J. Physiol. (1979), **287**, *pp.* 439–453
With 1 *plate and* 4 *text-figures*
Printed in Great Britain

HISTOCHEMICAL REACTIONS OF FIBRES IN A FAST TWITCH MUSCLE OF THE CAT

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(*Received 23 November 1977*)

SUMMARY

1. Serial sections of flexor digitorum longus muscle (f.d.l.) of the cat were examined histochemically for four enzyme systems: adenosine triphosphatase (ATPase) with alkaline and acid pre-incubation, phosphorylase and succinic dehydrogenase (SDHase).

2. The number of types into which fibres should be divided was assessed by estimating enzyme reaction intensity from measurements of light transmission through photomicrographs. It was concluded that in general the enzyme reaction intensities of fibres were distributed continuously. However, the distribution histograms showed two (phosphorylase and SDHase) or three (acid and alkaline ATPase) clear peaks. Eighteen combinations of reaction intensities (profiles) were seen of which eight were very rare. The distribution of profiles differed between individuals but were similar in right and left muscles.

3. Areas of fibres were measured from muscles which had been fixed at the length at which twitch tension was maximal. The variance in fibre area with any one profile was significantly less than the variance in fibre area of all fibres within a muscle. There were significant differences between the mean areas of fibres with different profiles.

4. If only three enzyme reactions are considered (acid and alkaline ATPase and phosphorylase) the majority of fibres fall into one of the three classes commonly accepted for other muscles. The remainder would fit into this classification with the minimal assumption of only one error of fibre typing resulting from the continuous distributions of enzyme reaction intensities. The SDHase reaction was not strongly correlated with the three classes and could be used to divide the fibres further into six groups. Differences between means of fibre areas were significant for all pairs out of these six groups except one.

5. The grouping may be considered to reflect a dual system of enzymes, the two systems being (*a*) ATPases and phosphorylase, (*b*) SDHase. A possible role of nervous activity in determining this dual system is discussed. The hypothesis involves two partly independent characteristics of motoneuronal activity: (*a*) the frequency of impulses, and (*b*) the total number of impulses.

6. The measurements are correlated with other physiological variables in the

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individual animals. The mean areas of fibres in all groups increased with body weight. There were changes in the proportions of light and dark SDHase fibres related to weight. The total area contributed by dark alkaline ATPase fibres decreased and that by intermediate alkaline ATPase fibres increased with increasing twitch time to peak.

7. Specific tension of the group of slower muscle fibres in f.d.l. was estimated to be 0·29 $N.mm^{-2}$ compared with 0·39 $N.mm^{-2}$ for the faster fibres.

INTRODUCTION

Flexor digitorum longus muscle (f.d.l.) of the cat has been used for a number of physiological studies particularly in the field of the influence of nerve on muscle (Buller, Eccles & Eccles, 1960). Olson & Swett (1966) and Prewitt & Salafsky (1970) have made histochemical studies of this muscle but they did not include in their work the reactions for adenosine triphosphatase (ATPase) which have been used in more recent histochemical work (Guth & Samaha, 1969). Davies & Gunn (1972) have made a fuller investigation of f.d.l. but the results are only very briefly recorded. One aim of our investigation was to describe the histochemical composition of f.d.l. for comparison with the known physiological and biochemical properties. A second aim of the work was to attempt to establish objective, semiquantitative criteria for describing the fibres, rather than fitting the results into an existing classification. In addition we have measured the areas of fibres at a standardized muscle length. We conclude that it is difficult to describe the population of f.d.l. in terms of the accepted three fibre types of fast muscle. We suggest that the most accurate description is in terms of two systems which are only partially dependent on each other. Some correlations are made with contractile properties of the muscles.

METHODS

(1) *Preparation of muscles*

Young adult cats from the University breeding colony, weighing 2–2·5 kg were anaesthetized with sodium pentobarbitone, 40 mg/kg injected I.P. In four experiments in which fibre diameters were to be measured f.d.l. muscle was prepared for isometric recording as described by Bagust, Knott, Lewis, Luck & Westerman (1973) except that gastrocnemius and plantaris muscles were removed and threads were used to draw the skin away from the muscle in order to achieve a large volume pool in which the muscle could be immersed with maximal exposure of its surface for rapid freezing. Two muscles received only sufficient twitch stimulation to let them be set to optimum length; two muscles, after establishment of this length, were later tetanized to deplete glycogen (see Results). Muscles were frozen, at the length which gave maximal twitch tension, by the following procedure. At the end of the recording period, liquid paraffin was washed away with warm Ringer–Locke solution. The pool was emptied and the muscle coated with talcum powder (Moline & Glenner, 1964) and then covered with liquid nitrogen immediately after the injection (I.P.) of a lethal dose of pentobarbitone. The level of liquid nitrogen was maintained whilst the leg was disarticulated at the hip. The limb was stored in the cryostat at − 20 °C until it was possible to separate the muscle. F.d.l. was then dissected free, working within the cabinet and using instruments which had been cooled in the cryostat. In other experiments in which fibre areas were not to be measured, muscles were removed from their origin in anaesthetized cats, covered with talcum powder and immersed in liquid nitrogen whilst held between forceps approximately at body length.

(2) *Preparation of sections*

In all experiments the middle third of the muscle was sectioned, except in one muscle in which two other regions were sectioned to test for uniformity. Serial sections of 10 μm were prepared at -20 °C. Twelve or sixteen serial sections were mounted individually on slides at room temperature, dried in the air and stained by one of four histochemical methods: acid or alkali pre-incubation techniques for ATPase (Guth & Samaha, 1970), phosphorylase (Eranko & Palkama, 1961) or SDHase (Nachlass, Tsou, de Souza, Cheng & Seligman, 1957). In preliminary experiments the procedures described by the original authors were modified in order to find conditions which gave maximal contrast between fibres. Sets of serial sections were stained each with one factor modified within the method. In this way determinations were made of optimal section thickness, pH of pre-incubaton medium (for ATPase), times and temperatures of incubation and pre-incubation, and substrate concentration. Care was taken to control temperature carefully and the water bath could be set to $\pm 0{\cdot}2$ °C by means of a thermistor-controlled heating circuit. This bath was insulated and covered to reduce temperature gradients. Details of these results are described elsewhere (Edjtehadi, 1974; G. D. Edjtehadi & B. Salafsky, in preparation), but briefly the following modifications were made to the original methods specified in the three papers quoted earlier in this paragraph. For alkaline ATPase the pre-incubation time was 15 min, incubation time 25–30 min, and the reaction jar containing solution was warmed at 37 °C for 10 min before the section was immersed. Acid pre-incubation for ATPase lasted for 5 min. Incubation for phosphorylase was 4 min. Incubation for SDHase was carried out at 30 °C in the water-bath.

(3) *Evaluation of results*

Area of fibres. The entire cross-sectional area was photographed with Ektachrome X colour film (at $\times 20$) and FP4 monochrome (at $\times 40$). Prints were made from the monochrome negatives at an over-all magnification of $\times 400$, so that areas of fibres could be measured by planimetry. Montages were also constructed from prints to obtain a picture of the whole section.

Two methods of evaluating reaction intensities were used, one subjective and one objective.

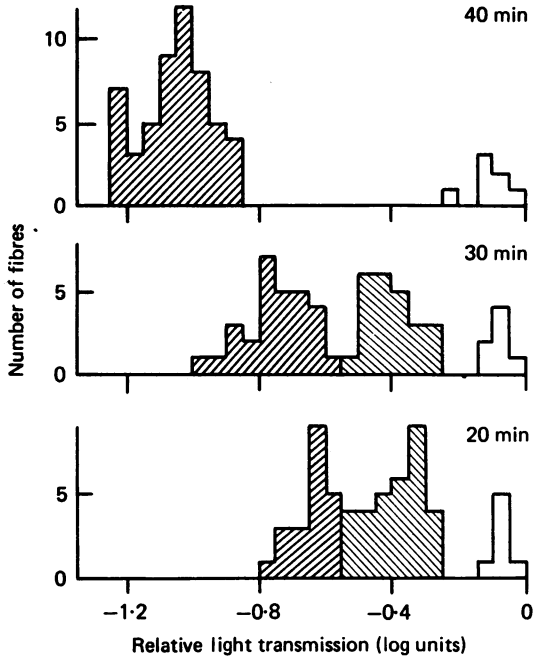
Subjective classification. Colour transparencies were projected against a white wall, and one or two fascicles, containing about sixty fibres, were selected from the central region of the slide. Samples of 600 and 900 (two muscles) or 1600–1700 (six muscles) fibres were taken from muscles estimated to consist of 20,000 fibres (Westerman, Lewis, Bagust, Edjtehadi & Pallot, 1974). Enlarged prints of the regions containing the chosen fascicles were used to ensure identification in the serial sections. All fibres in the chosen fascicles were examined and subjectively assigned to one of two or three types using colour transparencies (see Results).

Objective classification: light transmission. We justified our subjective choice of the number of types by measurements of light transmission of fibres in colour photomicrographs of representative sections which previously had been classified subjectively.

The total light transmitted by areas within individual fibres was measured with a variable resistance selenium photocell. The cell was connected as one arm of a Wheatstone bridge, the amplified output of which was measured by a digital voltmeter. The system was calibrated using neutral density filters. The amplification was set so that output voltage was linearly related to the logarithm of light transmission over a range of 2·3 density units. Colour transparencies of the sections were projected onto a screen for measurement of the transmission of individual fibres. The distance of the projector from the wall was used to adjust the output of the light measuring system so that the lightest fibre produced a voltage near one end of the log-linear range. Under these conditions the darkest fibre transmitted sufficient light to be within the calibrated portion of the photocell response. For measurements the photocell was mounted behind a circular hole cut in a white card. The projected image of the fibres could be seen on the card and the hole was centred on one fibre to allow its relative light transmission to be measured. The hole was cut so that it could just be positioned totally within the smallest fibre in the selected field. The illumination was not uniform over the whole field and measurements were restricted to bundles of about 100 fibres. Measurements were made in representative samples in three muscles.

Text-fig. 1 illustrates measurements made on three serial sections treated for alkaline ATPase reaction. Different periods of incubation in the substrate medium had been used in order to

decide the number of types into which the fibres could be assigned. In all three sections there was a distinct group of fibres with high transmission (i.e. pale reaction). At the longest incubation period (top) the darker fibres could not be subdivided. With shorter incubation periods the darker fibres still had a continuous distribution of light transmission, but two peaks were seen. The trough between the two peaks was clearer at the intermediate incubation time. On this basis we thought it reasonable to classify fibres with alkaline ATPase reaction into three types. Acid pre-incubation also allowed classification into three types. None of the variations in the histochemical techniques which were attempted could be used to classify fibres stained for



Text-fig. 1. Relative light transmission through photomicrographs of sixty fibres treated for ATPase with pre-incubation in alkaline solution. Three serial sections were used; the first (bottom) had been incubated for 20 min, the next for 30 min and the last (top) for 40 min. Before measuring the fibres, they had been typed subjectively; the types were dark (heavy hatching), intermediate (light hatching) and light (plain columns).

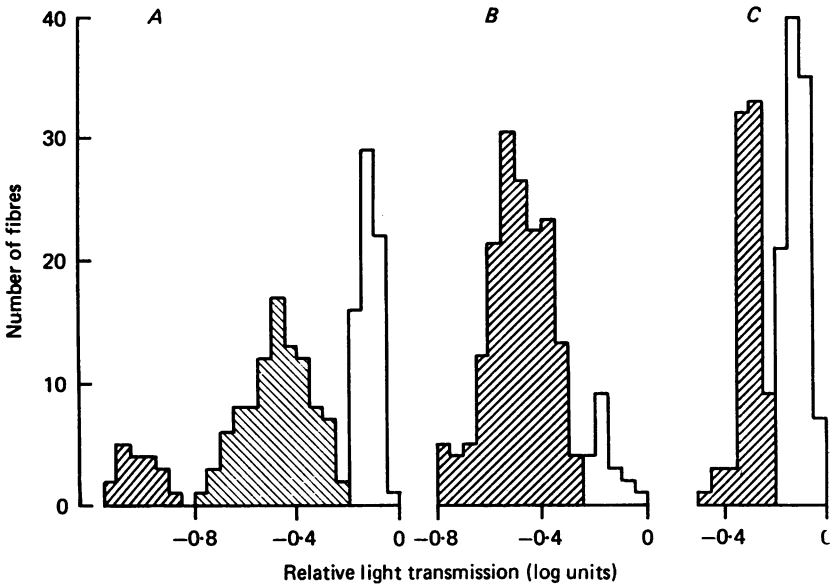
acid or alkaline ATPase reaction into more than three types. With the other two histochemical methods only two types of fibres could be separated (Text-fig. 2). In some of the larger fibres the photocell was moved to measure transmission over several (overlapping) parts of the fibre. Differences were seen across fibres but the variations usually were small compared with the width of the histogram groups, never more than one of the bins used in Text-figs. 1 and 2.

It should be noted from Text-figs. 1 and 2 that the initial subjective classification of the same fibres made by the main worker (G.D.E.) was in complete agreement with the subsequent objective measurements: this was true in all the sections tested. Moreover in another series of tests three observers independently examined six fascicles and disagreed on the subjective typing in less than 2% of the fibres.

Subjective classification therefore has been used for most of the results which follow.

RESULTS

Histological classification has been made of 8137 fibres from f.d.l. muscles in six cats. The distribution of staining reactions of fibres with the four techniques is shown in Text-figs. 1 (middle) and 2 in which the shading indicates the subjective classification of the fibres made before light transmission was measured. The fibres were classified subjectively into three types (light, intermediate or dark staining) for alkaline and acid ATPase and into two types (light or dark) for phosphorylase and SDHase. For convenience individual fibres were characterized by a set of four



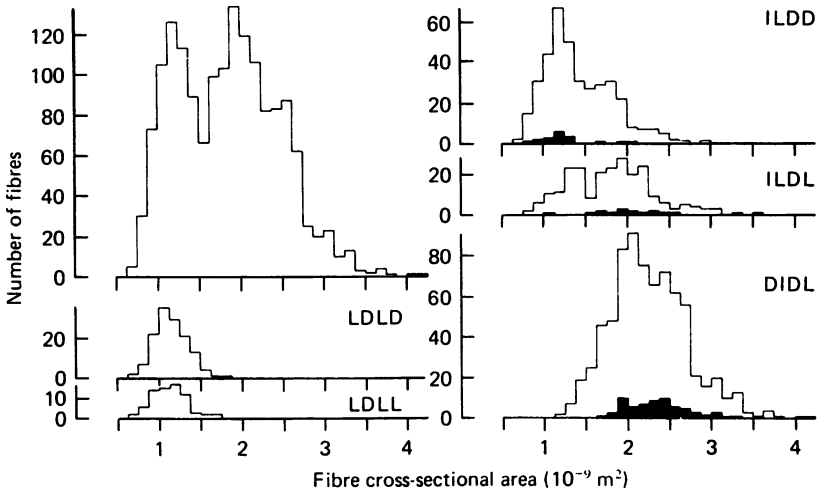
Text-fig. 2. Relative light transmission through photomicrographs of fibres in three serial sections treated for acid ATPase (*A*), phosphorylase (*B*) and SDHase (*C*). Scales and shading as Text-fig. 1. The sections were serial and the same 184 fibres were measured for each reaction; some of the fibres were used for Text-fig. 1.

initials corresponding to the reaction intensity in each of the four techniques. Thus a set of initials defines a histochemical profile. For example the profile DIDL indicates those fibres having a reaction which was dark for alkaline ATPase, intermediate for acid ATPase, dark for phosphorylase and light for SDHase. Examples of four types of fibre are illustrated in Pl. 1.

The maximum possible number of profiles in this classification would be thirty-six but only eighteen profiles were seen and the incidence of these is set out in Table 1(*c*). Only four of the profiles consisted of more than 10% of the fibres. Another three profiles had an incidence near 2%: it should be remembered that this represents some 400 fibres in one muscle. Care was taken to check that the less common profiles were not due to errors in classification: in each muscle more than one observer checked classification in some fibres and this was confirmed by the photometric method in some cases. One less common profile is seen in Pl. 1. In

Table 1 the ten most common profiles are set out individually, and these have been associated into six groups (i–vi) with the help of measurements of fibre areas (see below).

There was a strong correlation between three of the staining reactions. Fibres which were dark with alkaline ATPase were nearly all intermediate with acid ATPase and dark with phosphorylase (i.e. DID–). The other two common correlations for these three reactions were ILD– and LDL–. (Where a dash is substituted for one of the four letters it indicates that the reaction to the corresponding enzyme system has been ignored.)



Text-fig. 3. Distribution of fibre cross-sectional areas within one muscle (top left) and within the five most common histochemical groups composing that muscle. The shaded areas indicate fibres with uncommon profiles assumed to fall within the common groups.

The consistency of these correlations may be illustrated by the fact that 99·7% of fibres which were light with alkaline ATPase were dark with acid ATPase and none was light. Of the dark alkaline ATPase fibres 97·1% were intermediate for acid ATPase. The strong correlation also held for phosphorylase which produced a dark reaction in 99·5% of fibres which were dark or intermediate for alkaline ATPase and a light reaction in 98·8% of light alkaline ATPase fibres.

There was no comparable rigid correlation between SDHase reaction and any of the other three. Although there was a strong tendency for dark alkaline ATPase fibres to be light with SDHase (94·3%), only 88·1% of light alkaline ATPase fibres were dark with SDHase. The intermediate alkaline ATPase fibres were much more evenly split into dark (66·2%) and light SDHase reactions: this division was responsible for there being four commonly occurring profiles rather than three. The occurrence of two of the three profiles with an incidence between 1 and 10% was again due to the weaker correlation between SDHase and dark or light staining alkaline ATPase fibres.

The incidence of profiles varied between animals but in two cats right and left

muscles were compared and found to show much greater consistency (Text-fig. 4*B*). These pairs of muscles were processed independently.

Fibre areas

In four muscles the area was measured of each of the fibres submitted to histochemical analysis. Muscles were frozen at twitch optimum length to standardize the areas. An average of 1670 fibres were measured in each muscle. Distribution histograms from a typical muscle are illustrated in Text-fig. 3.

The mean fibre area varied between the four muscles and there were corresponding differences between the mean areas of fibres with similar histochemical profiles (Text-fig. 4*A*). To normalize the values, the mean area of fibres with one histochemical profile was expressed as a fraction of the mean area of all fibres measured in that muscle. Table 1(*d*) shows the normalized areas for the more common profiles and it can be seen from the standard errors of the mean that there was little variation between the six muscles.

In Table 1 some of the less common profiles (IIDL, DLDD, IIDD and DLDL) have been grouped under one or other of the four more common profiles. This grouping was done on the basis of mean fibre area, so that within any one group none of the profiles had significantly different areas. The mean area of each group was significantly different from that of every other group, except for groups (v) and (vi) (see legend to Table 1).

The groups were thus arranged by areas but an examination of profiles showed a further consistency in that there was never more than one point of difference between histochemical types within a group. For example in group (i) there were two profiles (DIDL and IIDL) between which the only difference was that the alkaline ATPase type was either dark or intermediate (adjacent on the intensity scale) and the other three reaction types were identical (–IDL). Since the transmission distribution curves of Text-figs. 1 and 2 were continuous, even if it is assumed that the population consisted of three or two distinct types for each histochemical reaction, there is sure to be some misclassification because of the regions of overlap. Without misclassification it may be assumed that all fibres in group (i) would have been DIDL.

'Misclassification' is used here and later not to imply an error in the estimate of reaction intensity of a fibre, but rather to describe the consequence of overlapping populations. For example, if it is assumed that for phosphorylase there are two types of fibres then some of the 'light fibres' will have stronger reactions than some of the 'dark fibres' (due to the overlap of reaction intensities) and will be misclassified as dark type.

No precise expectations of misclassification have been deduced from the distributions of light transmission because of the problems involved in interpretation of the simple technique of measurement, but two semiquantitative observations can be made. First, the most clear separation of types was between the light and intermediate fibres with alkaline ATPase and the dark and intermediate with acid ATPase: none of the differences within the groups of Table 1 was of this type. Secondly, the least clear separation was between the two types of SDHase fibres where the misclassification could have been some 5–10%. These values were estimated by fitting two log.-normal distributions to the bimodal SDHase histograms. For Text-fig. 2*C* the mean light transmission of the dark fibres measured from the line of division between light and dark fibres was 2·4 times the S.D. for the dark fibres. On the assumption of two log.-normally distributed classes of fibres this figure predicts that less than 2% of the dark fibres would fall on the light side of the line of division. For light fibres the corresponding

figures were: mean was 2·0 times the S.D. from the line of division; 2·5% of fibres misclassified. The total misclassification would have been 4·5% of fibres. In the worst case the prediction was that 8% of fibres would be misclassified for SDHase reaction.

Difference in the SDHase reaction was the only criterion for separating groups (v) and (vi), but to explain the incidence of these two groups would require a misclassification of some 14% of dark SDHase fibres as light. Although groups (i) and (ii) also only differed histochemically in their SDHase reaction there was also a significant difference ($P < 0·02$) between the mean fibre areas.

The fibres of the eight other groups were so rare that it was impossible to obtain reliable estimates of mean area. But it was possible to assign them to one of the six groups of Table 1 without more than one point of histochemical difference.

TABLE 1. *Numbers and areas of fibres in f.d.l. muscles*

(a) Group	(b) Profile	(c) Incidence (%) Mean	(c) S.E.	(d) Normalized areas Mean	(d) S.E.	(d) C.V.	(e) Total area (%) Mean	(e) S.E.
(i) DIDL	DIDL	46·1	2·3	1·22	0·008	0·25	—	—
	IIDL	1·5	0·6	1·23	0·072	0·23	—	—
	All	47·7	2·1	1·23	0·007	0·23	56·0	2·70
(ii) DIDD	*All*	2·2	0·6	1·10	0·069	0·23	1·95	0·78
(iii) ILDL	ILDL	12·0	2·2	0·98	0·012	0·28	—	—
	DLDL	0·7	0·2	1·02	0·039	0·22	—	—
	All	12·8	2·4	0·98	0·021	0·27	14·3	3·65
(iv) ILDD	ILDD	23·3	1·6	0·80	0·025	0·28	—	—
	DLDD	0·5	0·1	0·80	0·062	0·28	—	—
	IIDD	0·5	0·2	0·75	0·044	0·34	—	—
	All	24·4	1·7	0·80	0·025	0·28	18·7	2·37
(v) LDLL	*All*	1·5	0·2	0·60	0·019	0·17	1·0	0·60
(vi) LDLD	*All*	11·0	1·1	0·59	0·030	0·22	7·1	0·88
(i–vi)	eight profiles	0·7	0·2	0·91	—	0·37	0·8	0·10
All	—	100	—	1·00	—	0·36	100	—

The fibre groups and dominant profiles are shown in (*a*). Where appropriate subdivisions of the groups are indicated as profiles in (*b*) (see text); '*All*' indicates figures for all fibres within the group. The profile initials show the intensity of reaction (dark, intermediate or light) with the four histochemical reactions (alkaline and acid ATPase, phosphorylase and SDHase).

In (*c*), the number of fibres of each group has been expressed as a percentage of the total number examined in each of six muscles: the S.E. is calculated from the mean values for individual muscles.

In (*d*), the mean area per fibre of each set has been expressed as a fraction of the mean area per fibre of all fibres in the muscle: the S.E. of mean is calculated from the means of four muscles; C.V. is the average of the coefficient of variation (standard deviation/mean) in each of the four muscles, and is included to indicate the scatter of areas of fibres within groups.

Differences between group mean areas were: (v), (vi): not significant ($t = 0·24$); (ii), (iv): $P < 0·05$, $t = 2·1$, D.F. = 1008; (i), (ii): $P < 0·02$, $t = 2·3$, D.F. = 3329; all other pairs: $P < 0·001$, $t \not< 4·6$, D.F. $\not< 243$. Differences of areas within groups were not significant: largest difference between ILDL and DLDL, $t = 0·50$.

In (*e*) the total area of fibres in each of the groups is expressed as a percentage of the total area of all fibres measured and has been estimated as (*c*) times (*d*) for each of four muscles

Two muscles in Table 1 had been subjected to tetani. There were no systematic differences in the means or variances of the normalized areas between these and

the untetanized muscles. Therefore it was considered acceptable to pool results from all four muscles.

Although the mean diameters of five of the six groups were all significantly different from each other, there was considerable overlap between the fibres of groups: much greater than the overlap between histochemical reaction intensities. However, the variation within groups was considerably less than that in the muscle as a whole, as can be seen in Text-fig. 3. The coefficients of variation of diameter within the groups ranged from 17 to 28% (weighted mean 24·5%) and were all significantly less than that for all fibres in a muscle (35·7%); see Table 1*d*.

Uniformity of the muscles

The length of fibres in f.d.l. is only 32% of that of the muscle (Al-Amood & Pope, 1972) and the mid-belly transverse section can sample only 8500 of the 20,000 fibres, so it was essential to test for uniformity.

In one muscle the alkaline ATPase reaction of fibres was determined at three positions equally spaced along the muscle. Sections were processed together to minimize differences in treatment. In a second test for uniformity in another muscle, 8245 fibres of the normal transverse section were assigned to approximately equal deep and superficial parts. In both tests the fibres were classified into the three ATPase types and the frequencies of occurrence in different positions were compared by a χ^2 test. In neither test was there a significant difference due to position in the muscle (χ^2 = 2·72 and 5·75 with 4 and 2 degrees of freedom respectively). Similar tests showed that the differences in the incidence of the three alkaline ATPase types between four of the muscles were greater than could be accounted for by sampling errors (χ^2 = 89·6, 6 degrees of freedom, $P < 0{\cdot}001$).

Tetanization

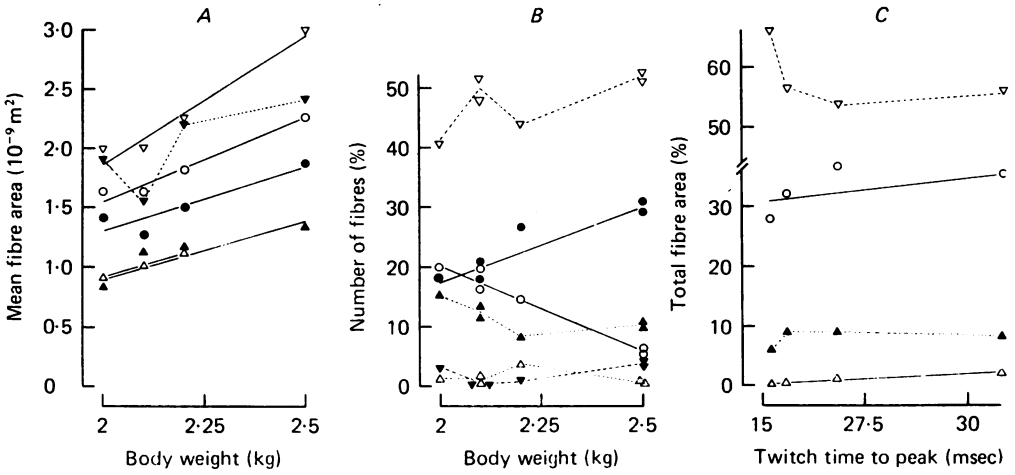
Two muscles were subjected to tetani at 100/sec for 1 sec every 10 sec. In one muscle forty-eight tetani were elicited, in the other ninety-six. In these two cases the contralateral muscle was prepared without eliciting tetani. Histochemical characterization of 1600–1700 fibres was performed in each of the tetanized muscles and the contralateral controls. There were no significant differences between the incidence of reactions to acid or alkaline ATPase or SDHase in the two groups. However, the phosphorylase sections showed three types of fibres in the tetanized muscle (by eye and photometrically). The new type consisted of fibres which were almost unstained and were also large; they may be considered to be equivalent to the fibres which were dark for phosphorylase in the untetanized muscles but had been depleted of glycogen by the tetani and could not develop the full phosphorylase reaction. If this assumption were made the distribution of fibre profiles was very similar in the tetanized and their contralateral controls. None of the 420 fibres which were light with alkaline ATPase and only 2·6% of the intermediate alkaline ATPase fibres showed a depleted phosphorylase reaction. The dark alkaline ATPase fibres were more extensively depleted: the percentage was significantly greater for the muscle which had received more tetani, 86% compared with 75% (χ^2 = 25, 1 degree of freedom, $P < 0{\cdot}001$). This percentage may be compared with estimates in cat gastrocnemius by Burke & Tsairis (1973, table 2) that 73% of fast twitch muscle fibres fatigue rapidly (i.e. were of the type described as FF).

Relation to contractile properties

Muscle tension increases with body weight without an increase in the number of fibres, and as would be expected this was seen as an increase of over-all mean fibre diameter with body weight in the present series of experiments. The increase

was seen in all six groups of fibres and is illustrated in Text-fig. 4*A*. (This is another expression of the consistency of normalized mean areas of groups already discussed in relation to Table 1.)

Correlations were also seen between the number of fibres in each group and body weight (Text-fig. 4*B*). The percentage of ILDD fibres progressively increased with body weight whilst the proportion of ILDL fibres decreased. No significant systematic trends were seen for other groups. Text-fig. 4*B* also demonstrates that side to side variation was less than variation between animals (the pairs of results at 2·1 and 2·5 kg represent left and right muscles).



Text-fig. 4. Relations between histochemical groups and physiological measurements. The number of fibres (*B*) in groups and their total area (*C*) are expressed as a percentage of all fibres in a muscle. The fibre groups DIDL, DIDD, ILDL, ILDD, LDLL and LDLD are indicated by the symbols ▽, ▼, ○, ●, △ and ▲ respectively in *A* and *B*. In *C* combined groups DID–, ILD– and LDL– are indicated by ▽, ○ and ▲ respectively. The continuous lines indicate fitted regressions where these were significant at the 0·05 level. Interrupted lines join other sets of points. The pairs of points at 2·1 and 2·5 kg in *B* indicate left and right muscles from one animal.

Twitch time to peak had also been measured in four muscles; it was not related to body weight. The total area contributed by dark alkaline ATPase fibres decreased with increasing time to peak whereas the area of intermediate ATPase fibres increased (Text-fig. 4*C*). There was no trend in the proportion of light ATPase fibres although if those of group LDLL were considered separately, their total area did increase with twitch time to peak; probably with a corresponding decrease in the proportion of LDLD fibres (not significant at the 5% level). It should be remembered that even in the slowest muscle the LDLL fibres contributed only 2·8% of the muscle cross-sectional area.

DISCUSSION

The classification into six histochemical groups is more complex than has been proposed by other workers, and it is necessary to examine the reasons behind this and to consider possible advantages. If only the first three enzyme systems had been used only three profile classes would have been required, the classification adopted by most other workers, whether or not using these three reactions. In one

class the fibres would have a dark reaction for alkaline ATPase, intermediate for acid ATPase and dark for phosphorylase (DID–). The other two commonly occurring combinations were ILD– and LDL–. Only 4·6% of fibres could not be included in one of these three classes and all could be incorporated if allowance is made for the overlap between the reaction intensities of the fibre types. In none of the 8137 fibres examined would it be necessary to assume more than one error of typing due to overlapping distributions or any error involving two histochemical types with non-overlapping densities.

There are also differences from other workers in the number of fibre types found for some of the enzyme systems. We have not found any objective evidence for more than two groups of fibres with the phosphorylase reaction even after systematically varying incubation conditions. Three phosphorylase types have been claimed on subjective evidence by other authors (Kugelberg & Edstrom, 1968; Brooke & Kaiser, 1969), but our division into two types is in agreement with Dubowitz & Pearse (1960) and Prewitt & Salafsky (1970). There may well be a real difference between muscles or species (only the last group used cat f.d.l.).

We have only distinguished two groups of fibres for the fourth reaction (SDHase) with the transmission measurements and separation between the two was less clear than for other reactions. In this division we again agree with Prewitt & Salafsky (1970) working with cat f.d.l. and also Dubowitz & Pearse (1960) and Davies & Gunn (1972). Others have described three groups. Yellin & Guth (1970) found that they could separate subjectively three groups of fibres in the cat but with more difficulty than in the rat. Stein & Padykula (1962) in the rat used the distribution of dark staining particles within the fibre cross-section to help in their three-type classification. In estimating total light transmission through a central sample of a fibre our photometric method would be insensitive to differences in distribution. Subjectively we saw differences in particle distribution in the dark fibres of our preparations but none which could be used with any confidence to separate the dark fibres into two groups for SDHase reaction. A quantitative measure of density and distribution of particles with a scanning microdensitometer might be able to resolve this problem.

The need to adopt six rather than three histochemical groups arises from the fact that it was not possible to correlate the two SDHase fibre types rigidly with the three classes defined by the other three enzyme systems and in this lies our essential difference from other workers. Table 2 sets out some classifications for comparison. It is true that 95% of fibres with the profile DID– had a light SDHase reaction and 88% of LDL– fibres were dark for SDHase. It is clearly possible that the 5 and 12% anomolous fibres could be accounted for by overlap between the light transmission of the SDHase types (Text-fig. 2). However, the third class of fibres (ILD–) was much more evenly split between light and dark SDHase types (approximately in the ratio of one to two) and it is unlikely that this can be accounted for by misclassification of SDHase types. So it is necessary to subdivide the ILD–fibres: altogether four classes of fibres. This agrees with Prewitt & Salafsky (1970) who also found four classes using only phosphorylase and SDHase reactions. Davies & Gunn (1972) in their table 3 indicate four profile groups for cat f.d.l., although one of the profiles contained only 2% of the fibres.

This uncommon profile probably corresponds to (D or I)–LD in our classification. (The ATPase reaction of Davies & Gunn distinguishes only two reaction levels and their high density fibres would be expected to give either dark or intermediate fibres with akaline ATPase) We saw no D–LD fibres; I–LD fibres occurred in only one muscle with an incidence of 0·5% in that muscle.

TABLE 2. Comparison of fibre type classifications of various authors. In some cases (enclosed in parentheses) the equivalence of two systems is speculative but included to introduce the names used to indicate difficulties of comparisons. The columns show labels used by various authors: Stein & Padykula (1962) (*a*); Brook & Kaiser (1969) (*b*); Peter *et al.* (1972) (*c*); Davies & Gunn, (1972) (*d*); Burke *et al.* (1973) (*e*); Yellin & Guth (1970), cat (*f*) and rat (*g*).

This paper	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)
DIDL	A	IIb	FG	Ah Ph Sl	FF	Aα	—
DIDD	—	(IIc)	—	—	FF	—	(Cα)
ILDL	—	—	—	—	FR	—	(A$\alpha\beta$)
ILDD	C	IIa	FOG	Ah Ph Sh	FR	C$\alpha\beta$	—
LDLL	—	—	—	—	—	—	—
LDLD	B	I	SO	Al Pl Sh	S	Bβ	(Bβ)
ILLD	—	—	—	(Ah Pl Sh)	—	—	—

If it is necessary to assume that intermediate alkaline ATPase fibres may give either a dark or light reaction for SDHase, then it is not unreasonable to suggest that the same may be true of dark and light alkaline ATPase fibres. It would then be unnecessary to assume that the two less common groups (DIDD and LDLL) are misclassified examples of common groups (DIDL and LDLD respectively). The mean fibre areas gave support for the separation of at least five groups. The classification into six groups of fibres would seem to involve fewest assumptions and is compatible with the reports of other authors who frequently report exceptions to simpler classifications (e.g. Stein & Padykula, 1962; 26% exceptions). Comparable motor unit studies have also found exceptions to a simple separation into three classes based on contractile properties and resistance to fatigue. The proportion of exceptions varies from 4% (Burke, Levine, Tsairis & Zajak, 1973) and 11% (Proske & Waite, 1974) to 28% (Stevens & Stuart, 1974).

The model which we propose to explain our classification is one involving two biochemical systems. One, represented by alkaline and acid ATPase and phosphorylase, is present in three different forms and gives rise to the three commonly described fibre classes in fast twitch muscle. The second, represented by SDHase, divides fibres into two types (in the cat). Combinations of these two systems would give the six groups some of which are linked loosely in that dark alkaline ATPase fibres are very likely to be light for SDHase and light alkaline ATPase fibres are likely to be dark for SDHase.

The separation of the two systems could occur if there were two control mechanisms which depended on each other only in part. It is known that many enzymes and their histochemical reactions can be modified by artificial stimulation. For example, prolonged stimulation of fast muscle at 10/sec causes a decrease in the reaction to alkaline ATPase and an increase to SDHase (Romanul, Sréter, Salmons & Gergely, 1974). However, Buller *et al.* (1960), in the first description of neuronal influence on muscle, discussed two aspects of activity which might bring about muscle

changes. The two aspects were the frequency of impulses and the aggregate number of impulses. One possible mechanism for the two semi-independent histochemical systems is that the ATPase-phosphorylase fibre type depends on the frequency of nervous impulses, whereas the SDHase type depends on the total amount of activity. The former has been demonstrated and it is not unreasonable that oxidative enzymes respond mainly to the total amount of activity demanded of a muscle fibre. Current ideas of motoneurone function suggest that frequency and total activity are related: a postural muscle is activated at low frequencies but is in such constant use that the total number of impulses is probably greater than in a phasic muscle. The complexity of use of motor units is such that such associations need not be rigid and a minority of rapidly firing motoneurones may be in more continuous use or some slowly firing ones recruited infrequently. The six groups of fibres would then depend on two semi-independent aspects of motoneurone activity which result in the combinations of two histochemical systems.

There is some evidence for the separation of two systems in the present results in the reciprocal changes in the proportions of ILDL and ILDD fibres (Text-fig. 4*B*) despite there being only small differences between right and left sides. Too few muscles were examined to be sure if the trends of Text-fig. 4*B* were a true change with body weight or age but they support the argument that SDHase reaction is not rigidly linked to alkaline ATPase reaction.

Similar suggestions have been discussed by Burke *et al.* (1973) working with cat gastrocnemius. The major difference is that we propose that SDHase activity is independent of ATPase activity for all ATPase types; Burke *et al.* restricted their suggestion to fast twitch motor units. Another point of difference is that although we agree that there is a continuous distribution of reaction intensities for SDHase we would suggest that it is bimodal and that SDHase activity can be used to separate two types of fibre with as much validity as ATPase activity can define three types. If nervous control of these types is accepted the presence of more than one mode in two semi-independent systems may have implications in the organization of motoneurone usage.

A brief report by Spurway (1978) is in general agreement with our suggestions in that it shows that in rat fast muscle there is also a poor correlation between some enzyme systems. It may also be possible to reduce differences between species. For example, Yellin & Guth (1970) showed that A fibres (light SDHase) are dark for alkaline ATPase in cat (our profile DIDL) whereas in the rat they are intermediate for alkaline ATPase, possibly corresponding to ILDL fibres which were less common but present in the cat (see Table 2).

Kugelberg (1976) has demonstrated transformation of fibre types even in fully mature rats and transitional forms could have complicated the present results. Although there were differences between the incidences of profiles between animals, in every one of the six animals the same pattern of groups was seen.

Histochemical differences have been related to contractile properties of different muscles (e.g. Davies & Gunn, 1972). Within one type of muscle, histochemistry and twitch responses are correlated. The variation of the relative areas of the histochemical groups with twitch time to peak is another illustration that small differences between samples of one muscle are related to the proportions of fibres. This modu-

lation was not brought about by variation in the total area of LDL– fibres (slow twitch: Burke *et al.* 1973) which form too small a fraction of the muscle to influence its total response. What was found was a change in the proportion of DID– and ILD– fibres. These may be assumed to be comparable with Burke's FF and FR motor units which have mean twitch times to peak of 35 and 48 msec respectively in gastrocnemius (from Burke, 1974; text-fig. 2). The changes in the proportion of dark to intermediate alkaline ATPase fibres with body weight may be compared with the late developmental changes described by Kugelberg (1976).

It is possible to derive the specific tensions contributed by some of the groups in f.d.l. The two groups LDLL and LDLD may be assumed to be equivalent to S-type motor units (Burke *et al.* 1973). In f.d.l. it is possible to distinguish a set of slow motor units and to assess the total tension contributed by them to the whole muscle. A study has been made of f.d.l. motor units in cats from the same inbred colony used for the present experiments (unpublished observations with H. J. Finol & Sandra N. Webb) and we estimate that the slow motor units contribute 6·2% of the total tetanic tension of the muscle. The LDL– fibres contribute 8·1% of the total fibre cross-sectional area and f.d.l. develops 0·38 N/mm^2 (Kean, Lewis & McGarrick, 1974). From these figures it is possible to deduce that LDL– fibres would have a specific tension of 0·29 N/mm^2 which is similar to that of cat soleus muscle (0·25 N/mm^2). This figure is within the range expected for skeletal muscle and contrasts with very low values of 0·06 N/mm^2 obtained by Burke & Tsairis (1974) for S-type motor units in cat gastrocnemius. The specific tension of the DID– plus ILD– fibres calculated as above would be 0·39 N/mm^2, not very different from the whole muscle. It is greater than the values quoted for FF motor units (0·14, 0·20 N/mm^2) or for a FR unit (0·27 N/mm^2) calculated by Burke & Tsairis (1973). The estimates of Kean *et al.* (1974) derived muscle cross-sectional area from muscle mass and fibre length and would be the total muscle area (fibres plus connective tissue) and should be lower than motor unit specific tensions in which muscle fibre areas are measured directly (Burke & Tsairis, 1973).

We would like to thank Dr B. Salafsky for helpful discussion and assistance and Mrs Janis Taberner for technical assistance. We are grateful to the Muscular Dystrophy Association of America for financial help.

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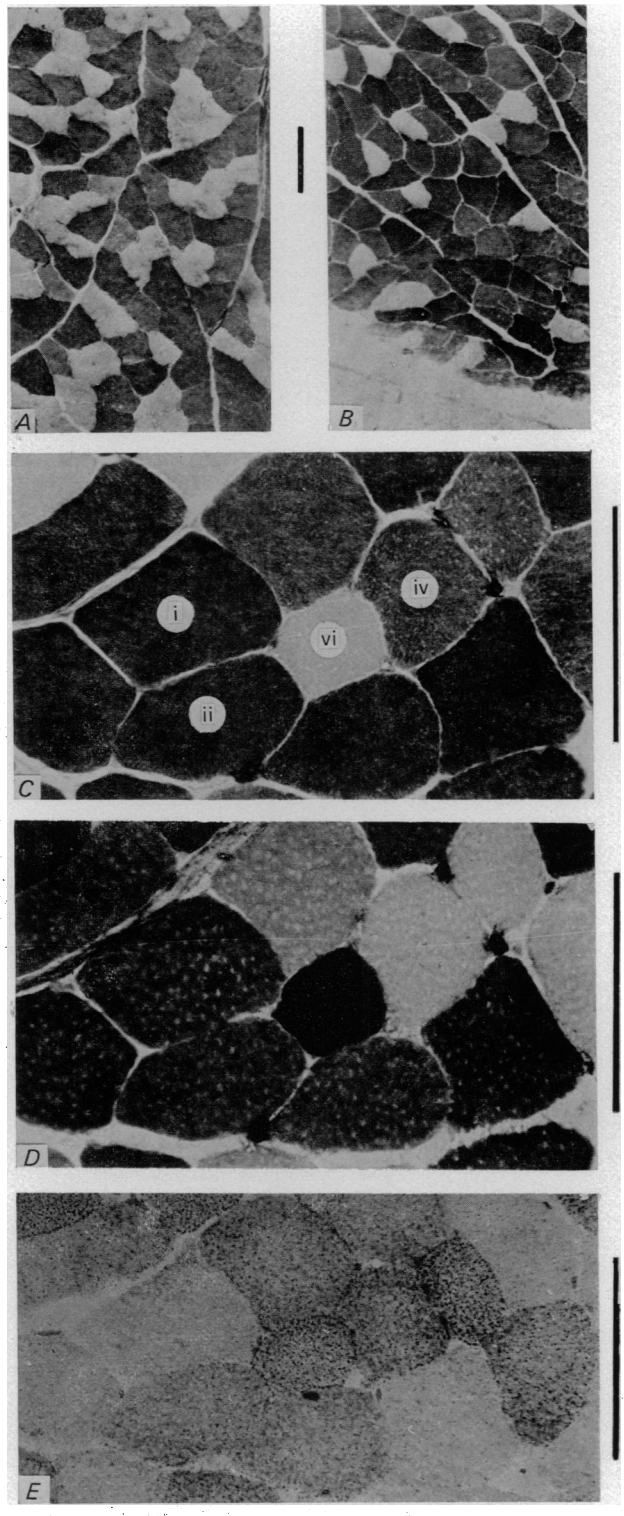

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EXPLANATION OF PLATE

Histochemical reactions of muscle fibres from cat FDL Above, alkaline ATPase reaction of fibres near the superficial surface (*A*) and the deep surface (*B*) of the muscle. The two samples are from one section of the muscle. At higher power are three histochemical reactions: *C*, alkaline ATPase; *D*, acid ATPase; *E*, SDHase. *C* and *D* were serial and there was one 10 μm section between *D* and *E*. Four fibres of four groups are indicated in *C*; the profiles are: (i) DIDL; (ii) DIDD; (iv) ILDD and (vi) LDLD. All calibration bars are 100 μm.