATP, the rate of dinitrophenylation of myosins of N-EDL, S-EDL, and X-SOL muscles decreases to 68–74 % of that of the control ('No additions' column). On the other hand, the rate of dinitrophenylation of myosins of X-EDL, N-SOL and S-SOL muscles in the presence of $MgCl_2$ + ATP decreases only to 90–97 % of that of the control. The accelerating effect of metal-free ATP (EDTA + ATP addition) on the rate of dinitrophenylation is greater in the case of myosins of N-EDL, S-EDL, and X-SOL (150–184 % of the control) than in the case of myosins of X-EDL, N-SOL, and S-SOL (121–128 % of the control). Accordingly, myosin of the X-EDL exhibits a dinitrophenylation pattern characteristic of slow muscle myosin, and myosin of the X-SOL has a pattern common to that of the fast muscle myosin.

DISCUSSION

The results of this paper demonstrate clearly the transformation of myosin in cross-innervated rat muscles. This transformation takes place in both directions, that is, myosin of X-EDL is very similar to that of N-SOL, and myosin of X-SOL resembles that of N-EDL. The following biochemical characterizations support this conclusion. (i) The ATPase activities of myosin and actomyosin of X-EDL decrease to the level of those of N-SOL, and the ATPase activities of X-SOL approach those of N-EDL (Tables 7–9). This is based on four or five different ATPase assays (Tables 7 and 8), and on determinations at three different pH values (Table 9). (ii) The contractile properties of myosin of cross-innervated muscles change in the same direction as their ATPase activities (Fig. 1). (iii) Sensitive chemical methods reveal that the structure of myosin of X-EDL is altered to that of N-SOL, and the structure of myosin of X-SOL is modified to that of N-EDL (Fig. 2 and Table 10). (iv) These results were obtained with most of the pure myosin of the rat muscles (Table 6) and with the remaining myosin isolated in the form of actomyosin. Therefore, these conclusions are valid for all the myosin molecules in cross-innervated and normal rat muscles. It is important to note that self-innervation has no effect on the characteristic properties of myosin of EDL and SOL muscles.

The changes in ATPase activities of myosins of cross-innervated muscles are related to changes in force:velocity properties of these muscles. The intrinsic speed of shortening of N-EDL is about 2 times higher than that of X-EDL, and X-SOL contracts about 1·7 times faster than N-SOL (Close, 1969). Of the various ATPase activities, the actin- and Mg^{2+}-

activated ATPase activity of myosin and the Mg^{2+}-activated ATPase activity of actomyosin show the best fit to the changes in contraction velocities. Thus, the rate of ATP hydrolysis catalysed by myosin or actomyosin of N-EDL is 1·8–2·1 times faster than that of X-EDL, and the rate is 1·6–1·9 times faster for myosin or actomyosin of X-SOL than that of N-SOL. The relation between intrinsic speed of shortening (Table 1,

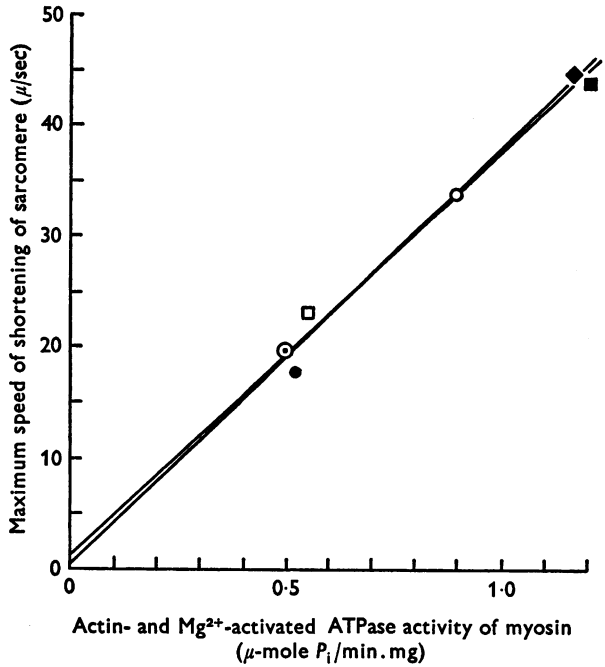

Fig. 3. The relation between intrinsic speed of shortening of sarcomeres and actin- and Mg^{2+}-activated ATPase activity of myosin of N-EDL (◆), S-EDL (■), X-EDL (□), N-SOL (○), S-SOL (●) and X-SOL (○) muscles of rats. The lines drawn in this graph were fitted by the method of least squares for the regression of one variable on the other. Temperature = 35° C.

Close, 1969) and the actin- and Mg^{2+}-activated ATPase activity of myosin (Table 7, above) for the six groups of muscles is shown in Fig. 3. Concerning other ATPase activities, only the K^+- and EDTA-activated ATPase activity of myosin (but not that of actomyosin) follows the pattern in the change of muscle speed. The Ca^{2+}-activated ATPase, especially at high ionic strength, shows a poor relation. However, the changes in the speed of superprecipitation of the myosins are consistent with the changes in the intrinsic speed of the muscles, i.e. the myosin of N-EDL in the presence of actin produces turbidity changes 1·8 times faster than the myosin of X-EDL, and the myosin of the X-SOL is 2·0 times faster than that of N-

SOL. These results confirm and extend the previous work of Bárány (1967), viz. that the ATPase activity of myosin is correlated with the speed of muscle shortening. They show, furthermore, that of all the activators of the myosin ATPase, the physiological one, actin, gives the best correlation.

Recently, Buller, Mommaerts & Seraydarian (1969) and Samaha, Guth & Albers (1970*b*) reported changes in the ATPase activities of myosin and myofibrils after cross-innervation of fast and slow muscles of the cat. The results obtained on rat muscles are qualitatively similar to those of Samaha *et al.* (1970*b*) with regard to the changes in ATPase activity of myosin and the alkali lability of slow muscle myosin ATPase. Buller *et al.* (1969) found a significant decrease in ATPase activity of myosin and myofibrils of cross-innervated flexor digitorum longus, as compared to those of the normal flexor. However, their results showed only a slight increase for the ATPase activity of X-SOL over that of N-SOL, and in this respect their results differ from those of the present work and those of Samaha *et al.* (1970*b*).

The results of the present work show for the first time the direct proportionality between intrinsic speed of shortening of sarcomeres and actin-activated ATPase activity of myosin of normal and cross-innervated fast and slow muscles of mammals. This provides strong evidence in support of the hypothesis that neural influences determine the fundamental dynamic properties of the contractile material through an effect on the ATPase site of myosin.

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