Histochemical Journal **18**, 487–496 (1986)

Calcium-stimulated myofibrillar ATPase activity correlates with shortening velocity of muscle fibres in *Xenopus laevis*

W. J. VAN DER LAARSE[1]*, P. C. DIEGENBACH[2] and M. A. HEMMINGA[1]

[1]*Laboratory for Physiology, Free University, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands*
[2]*Department of Zoology, University of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, The Netherlands*

Received 11 March 1986 and in revised form 18 June 1986

Summary

The iliofibularis muscle of *Xenopus laevis* is reported to contain five types of fibres which have different force–velocity relationships. Ten fibres of each type were selected on the basis of succinate dehydrogenase activity, cross-sectional area and location in the muscle, in order to assess the validity of the fibre type classification.

Maximum calcium-stimulated myofibrillar ATPase activity (V_{max}) and apparent Michaelis constant (K_m) for ATP were determined for these 50 fibres from serial sections. The values obtained varied according to the type of fibre. Type 1 had the highest and type 5 the lowest values for K_m and V_{max}.

In a separate experiment, single freeze-dried fibres were used to determine the relationship between their ATP content and apparent K_m for ATP. There was a tendency for high ATP concentrations in fibres with high K_m values.

When myofibrillar ATPase activity was related to the maximum velocity of shortening of the five fibre types, a significant correlation was found. It is concluded that calcium-stimulated myofibrillar ATPase histochemistry allows an estimate of the maximum shortening velocity of muscle fibres from *Xenopus laevis*.

Introduction

Myofibrillar ATPase determined histochemically with acid or alkaline preincubation is often used to classify muscle fibres into one slow and two fast types (Brooke & Kaiser, 1970; for a review see Kahn, 1976). It has been demonstrated that different skeletal muscles from a large number of species contain these types of fibres in different proportions (Ariano *et al.*, 1973; Saltin & Gollnick, 1983). A classification into three types of fibres, however, is not in agreement with the continuous distribution of contractile characteristics of motor units from one muscle (Burke, 1978). Furthermore, there are large contractile differences between comparable fibre types in different species: small animals have faster muscle fibres (Close, 1972) and higher myosin ATPase activities than large animals (Bárány, 1967). It may therefore be concluded that myofibrillar ATPase determined histochemically at unphysiological pH and after drastic pH treatment has little value as predictor of contractile characteristics of muscle fibres. On the other hand, however, a close relationship exists between velocity of muscle shortening and myosin ATPase activity determined biochemically at physiological pH (Bárány, 1967).

To resolve this discrepancy we have previously developed a quantitative histochemical method for the determination of calcium-stimulated myofibrillar ATPase activity at pH 7.2 (van der Laarse *et al.*, 1984). This method is based on the lead-precipitation technique described by Meijer (1970). It does not require fixation, or acid or alkaline preincubations of the sections. A linear relationship exists between the absorbance of the final lead sulphide precipitate in the section and the incubation time, and the method therefore allows the determination of the relative activity of the enzyme (van der Laarse *et al.*, 1984). To assess the physiological significance of this ATPase determination in more detail, at least two problems require further study: (i) how does calcium-stimulated myofibrillar ATPase activity compare with contractile muscle fibre characteristics? (ii) how can absolute values for the enzyme activity be obtained? The latter problem can be studied, for instance, by using the technique described by Mabuchi & Sréter (1980) for measurement of actomyosin ATPase in cryostat sections, but the former is more difficult to approach.

The most straightforward way to establish the relationship between enzyme activity measured using quantitative histochemical methods and the contractile characteristics of a muscle fibre, is to determine both features in single muscle fibres which vary widely in their properties. The present study, however, follows a somewhat easier route, which is based on results of Lännergren and co-workers on

*To whom all correspondence should be sent.

0018-2214/86 $03.00 + .12 © 1986 Chapman and Hall Ltd.

the illiofibularis muscle of *Xenopus laevis* (Lännergren, 1978, 1979; Lännergren *et al.*, 1982; Lännergren & Hoh, 1984). In these studies, the force–velocity relationships of five different types of single fibres from the iliofibularis muscle are described (for a brief summary, see Lännergren & Hoh, 1984). In a cross-section of the muscle, the five types of fibres can be distinguished using succinate dehydrogenase activity and diameter as classification parameters (Lännergren & Smith, 1966; Smith & Ovalle, 1973) (see Fig. 2a–c). It has been claimed that the number of mitochondria and fat droplets are responsible for the light-scattering properties of the fibres under dark-field illumination. This criterion, together with the diameter of the fibres and their location in the muscle, is used to select single fibres for the physiological experiments (for discussion see Lännergren & Smith, 1966). The histological appearance of the five fibre types has been described in detail by Smith & Ovalle (1973). Type 1 fibres have the largest diameters and low succinate dehydrogenase activity. Type 3 fibres are relatively thin and have the highest succinate dehydrogenase activity, and type 2 fibres have intermediate characteristics between types 1 and 3; they are thick and have high succinate dehydrogenase activity. Type 4 fibres are thin and have low to intermediate succinate dehydrogenase activity, but no tendency for increased subsarcolemmal activity of the enzyme. Finally, type 5 fibres are also thin and have very low succinate dehydrogenase activity.

The maximum velocity of shortening of these fibre types ranges about tenfold (Lännergren & Hoh, 1984). The mean maximum shortening velocities of these types, calculated as the mean of the range given for each type by Lännergren & Hoh (1984) are 7.8, 5.8, 4.9, 2.3 and 1.0 lengths/s at 20° C for types 1, 2, 3, 4 and 5, respectively. These values are used in the present study to investigate how maximum shortening velocity compares with calcium-stimulated myofibrillar ATPase activity determined by means of the quantitative histochemical method described previously (van der Laarse *et al.*, 1984).

Materials and methods

Xenopus laevis females, 8–12 cm, were used for this study. They were killed by decapitation. The iliofibularis muscle was excised, stretched just above slack length, and either quickly frozen in Freon 22, cooled with liquid nitrogen, or kept for 2 h in oxygenated Ringer solution (NaCl, 116.5 mM; KCl, 2.0 mM; $CaCl_2$, 1.9 mM; NaH_2PO_4, 2.0 mM; EGTA, 0.1 mM; adjusted to pH 7.0 with NaOH) at room temperature and then frozen as described above. The latter muscles were subsequently freeze dried and used to dissect single dried fibres in which ATP content was measured.

Histochemistry

Cryostat sections were cut at −20° C, 10 and 16 μm thick for ATPase and succinate dehydrogenase histochemistry, respectively. The sections were collected on coverslips coated with 0.1% poly-L-lysine (molecular mass 350 000 Da). Calcium-stimulated myofibrillar ATPase histochemistry was carried out as described previously (van der Laarse *et al.*, 1984) in a medium consisting of 80 ml Tris-maleate buffer, pH 7.2, 6 ml 45 mM lead nitrate and 10 ml 68 mM calcium chloride. The gelatin was omitted from the medium. Serial sections were incubated for 10 min at 20° C in media containing the following ATP concentrations: 0.375, 0.75, 1.5, 3.0 and 6.0 mM. After the incubation, the sections were washed in distilled water, immersed in 1% sodium sulphide, pH 7.5, and washed again.

Short sections (2 mm length) of individual fibres dissected from the freeze dried muscles for the determination of ATP content (see below), were embedded in 15% gelatin in 0.1 M Tris-maleate buffer containing 5 mM EGTA, pH 7.2. These preparations were rapidly cooled to 4° C, frozen and assayed for myofibrillar ATPase as described above.

The absorbance of the final lead sulphide precipitate in individual fibres was determined with a Zeiss cytospectrophotometer at 550 nm. A circular measuring field (diameter 5 μm at the section level) and × 40 objective were used. The cells were not scanned because of the small distribution error (van der Laarse *et al.*, 1984).

Succinate dehydrogenase activity was determined as described by Pool *et al.* (1979). This method uses scanning and integrating cytospectrophotometry to minimize distribution errors, and includes the determination of all subsarcolemmal succinate dehydrogenase activity as well as the cross-sectional area of the fibres. The sections were incubated at 20° C for 45 min in a medium consisting of 37 mM sodium phosphate buffer, pH 7.6, 74 mM sodium succinate and 0.4 mM tetranitro blue tetrazolium. After incubation the sections were washed once in 0.01 M HCl and twice in distilled water. The sections were scanned with a stepsize of 6 μm and a measuring spot of 5 μm diameter at 660 nm. Rectangular areas were scanned, and measuring points outside the fibre were set to background value by image editing.

All sections were mounted in glycerin–gelatin. Four determinations per cell for each incubation were obtained from four (ATPase) or two serial sections (succinate dehydrogenase, two determinations in each section). If not indicated otherwise, all absorbance values are the mean of four determinations.

ATP content of single muscle fibres

Single muscle fibres were isolated by dissection from four freeze dried muscles of four different animals. A part of the fibre was used for ATPase histochemistry as described above. The other part was weighed with a Cahn 29 electrobalance and homogenized in 0.6 M perchloric acid. After neutralization with 3 M $KHCO_3$, the extract was centrifuged. ATP concentration in the supernatants was measured by ion-pair reverse-phase high-performance liquid chromatography, using tetrabutyl ammonium phosphate as the ion-pairing agent (Juengling & Kammermeier, 1980). A Lichrosorb RP-18 column (Merck) and an isocratic solvent were used: 15% (v/v) acetonitril, 9.6 mM tetrabutyl ammonium phosphate, 50 mM KH_2PO_4, pH 5.8. The flow rate was 1.5 ml/min. (The contents of ADP and AMP were determined as well, but will not be reported in detail here: they ranged from 1.7 to 3.8 and from 0.1 to 0.7 μmol/g dry weight, respectively.)

The ATP concentration in the fibres was calculated from the relationship between the water content of the fibre and its dry weight. The latter was obtained from the relationship between

the volume of 20 living single muscle fibres of *Xenopus laevis* and their dry weight (dry weight in mg = 0.28 × vol in mm^3, unpublished observation), and the density of skeletal muscle (1.05 g/cm^3; Hill, 1965), which yields a water content of 77% (v/v) and the following relationship between water content and muscle dry weight: volume of water = 0.77/0.28 × dry weight = 2.75 × dry weight. It was assumed that there are no diffusional or permeability barriers for ATP in the fibres.

Cluster analysis

Cluster analysis was used to obtain an objective classification of the 50 selected fibres (Spurway, 1981). The analysis was carried out with the Clustan program (Wishart, 1975) as implemented on the University Computer Centre SARA. Of the many available algorithms, we choose Ward's hierarchical method.

Kinetics of calcium-stimulated myofibrillar ATPase

The absorbance values of the final lead sulphide precipitate in the myofibrillar ATPase reaction were fitted to the first-order Michaelis–Menten equation with the direct linear plot method (Eisenthal & Cornish-Bowden, 1974; a Pascal translation of their Fortran program was used). A simplified example of such a plot is shown in Fig. 1, where mean absorbance of the final lead sulphide precipitate ($n = 4$ for each ATP concentration) is plotted against the ATP concentration in the incubation medium. In reality, median values for maximum calcium-stimulated myofibrillar ATPase activity (V_{max}) and apparent Michaelis constant (K_m) for ATP of the myofibrillar ATPase in each fibre were calculated from 160 intersections (Cornish-Bowden *et al.*, 1978).

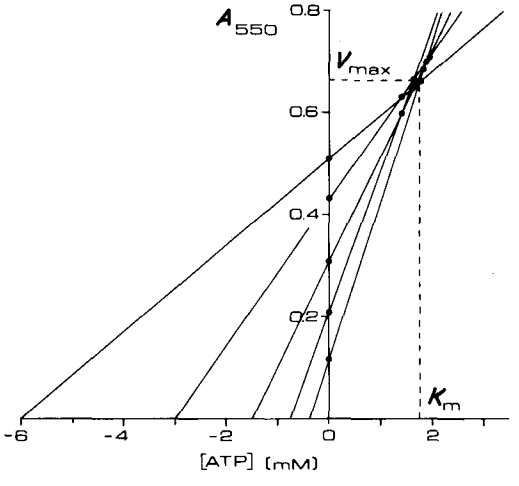

Fig. 1. Direct linear plot for the estimation of maximum myofibrillar ATPase activity (V_{max}) and the apparent Michaelis constant (K_m) for ATP of the ATPase in a type 1 fibre.

Results

Fibre typing

Examples of the five different fibre types found in the iliofibularis muscle of *Xenopus laevis* and in serial sections stained for succinate dehydrogenase and calcium-stimulated myofibrillar ATPase are shown in Fig. 2. Ten fibres of each type were visually selected from a cross-section of the muscle stained for succinate dehydrogenase, using the intensity of the staining, the cross-sectional area and the location of the fibre in the muscle as classification parameters (cf. Lännergren & Smith, 1966; Smith & Ovalle, 1973). Type 1 fibres are found in the so-called outer clear zone of the muscle. Approximately 50% of the fibres in the muscle are of this type. The dark staining zone contains the remaining fibre types. Type 5 fibres can be easily recognized as the most lightly staining fibres in the centre of this zone. Type 4 fibres are classified on the basis of an even distribution of succinate dehydrogenase activity. Type 3 fibres are the most darkly staining fibres in the central zone of the muscle, and type 2 fibres form a transition zone between the type 1 fibres and the central dark zone (Fig. 2d). Fig. 3a shows the absorbance of the formazan deposit and the diameter of these 50 fibres. It can already be seen in Fig. 3a that a simple subdivision into five types cannot be made on these grounds. This conclusion was confirmed by cluster analysis (Fig. 3b). Fibres with intermediary characteristics between so-called typical types 4 and 5, types 3 and 4, and types 3 and 2 were also present in the sample, although it was attempted to select only typical fibres, as described by Smith & Ovalle (1973).

It can be seen in Fig. 2d that fibres with characteristics intermediate between types 1 and 2 are also present in the muscle, but were not selected. Interestingly, there are no fibres intermediate between types 1 and 4 or 5. The only fibre type which clearly forms a single cluster is type 1 (cluster a in Fig. 3b). Two of the type 2 fibres (cluster c) have a higher succinate dehydrogenase activity than the other eight type 2 fibres (cluster b). The 20 type 3 and 4 fibres form three clusters (d, e and f). Cluster d contains seven type 3 fibres, cluster e three type 3 and three type 4 fibres and cluster f contains seven type 4 and one type 5 fibres. Cluster g contains the remaining nine type 5 fibres.

These results do not change drastically when parameters of myofibrillar ATPase activity (V_{max} and K_m, see below) are also included in the cluster analysis (Fig. 3c). Cluster h contains all ten type 1 fibres, cluster i contains nine type 2 fibres, cluster j one type 2 and seven type 3 fibres, cluster k three type 3 and three type 4 fibres, cluster l seven type 4 and one type 5 fibres, and cluster m contains the remaining nine type 5 fibres. Thus, including V_{max} and K_m of myofibrillar ATPase causes only one type 2 fibre to be incorporated in the type 3 cluster and, apart from the clustering levels, nothing else changes. However, it can be learned from the results presented in Fig. 6 (see below) that fibre typing on the basis of myofibrillar ATPase activity alone will lead to larger changes of the classification.

On the basis of these results, it is necessary in order to study the myofibrillar ATPase in the different fibre types to decide at this stage which fibres should be considered to represent the typical 'types', although it requires further discussion (see below) as to how realistic is the use of the word 'type' in this context. The choice that was made is given by the hatched areas in Fig. 3a. The remaining fibres

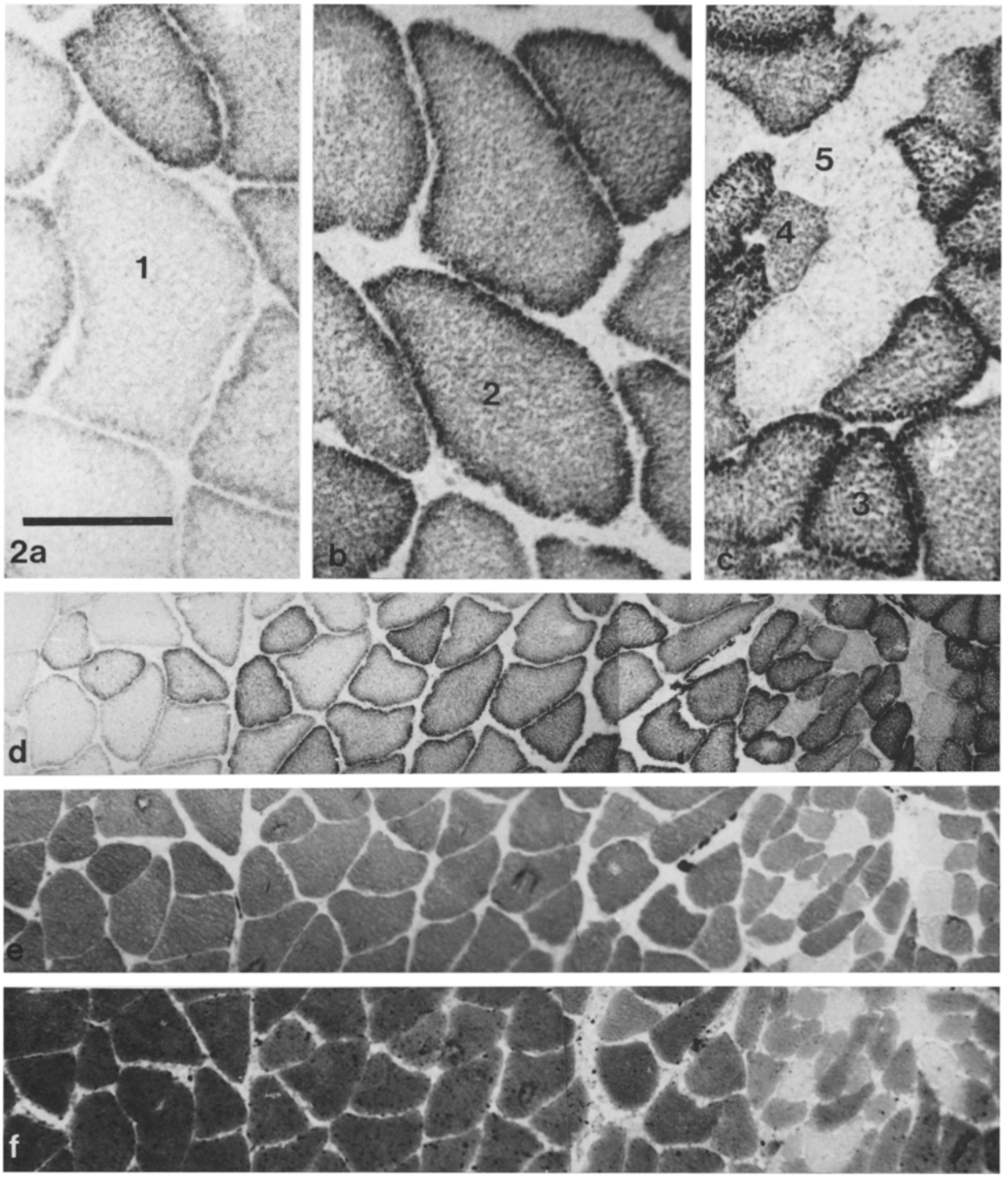

Fig. 2. Cross-sections of the iliofibularis muscle of *Xenopus laevis*. (a–c) Fibre types are classified on the basis of staining intensity for succinate dehydrogenase and cross-sectional area (cf. Lännergren & Smith, 1966; Smith & Ovalle, 1973) and are indicated by numbers in (a) type 1, (b) type 2, (c) types 3, 4 and 5. (d–f) Serial sections stained for (d) succinate dehydrogenase, (e) calcium-stimulated myofibrillar ATPase at 0.75 mM ATP and (f) at 6 mM ATP. Scale bar (a–c) 100 μm and (d–f) 310 μm.

are considered to be 'non-typical', and will not be used for the subsequent analysis.

Myofibrillar ATPase histochemistry

Serial sections incubated with 0.75 and 6 mM ATP, are shown in Figs. 2e,f, respectively. For comparison, the type 5 fibres (the light staining fibres at the right-hand side in Figs. 2e,f) have the same absorbance, A ($A_{550} = 0.11$) when incubated with these ATP concentrations. There was some non-specific staining on the surface of the sections

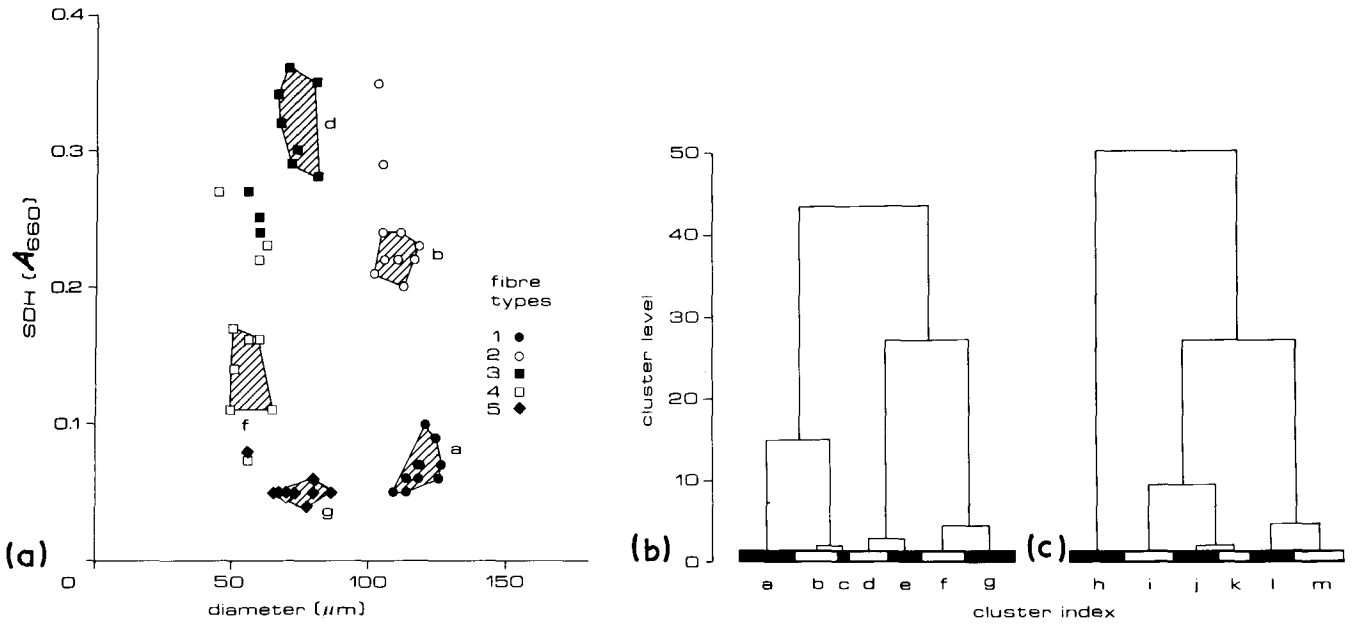

Fig. 3. (a) Succinate dehydrogenase activity (SDH) plotted against the diameter of the 50 selected fibres. The diameter was calculated as $2(A/\pi)^{0.5}$, where A is the cross-sectional area of the fibre. The visual fibre classification is indicated by different symbols. The fibres in the hatched areas are considered to be typical type 1, 2, 3, 4 and 5 fibres, respectively. The letters a, b, d, f and g correspond to the cluster indices in (b). (b) Dendrogram showing the results of cluster analysis on the data shown in (a). (c) Dendrogram showing the results of cluster analysis including, apart from succinate dehydrogenase activity and diameter, also maximum calcium-stimulated myofibrillar ATPase activity and apparent Michaelis constant for ATP of the myofibrillar ATPase.

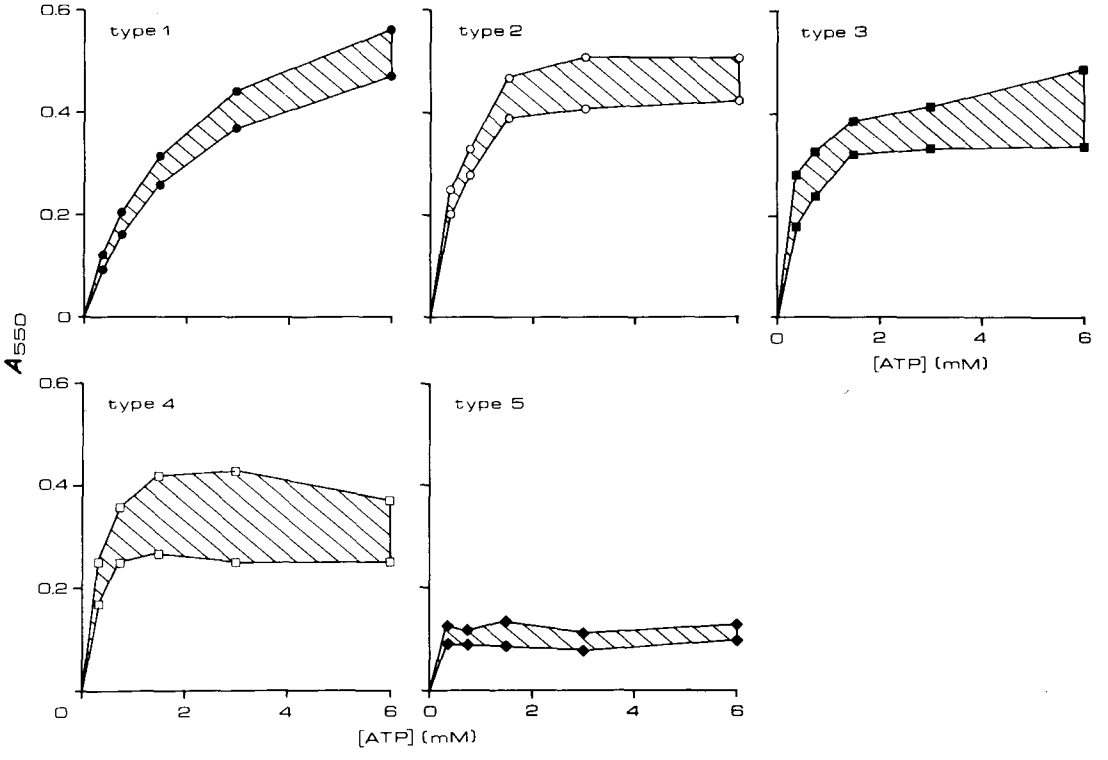

Fig. 4. Relationship between the absorbance (A_{550}) of the final lead sulphide precipitate in the myofibrillar ATPase reaction and the ATP concentration in the incubation medium. The data are from fibres in the hatched areas in Fig. 3a only. (a) 10 type 1 fibres, (b) 8 type 2 fibres, (c) 7 type 3 fibres, (d) 6 type 4 fibres, (e) 9 type 5 fibres. The hatched areas in the five panels encompass all absorbance values.

that were incubated at 3 and 6 mM ATP (Fig. 3f). Meijer (1970) added gelatin to the incubation medium to prevent this, but we found that the ATPase is inhibited by the gelatin. It seems likely that the inhibition results from reduced diffusion of reactants in media containing gelatin because the phenomenon occurred mainly in fibre types 1 and 2, and only at the high ATP concentrations, i.e. if ATPase activities are relatively high. To avoid inhibition it was decided to omit the gelatin from the medium. Care was taken not to determine non-specific precipitate in the absorbance measurements.

Fig. 4 shows the relationship between the myofibrillar ATPase activity and the ATP concentration in the incubation medium. These relationships are clearly different in the five fibre types. Analyses of variance, carried out on each fibre type at each ATP concentration showed that significant differences exist between the individual fibres of type 4 only ($F_{5,18}=3.70$, $P<0.025$ and $F_{5,18}=3.34$, $P<0.05$, at 1.5 and 6 mM ATP, respectively). This indicates that the individual fibres of each type (except type 4) all have similar, but not necessarily the same, myofibrillar characteristics.

Fig. 5 compares the mean relationships between the myofibrillar ATPase activity and the ATP concentration

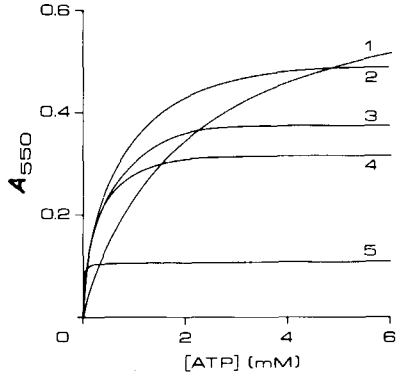

Fig. 5. Average relationships between calcium-stimulated myofibrillar ATPase activity and ATP concentration. The hyperbolas are determined by the mean V_{max} and the mean K_m found for each fibre type (see Table 1). Numbers indicate fibre types.

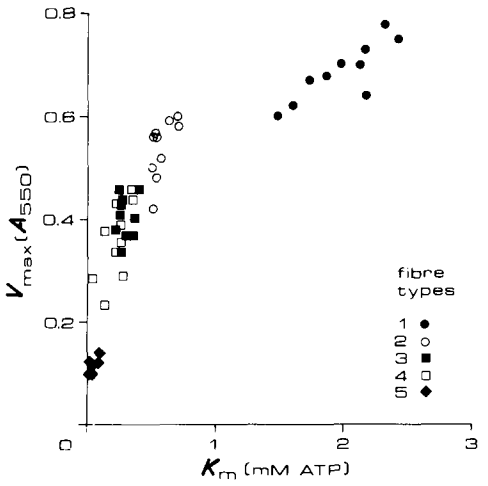

Fig. 6. Maximum calcium-stimulated myofibrillar ATPase activity (V_{max}) plotted against the apparent Michaelis constant (K_m) for ATP of the myofibrillar ATPase. The data are from all fibres in Fig. 3a. Note that many type 4 fibres have myofibrillar characteristics similar to those of type 3 fibres. A number of data points for type 5 fibres overlap. (Negative values for K_m were found in three type 5 fibres, which is due to the fact that the range of ATP concentrations used in the present study is not suited to determine accurately the K_m in these fibres. The negative values were set to zero.)

for each of the five fibre types. The data for all individual fibres were fitted by the first-order Michaelis–Menten equation, making use of the linear approach shown in Fig. 1. In Fig. 6, the maximum myofibrillar ATPase activity (V_{max}) is plotted against the apparent Michaelis constant (K_m) for ATP of the ATPase. Type 1 and 5 fibres form different clusters, but the data for the other types overlap to some extent. Mean values for maximum myofibrillar ATPase activity and the apparent Michaelis constant for ATP in the five fibre types are given in Table 1. Type 1 fibres have the highest V_{max} and K_m and type 5 fibres the lowest.

ATP content of single fibres

Since we found different K_m values for different fibres, it was investigated whether the ATP concentration at rest

Table 1. Maximum calcium-stimulated myofibrillar ATPase activity and apparent Michaelis constant for ATP in different fibre types from *Xenopus laevis*.

	Fibre type[a]				
	1	*2*	*3*	*4*	*5*
Number of fibres	10	8	7	6	9
Maximum myofibrillar ATPase activity (A_{550})[b] $m \pm$ S.D.	0.69 ± 0.06	0.55 ± 0.04	0.40 ± 0.04	0.34 ± 0.05	0.11 ± 0.01
Apparent K_m (mM ATP)[b] $m \pm$ S.D.	1.99 ± 0.31	0.57 ± 0.08	0.33 ± 0.06	0.19 ± 0.11	0.02 ± 0.03

[a]Fibres from the hatched areas in Fig. 3a only.
[b]Calculated from outcomes of direct linear plots (see Methods); the corresponding hyperbolas are shown in Fig. 5.

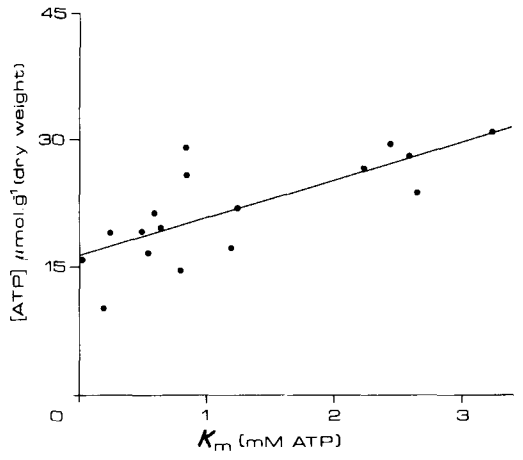

Fig. 7. ATP concentration at rest in 17 single muscle fibres plotted against the apparent Michaelis constant (K_m) for ATP in the same fibre. The equation of the line is as follows: $[ATP] = 16.4 + 4.4 \times K_m$, where K_m is in mM ATP and [ATP] in μmol/g dry weight.

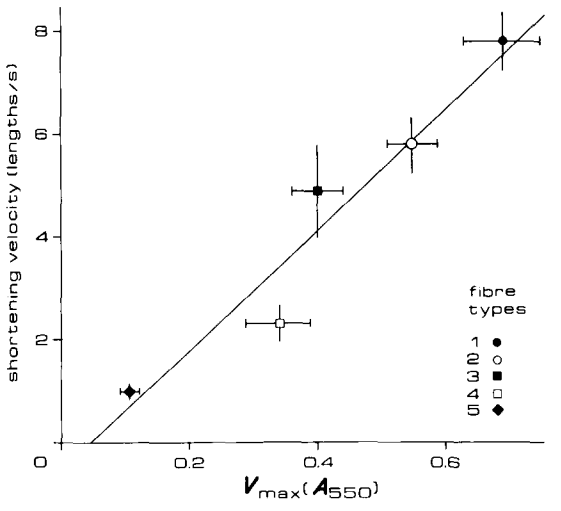

Fig. 8. Maximum velocity of shortening of five different fibre types plotted against the maximum calcium-stimulated myofibrillar ATPase activity ($m \pm$ S.D. see Table 1) of these types. The data for shortening velocity were taken from Lännergren & Hoh (1984, see Introduction). The range of shortening velocities for each type is also shown. The equation of the line (best fit with the mean values) is as follows: shortening velocity $= -0.65 + 12 \times V_{max}$, where V_{max} is an absorbance value (A_{550}) and shortening velocity is in lengths/s, both obtained at 20° C.

also differed. There appeared to be a tendency for higher ATP concentrations in fibres with high K_m values (Fig. 7; $r_s = 0.78$, $P < 0.01$; Spearman rank correlation coefficient, Siegel, 1956). Because we could not control the sarcomere length in the fibre it was not possible to determine the maximum myofibrillar ATPase activity in these experiments. It may be expected, however, that K_m is not affected by the number of sarcomeres in the section. The range of K_m values in Fig. 7 compares well with the range shown in Fig. 6. Making use of the relationship between water content and fibre dry weight (see Materials and methods), the relationship between ATP concentration at rest and K_m ([ATP] and K_m in mM) equals

$$[ATP] = 6.0 + 1.6 \times K_m$$

Table 2 gives the maximum shortening velocity of the five different fibre types as well as the maximum myofibrillar ATPase (V_{max}) and the myofibrillar ATPase activity ($V_{[ATP]}$) which was calculated using the relationship between the ATP concentration and K_m. A close relationship exists between maximum calcium-stimulated myofibrillar ATPase activity and maximum speed of shortening (Fig. 8). Considerable variation in both maximum shortening velocity and myofibrillar ATPase activity is present for all fibre types, except type 5. The correlation of the mean maximum shortening velocity and the mean V_{max}, as given in Table 2, is statistically significant ($r = 0.97$, $P < 0.01$). The same holds true for the correlation of shortening velocity and $V_{[ATP]}$ ($r = 0.95$, $P < 0.05$).

Discussion

The aim of the present study was to find out whether myofibrillar ATPase activity, determined with a quantitative histochemical method in individual muscle fibres, correlates with the maximum shortening velocity of these

Table 2. Shortening velocity and calcium-stimulated myofibrillar ATPase activity in five fibre types from *Xenopus laevis*.

Fibre type	V_o[a] *(lengths/s)*	K_m[b] *(mM ATP)*	*[ATP]*[c] *(mM ATP)*	V_{max}[b] A_{550}	$V_{[ATP]}$[d] A_{550}
1	7.8	1.99	9.2	0.69	0.57
2	5.8	0.57	6.9	0.55	0.51
3	4.9	0.33	6.5	0.40	0.38
4	2.3	0.19	6.3	0.34	0.33
5	1.0	0.02	6.0	0.11	0.11

[a] Maximum shortening velocity. The data were taken from Lännergren & Hoh (1984), see Introduction.
[b] Data from Table 1.
[c] $[ATP] = 6.0 + 1.6 \times K_m$.
[d] $V_{[ATP]} = [ATP] \times V_{max} / ([ATP] + K_m)$.

fibres. The results in Table 2 and Fig. 8 indicate that this is indeed the case.

One may argue that these results depend on the classification of the muscle fibres, which is to some extent a subjective procedure; (it should be noted that myofibrillar characteristics were not included in the classification of the fibres in the present study). The visual selection of the 50 fibres from a cross-section of the muscle was based on the criteria of Lännergren & Smith (1966) and Smith & Ovalle (1973). A more detailed analysis of these 50 fibres on the basis of diameter and succinate dehydrogenase activity did not, however, yield a simple subdivision into five types (Fig. 3). The major difficulty here was to distinguish between type 3 and type 4 fibres.

Three type 4 fibres, which have no tendency for increased subsarcolemmal succinate dehydrogenase activity, and three type 3 fibres had similar overall succinate dehydrogenase activity. These fibres were considered to be non-typical because the visual fibre classification and the results of cluster analysis were not in agreement. Similar problems occurred with the classification of one type 5 and one type 4 fibre, visually classified as such, but with almost equal succinate dehydrogenase activity, and with two type 2 fibres which had succinate dehydrogenase activities comparable to those of type 3 fibres.

If one assumes the existence of five fibre types, it is possible to classify all 50 fibres using the results of cluster analysis in Fig. 3b. Five types are obtained between cluster levels 3 and 4.5. Using this classification, the following subdivision of the 50 fibres is obtained:

ten type 1 fibres, cluster a;
ten type 2 fibres, clusters b and c;
thirteen type 3 fibres, clusters d and e;
seven type 4 and one type 5 fibre, cluster f;
nine type 5 fibres, cluster g.

Thus, three fibres which were visually classified as type 4 are now classified as type 3, and one type 5 fibre as a type 4. Analyses of variance, carried out on absorbance values obtained at 6 mM ATP in the myofibrillar ATPase reaction of these new groups, detected significant differences between the individual type 2 fibres ($F_{9,30} = 4.00$, $P < 0.005$) and between the individual type 4 fibres ($F_{7,24} = 8.20$, $P < 0.001$). This new classification therefore leads to considerably more scatter than that of the data shown in Figs. 4b,d. However, only minor changes result with respect to the data given in Table 2, and the correlation of maximum speed of shortening and maximum ATPase activity, as well as the correlation of maximum speed of shortening and calculated ATPase activity, remain statistically significant ($r = 0.97$, $P < 0.01$ for both cases). The initial selection of the 50 fibres, based only on the criteria used by Lännergren & Smith (1966) and by Smith & Ovalle (1973), yields similar results. The correlation coefficients in this case are $r = 0.96$ ($P < 0.01$) for shortening velocity and maximum ATPase activity, and $r = 0.94$ ($P < 0.05$) for shortening velocity and calculated ATPase activity. These results imply that the correlation between ATPase activity and shortening velocity, as described in the present study, is valid if the classification of muscle fibres that was made by Lännergren & Smith (1966) and by Smith & Ovalle (1973) on the one hand and the present selection of the fibres on the other are in agreement. We do not find much reason to doubt this.

Apparent Michaelis constant for ATP

To our knowledge, there has been only one report (rabbit psoas muscle, Mabuchi & Sréter, 1980) on the relationship between ATPase activity in cryostat sections and the ATP concentration. The value for K_m in that study was about 0.9 mM ATP at 37° C and pH 9 (K_m calculated from their Table 3). Glyn & Sleep (1985) found a K_m of 16 μM MgATP at 15° C in skinned rabbit psoas fibres, a much lower value. They demonstrated that K_m depends strongly on the composition of the medium. Another factor which may be partly responsible for these different K_m values is the difference in temperature. For fast mouse skeletal muscle fibres we found, using the technique described in the present study, a K_m of about 0.26 mM ATP at 18° C and about 1.5 mM ATP at 37° C, which indicates that the K_m for ATP is temperature-dependent with a Q_{10} of about 2.6 (unpublished observation).

The values for K_m in fast twitch *Xenopus* fibres (types 1 and 2) presented in this study are higher than those for K_m in fast mouse muscle fibres at 18° C. Thus it may be expected that the apparent Michaelis constant for ATP of the myofibrillar ATPase varies not only between different muscle fibres of an individual but also between species. Ferenczi *et al.* (1984) found a K_m for MgATP of maximum velocity of shortening in frog fibres at 0–5° C of 0.47 mM. Assuming Q_{10} for K_m is 2.6, this value agrees well with the K_m for ATP of calcium-stimulated myofibrillar ATPase at 20° C in type 1 fibres from *Xenopus*, which is about 2 mM (Table 1). However, there is no *a priori* reason to assume that the K_m for MgATP and CaATP are the same. For this reason, the values for K_m presented in this study should be interpreted with great care.

The results indicate that the ATP concentration in *Xenopus* fibres depends on the type of fibre. The ATP concentration in type 1 fibres (i.e. with $K_m > 1.5$ mM ATP, see Fig. 7) was 27.2 ± 2.7 μmol ATP/g dry weight ($m \pm$ S.D., $n = 5$) while in the other types (i.e. with $K_m < 1.5$ mM ATP) it equalled 18.9 ± 4.9 μmol ATP/g dry weight ($m \pm$ S.D., $n = 12$). The difference between these groups is statistically significant ($F_{1,15} = 12.75$, $P < 0.005$). Similar differences between fast and slow mammalian muscle have been reported (Hintz *et al.*, 1982, Edström *et al.*, 1982, Saltin & Gollnick, 1983). The ATP concentrations in *Xenopus* fibres are also similar to the values published for frog (*Rana*) muscle: 14 to 28 μmol ATP/g protein (for review see Nassar-Gentina *et al.*, 1978; the protein content of *Rana* muscle equals 84–97% of muscle dry weight).

The ATP concentration at rest was about 4.5 times

higher than the apparent K_m for ATP of the myofibrillar ATPase for type 1 fibres and at least 12 times higher for the other types (Table 2). These results imply that small changes of the ATP concentration will hardly affect the ATPase activity, and consequently the performance of the fibres. Considerable changes of the ATP concentration will, however, affect the performance of type 1 fibres to a substantial degree. These fibres have the highest myofibrillar ATPase activity and low oxidative capacity (Fig. 3) as well as low anaerobic capacity (Rowlerson & Spurway, 1985). It is likely therefore that a considerable decrease of the ATP concentration occurs in these fibres during prolonged activity. Interestingly, type 1 fibres have little resistance to fatigue relative to types 2 and 3 (Lännergren & Smith, 1966).

Conclusions

It is concluded that the quantitative histochemical method for calcium-stimulated myofibrillar ATPase allows an estimate of the maximum velocity of shortening of muscle fibres from *Xenopus laevis*. Apart from differences between fibres in maximum ATPase activity, differences are present for the apparent Michaelis constant for ATP of the myofibrillar ATPase and the ATP concentration at rest in the fibres. The quantitative histochemical data suggest that a subdivision of the fibres in the iliofibularis muscle of *Xenopus laevis* into five types may be a simplification. Therefore, more detailed studies on the relationship between velocity of shortening and myofibrillar ATPase activity require paired observations on different single muscle fibres.

Acknowledgements

We thank Professor Dr G. Elzinga for reading and criticizing the manuscript, and R. Zaremba for technical assistance. This study was supported, in part, by a grant from FUNGO with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

ARIANO, M. A., ARMSTRONG, R. B. & EDGERTON, V. R. (1973) Hindlimb muscle fibre populations of five mammals. *J. Histochem. Cytochem.* **21**, 51–5.

BÁRÁNY, M. (1967) ATPase activity of myosin correlated with speed of muscle shortening. *J. Gen. Physiol.* **50**, 197–218.

BROOKE, M. H. & KAISER, K. K. (1970) Three 'myosin adenosine triphosphatase' systems: the nature of their pH lability and sulphydryl dependence. *J. Histochem. Cytochem.* **18**, 670–2.

BURKE, R. E. (1978) Motor units: physiological/histochemical profiles, neuronal connectivity and functional specialization. *Am. Zool.* **18**, 127–34.

CLOSE, R. I. (1972) Dynamic properties of mammalian skeletal muscles. *Physiol. Rev.* **52**, 129–97.

CORNISH-BOWDEN, A., PORTER, W. R. & TRAGER, W. F. (1978) Evaluation of distribution-free confidence limits for enzyme kinetic parameters. *J. Theoret. Biol.* **74**, 163–75.

EDSTRÖM, L., HULTMAN, E., SAHLIN, K. & SJOHOLM, H. (1982) The contents of high-energy phosphates in different fibre types in skeletal muscles from rat, guinea-pig, and man. *J. Physiol. (Lond.)* **332**, 47–58.

EISENTHAL, R. & CORNISH-BOWDEN, A. (1974) The direct linear plot. A new graphical method for estimating enzyme kinetic parameters. *Biochem. J.* **139**, 715–20.

FERENCZI, M. A., GOLDMAN, Y. E. & SIMMONS, R. M. (1984) The dependence of force and shortening velocity on substrate concentration in skinned muscle fibres from *Rana temporaria*. *J. Physiol. (Lond.)* **350**, 519–43.

GLYN, H. & SLEEP, J. (1985) Dependence of adenosine triphosphatase activity of rabbit psoas muscle fibres and myofibrils on substrate concentration. *J. Physiol. (Lond.)* **365**, 259–76.

HILL, A. V. (1965) *Trails and Trials in Physiology*, p. 245. London: Edward Arnold.

HINTZ, C. S., CHI, M. M.-Y., FELL, R. D., IVY, J. L., KAISER, K. K., LOWRY, C. V. & LOWRY, O. H. (1982) Metabolite changes in individual rat muscle fibres during stimulation. *Am. J. Physiol.* **242**, C218–28.

JUENGLING, E. & KAMMERMEIER, H. (1980) Rapid assay of adenine nucleotides of creatine compounds in extracts of cardiac tissue by ion-paired reverse-phase high-performance liquid chromatography. *Anal. Biochem.* **102**, 358–61.

KAHN, M. A. (1976) Histochemical characteristics of vertebrate striated muscle: a review. *Prof. Histochem. Cytochem.* **8** (4), 1–48.

LÄNNERGREN, J. (1978) The force-velocity relation of isolated twitch and slow muscle fibres of *Xenopus laevis*. *J. Physiol. (Lond.)* **283**, 501–21.

LÄNNERGREN, J. (1979) An intermediate type of muscle fibre in *Xenopus laevis*. *Nature (Lond.)* **279**, 254–6.

LÄNNERGREN, J. & SMITH, R. S. (1966) Types of muscle fibres in toad skeletal muscle. *Acta Physiol. Scand.* **68**, 263–74.

LÄNNERGREN, J. & HOH, J. F. Y. (1984) Myosin isoenzymes in single muscle fibres of *Xenopus laevis*: analysis of five different functional types. *Proc. R. Soc. Lond.* **B222**, 401–8.

LÄNNERGREN, J., LINDBLOM, P. & JOHANSSON, B. (1982) Contractile properties of two varieties of twitch muscle fibres in *Xenopus laevis*. *Acta. Physiol. Scand.* **114**, 523–35.

MABUCHI, K. & SRÉTER, F. A. (1980) Actomyosin ATPase. I. Quantitative measurement of activity in cryostat sections. *Muscle Nerve* **3**, 227–32.

MEIJER, A. E. F. H. (1970) Histochemical method for the demonstration of myosin adenosine triphosphatase in muscle tissues. *Histochemie* **22**, 51–8.

NASSAR-GENTINA, V., PASSONNEAU, J. V., VERGARA, J. L. & RAPOPORT, S. I. (1978) Metabolic correlates of fatigue and of recovery in single frog muscle fibres. *J. Gen. Physiol.* **72**, 593–606.

POOL, C. W., DIEGENBACH, P. C. & SCHOLTEN, G. (1979)

Quantitative succinate dehydrogenase histochemistry. I. A methodological study on mammalian and fish muscle. *Histochemistry* **64**, 251–62.

ROWLERSON, A. & SPURWAY, N. C. (1985) How many fibre types in amphibian muscles? A comparison of *Rana* and *Xenopus*. *J. Physiol. (Lond.)* **358**, 78P.

SALTIN, B. & GOLLNICK, P. D. (1983) Skeletal muscle adaptability: significance for metabolism and performance. In *Handbook of Physiology: Skeletal Muscle* (edited by PEACHEY, L. D., ADRAIN, R. H. and GEIGER, S. R.), pp. 555–631. Baltimore: Williams & Wilkie.

SIEGEL, S. (1956) *Non-parametric Statistics for the Behavioral Sciences*. pp. 312. New York: McGraw-Hill.

SMITH, R. S. & OVALLE, W. K. (1973) Varieties of fast and slow extrafusal fibres in amphibian hind limb muscles. *J. Anat. (Lond.)* **116**, 1–24.

SPURWAY, N. C. (1981) Objective characterization of cells in terms of microscopical parameters: an example from muscle histochemistry. *Histochem. J.* **13**, 269–317.

VAN DER LAARSE, W. J., DIEGENBACH, P. C. & MASLAM, S. (1984) Quantitative histochemistry of three mouse hind-limb muscles: the relationship between calcium-stimulated myofibrillar ATPase and succinate dehydrogenase activities. *Histochem. J.* **16**, 529–41.

WISHART, D. (1975) *Clustan Manual* University College, London: Computer Centre.