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THE NON-SELECTIVE INNERVATION OF MUSCLE FIBRES AND MIXED COMPOSITION OF MOTOR UNITS IN A MUSCLE OF NEONATAL RAT

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SUMMARY

1. Motor-unit size was measured by tension recording in neonatal (3–5 day) rat skeletal muscle (fourth deep lumbrical muscle). Each unit was then depleted of glycogen and its fibres studied in mid-belly frozen sections, by staining for glycogen (periodic acid–Schiff reagent and antibody labelling for slow myosin. The contralateral muscle acted as control, and further controls for the method are described.

2. All the motor units contained both slow-myosin-containing (S; antibody-positive) and slow-myosin-free (F; antibody-negative) fibres.

3. The proportion of each unit that was made up of S fibres was compared with the whole muscle. Of the twelve units studied seven were not selectively innervated, four may have been selectively innervated in favour of F fibres, and one was selectively innervated in favour of S fibres. The last unit was much smaller than the others.

4. Fibre cross-sectional areas were measured in units and in the whole muscles. Mean cross-sectional areas for individual F fibres in all the motor units were smaller than in the corresponding whole muscles (ratio 0·71), implying that small fibres have higher levels of polyneuronal innervation than larger ones (each small fibre occurring in more overlapping units than each larger fibre). There was no such difference in S fibres (ratio 0·96).

5. Motor-unit sizes (as a percentage of whole muscle) were smaller when obtained from summed fibre cross-sectional areas than from fibre counts (this follows from 4, above). Comparisons with unit sizes from tension recording are discussed.

6. Controls show that there is little, if any, non-specific fatigue of muscle fibres that are not part of the unit subjected to glycogen depletion.

7. Evidence is given that muscle fibre conduction block occurs during the depletion regime, leading to less glycogen depletion towards the ends of the muscle fibres than in the end-plate zone.

INTRODUCTION

In adult mammalian skeletal muscle there is abundant evidence, from cross-innervation and stimulation studies, that the physiological and biochemical characteristics of the muscle fibres are determined by the motoneurones innervating them (Jolesz & Sreter, 1981). In adult muscle each muscle fibre receives its motor supply from one motoneurone only. On the other hand, in rat neonatal muscle each fibre is polyneuronally innervated (Redfern, 1970). Subsequent synapse elimination leads to the adult state of mononeuronal innervation. If during early development muscle fibre differentiation is dependent upon motoneuronal influences then one might expect that muscle fibres differentiate late in, or after, the period of developmental synapse elimination. In fact this is not so. At least so far as the presence or absence of slow myosin is concerned, muscle fibres are differentiated before birth in rat limb muscles (Rowlerson, 1980; and see Lyons, Haselgrove, Kelly & Rubinstein, 1983); although in other respects myosin polymorphs have not yet reached the adult condition (Whalen, 1985). One might therefore expect, if fibre differentiation were dependent upon motoneuronal influences, that even during polyneuronal innervation single motor units would consist of one type of muscle fibre only; that is, segregation of fibres into homogeneous motor units occurs by some earlier process.

In this paper we present evidence that no such earlier segregation takes place. Instead we found that all the motor units we studied in neonatal muscle were mixed with respect to fibre types, and most appeared to contain muscle fibres randomly selected from within the whole muscle. From this we conclude that at this stage of development the motor axons are not determining the muscle-fibre properties. Furthermore, the production of homogeneous motor units occurs by some process operating during a period roughly corresponding to that of synapse elimination.

In the experiments single motor units were depleted of their glycogen by prolonged stimulation (Edstrom & Kugelberg, 1968) to allow subsequent identification of the muscle fibres in the unit in frozen sections. Neighbouring sections were treated with an antibody that binds specifically to slow myosin. Examination of the sections showed that all the motor units studied contained both antibody-positive (slow-myosin-containing; S) and antibody-negative (slow-myosin-free; F) muscle fibres. Preliminary accounts of some of this work have been given earlier (Jones, Ridge & Rowlerson, 1985; Rowlerson, Jones & Ridge, 1986).

METHODS

The methods used for preparation of and recording from the muscle, and for isolation of single motor units, are described in the preceding paper (Jones & Ridge, 1987). Glycogen depletion was achieved by continuous stimulation of the nerve at 5 impulses/s, in glucose-free Ringer solution containing 5 mM-Ca^{2+} at 30 °C, until twitch tension was reduced to zero. Stimulation was then stopped for 5 min, resumed until tension again reduced to zero, and this cycle repeated, ideally until recovery after 5 min was small and short-lived. Usually tetani of 1–2 s at 40 impulses/s were also given at this stage. In fact, in many cases the axon blocked before completion of the sequence, in which case the muscle was frozen if it had undergone marked fatigue at the time of the block. Finally the muscle and its contralateral, unstimulated control (which had been immersed in the muscle bath throughout the experiment) were removed from the bath and pinned out in a Sylgard dish under glucose-free Ringer solution. Silk ties were then made at both myotendinous junctions and

the muscles were frozen in isopentane pre-cooled in liquid nitrogen. Frozen sections were cut in a cryostat at 15 μm thickness. Sections were taken off individually and mounted on numbered cover-slips or slides. Alternate sections were stained for glycogen with periodic acid–Schiff reagent (PAS) and the other sections were treated with anti-slow-myosin antibody.

The anti-slow-myosin antibody used is polyclonal, and was raised against myosin obtained from cat soleus muscle, which is exclusively of slow-twitch (type I) muscle fibres in the adult (Guth & Samaha, 1969). Methods of myosin extraction, immunization and antibody specificity testing are described by Rowlerson, Pope, Murray, Whalen & Weeds (1981). The GEDELISA test described in Rowlerson *et al.* (1981) was also used to examine the specificity of the anti-slow-myosin antibody for all the main myofibrillar proteins, and showed that although there is a small reaction against the light chains of slow myosin, most of the activity is against the slow myosin heavy chains. There is also a small reactivity against another fairly high molecular weight protein, possibly C-protein, but no significant reaction with other myofibrillar proteins including fast myosin heavy and light chains. Immunohistochemical staining of frozen sections of a variety of adult and developing skeletal muscles confirmed that this antibody is specific for fibres containing slow myosin (types I and IIC, Brooke & Kaiser, 1970) and does not react significantly with any of the adult fast myosins or with neonatal or embryonic myosins.

Immunocytochemical staining (indirect immunoperoxidase method) was carried out as follows. (i) Muscle sections were incubated overnight in a drop of specific (i.e. anti-slow-myosin) antibody diluted in phosphate-buffered saline (PBS; 0·145 M-NaCl in 0·01 M-phosphate buffer, pH 7·4) containing 1 % (w/v) bovine serum albumin (BSA). (ii) Unbound antibody was removed by 3 × 5 min washes in PBS containing 0·025 % Tween 20. (iii) The sections were then incubated for 2–3 h in peroxidase-labelled anti-IgG (goat anti-rabbit, Miles; affinity-purified) also diluted in PBS–BSA. (iv) Unbound peroxidase-labelled second antibody was removed by 3 × 5 min washes in PBS–Tween 20. (v) Bound peroxidase-labelled anti-IgG was visualized by incubating the sections in PBS containing diaminobenzidine (*ca.* 50 μg/ml) and 0·03 % H_2O_2 (all incubations at room temperature). The stained sections were then dehydrated, cleared and mounted in Piccolyte. Control experiments established that there was no endogenous peroxidase activity visible in the muscle sections and no staining attributable to the second antibody alone.

Our results with the antibody differentiate only between slow-myosin-containing (S) and slow-myosin-free (F) muscle fibres. They do not distinguish between subtypes of fast fibres, or between type I slow fibres and transitional forms containing slow myosin (IIC). Further histochemistry would be required to investigate these aspects. However, such considerations do not affect the conclusions drawn in this paper.

A camera lucida drawing of an antibody-stained section from the mid-belly was then made (e.g. Fig. 1), and the S fibres marked (there is sufficient background staining for all the fibres to be clearly visible). A neighbouring section stained for glycogen was then examined and each fibre profile matched to a fibre in the drawing. To do this it was essential to work with exactly neighbouring sections. Any further separation is associated with sufficient relative movement of fibre positions to make it impossible to relate all the fibre profiles in the two sections (fibre-packing density is much lower in neonatal than in adult muscle). The fibres with no glycogen could then be identified on the drawing. The control muscle was treated identically. In all controls 5–10 % of the fibres (or cell profiles) did not stain for glycogen. These profiles were scattered throughout the muscle and tended to be small. Their distribution is described in the Results.

One muscle we took as a control immediately after killing the rat. Of the 729 fibres in this muscle 10·2 % were glycogen-free. Of the slow fibres (89) 2·2 % were glycogen-free, and of the F fibres (640) 11·3 % were glycogen-free. We conclude from this that our individual control muscles had not been affected by the time they spent in the muscle bath; rather, their glycogen-free fibres had been in this condition throughout.

As well as fibre counts for whole muscles and motor units we measured fibre cross-sectional areas with a digitizing pen. In this paper we describe the mean and summed total cross-sectional areas for S and F fibres in these.

Data for whole muscles and motor units are given uncorrected and corrected for the values obtained from controls. Fibre counts were corrected by subtracting the proportion of S and F fibres that was found without glycogen in the control muscle (or, for some experimental muscles, mean values from a number of controls; see Results). Mean fibre areas were not corrected, but summed fibre cross-sectional areas of motor units and whole muscles were corrected by the appropriate proportional area derived from control muscle area measurements.

In all PAS-stained muscles there was a range of staining intensity in different fibres. Our criterion for depletion was that there was no discernible pink stain in the fibre profile. The strictness of this criterion could have led to undercounting in some units, especially in any units where depletion had not run its full course.

RESULTS

Glycogen depletion

We adopted the stimulus regime for depletion reported in the Methods because it produced the most effective depletion of whole muscles when applied to the muscle nerve, all but a few fibres being depleted in mid-belly sections from the muscle. An example of such a section is shown in Pl. 1*A*. In this case in a total of about 660 fibres 19 failed to be depleted (2·9%). Using this regime we successfully depleted twelve motor units that also had satisfactory controls (see below), and the data from these are given.

The most common cause of failure in these experiments was a sudden cessation of the unit response (presumably because of axonal conduction block). If this occurred when the muscle already appeared to be approaching exhaustion it and its control were frozen and processed. In some cases histology revealed that glycogen depletion had been achieved, and eight out of our twelve good units were from this class. In other cases there was no appearance of glycogen depletion. Thus it appears that effective depletion develops rapidly and late in the depletion regime. In agreement with this are the results of a small set of controls where we stimulated the control-muscle nerve every time we stimulated the experimental unit except during the depletion regime. These control muscles were indistinguishable from unstimulated controls.

Contralateral controls

With each muscle subjected to glycogen depletion the contralateral muscle was mounted in the bath as a control. In the majority of cases the control was not stimulated (but see previous section). It was frozen and processed in the same way as the experimental muscle. A control was considered satisfactory if about 10% (or less) of cell profiles in mid-belly sections showed no staining for glycogen. In a few cases there was more general lack of PAS staining and we rejected both experimental and control muscles.

On examining electron micrographs of muscles at this age (Jones, Ridge & Rowlerson, 1987) one sees a small proportion of cell profiles that do not contain any myofilaments. Some of these may be immature myotubes cut through a nucleus. Others may be of cell types other than myotubes. These may be glycogen-free and would have been counted in these controls, since they could not be identified in the light microscope.

All the twelve units analysed here had satisfactory controls. In the controls of eight of the twelve units detailed counts of glycogen-free fibres were made. In these cases muscle-fibre counts and summed cross-sectional area measurements were corrected in the experimental muscle on the assumption that the proportion of glycogen-free fibres in the control was the same as the proportion before glycogen depletion in the experimental muscle. This interpretation is supported by measurements made on one

control muscle that was frozen immediately after removal from the animal (see Pl. 1*B*). In the remaining four control muscles it was not possible to obtain detailed counts and fibre typing from mid-belly sections because we did not obtain exactly adjacent sections of PAS and antibody staining of sufficient quality. In these cases corrections have been applied deriving from mean values from the eight countable controls. As controls do not vary widely from one another we consider this acceptable. Both corrected and uncorrected data are given in the Tables.

Antibody labelling

In all sections labelled with the slow-myosin-specific antibody, labelled fibres (S) occurred scattered fairly evenly throughout the section. In Pl. 2 a composite section is shown of a neonatal fourth deep lumbrical and part of an adult extensor digitorum longus (e.d.l.) muscle. The adult e.d.l. shows the characteristic small proportion of S fibres that has been observed by others (e.g. Rubinstein & Kelly, 1981) in this preponderantly fast muscle. In the neonatal lumbrical muscle also the antibody is bound to a minority of fibres (in a series of muscles between 70 and 103 fibres; Jones *et al.* 1987).

Glycogen-depleted motor units

Adult units. We depleted two adult motor units and observed the well-known scattered distribution of muscle fibres, which in this small muscle is throughout the whole mid-belly section. Both units consisted exclusively of F muscle fibres. These observations validated our methods, which produced quite different results for neonatal motor units.

Neonatal units. Examples are shown in Pl. 3 of neighbouring mid-belly sections of a muscle stained with PAS (*A*) and labelled with antibody (*B*) in which a unit had been depleted of glycogen. It will be seen in *A* that there is an area in the section where most of the fibres are depleted, but that depleted fibres are also scattered throughout the section beyond this area. Fig. 1 is of two camera lucida drawings, each made from sections similar to those shown in Pl. 3, but from two different units. Fig. 1*A* shows a unit where grouping of depleted fibres is particularly marked, and Fig. 1*B* shows a unit where there is rather little grouping.

Plate 3*B* shows that S fibres are distributed throughout the cross-section of the muscle, including the area of most depleted fibres (Pl. 3*A*). From this it appears that the unit contains both F and S muscle fibres. That this is so is shown in Pl. 4, where a small group of fibres from another unit is shown at higher magnification. Examples of both F and S fibres that have been depleted are present. The drawings in Fig. 1 show examples of the same thing in two other units. In all twelve motor units analysed here, and in others not analysed in detail, the units contained both F and S fibres. In the age range 3–5 days we have not seen any units composed solely of F or S fibres.

Non-specific depletion

In some units (e.g. those in Fig. 1*A*) most neighbouring fibres in one part of the muscle were depleted. We wondered if depletion of fibres other than those in the motor unit could have been occurring, especially as in two units the histologically

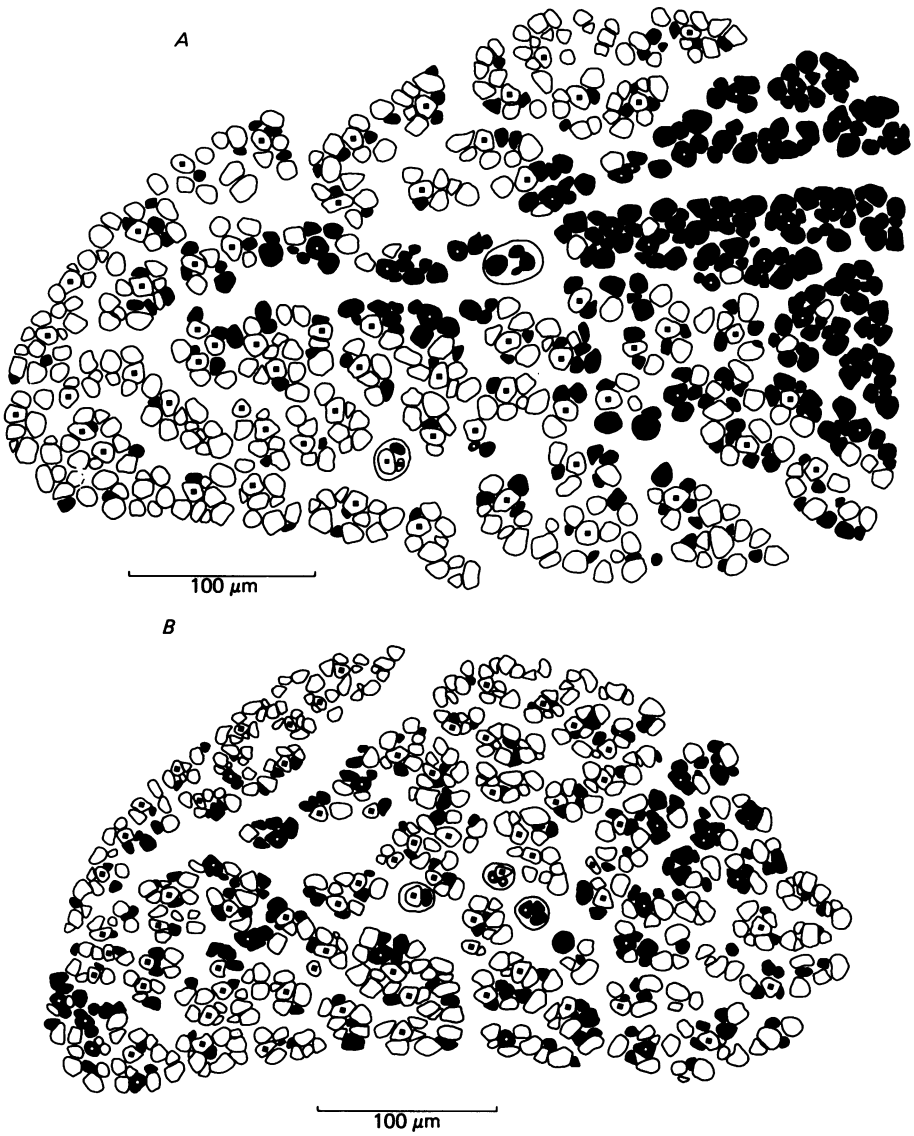

Fig. 1. Camera lucida drawings of mid-belly sections from two muscles (4 days). A single unit in each muscle was depleted of glycogen. The sections were stained with PAS and neighbouring sections labelled with anti-slow-myosin antibody. Depleted fibres are drawn black. Squares (black on white or the reverse) mark fibres staining positively for the antibody. Where identifiable, spindle capsules enclosing intrafusal fibres are drawn in. The unit in *A* (unit 9) shows marked grouping of depleted fibres, though scattered fibres outside the group are also depleted. *B* (unit 11) shows less grouping.

derived motor-unit size by larger than that derived from tension measurements. This might be brought about by electrical coupling, or some non-specific metabolic effect due, perhaps, to developing hypoxia in the region of active fibres that were members of the motor unit.

We cannot resolve completely how much effect electrical coupling may have in

these muscles. Schmalbruch (1982) found gap junctions in electron micrographs from fourth deep lumbrical muscles of new-born rats. At this time muscle fibres and myotubes were in clusters of one to four, each cluster being surrounded by a basal lamina. By 1 week after birth gap junctions were absent and muscle fibres were not

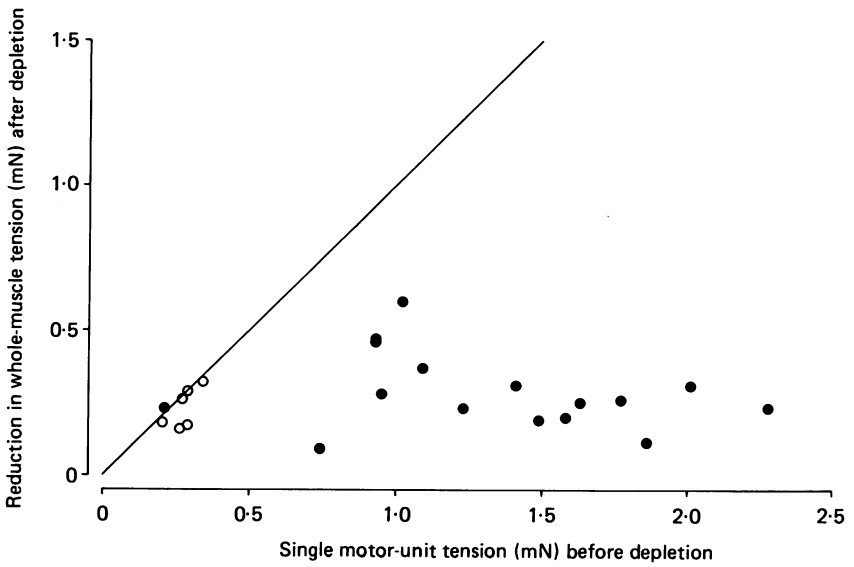

Fig. 2. Graph of reduction in whole-muscle tension on fatiguing a single motor unit plotted against initial motor unit tension. The line represents simple subtraction of motor-unit tension after fatique. ○, from twitch measurements, ●, from tetani.

in clusters. Extensive electrical coupling has been observed in intercostal muscles in embryonic rats, and weak coupling persisted between some pairs of fibres 'in the first few days after birth' (Dennis, Ziskind-Conhaim & Harris, 1981). How effective this coupling is post-natally is unknown. Presumably it is associated with gap junctions. The only data we have for the 3–5 day age range are from intracellular recordings (Jones & Ridge, 1987) which gave a level of about 12 % of fibres electrically coupled to muscle fibres in a single motor unit. Electron micrographs from fourth deep lumbrical muscles of a new-born and a 4 day animal show that the clusters in the new-born (Betz *et al.* 1979) have largely disappeared in the 4 day muscle (Jones *et al.* 1987), which implies that levels of electrical coupling are probably low by 4 days. Electrical coupling at 12 % could not account for the presence of S fibres in at least six of the twelve units, even if all the coupling was from F to S fibres (see Table 1). It is most unlikely that this is the commonest direction of coupling, since S fibres are larger in cross-section than many F fibres (see Table 3), which would present problems of electrical matching. Further, there are many examples of non-depleted S fibres with depleted F fibres as near neighbours (for example see Fig. 1*A* and *B*). Therefore we conclude that electrical coupling is most unlikely to account for our main finding: that all the motor units contained both S and F fibres. However, the more detailed quantitative analyses may contain an error of imprecisely known magnitude due to electrical coupling of muscle fibres.

In order to investigate the possibility of non-specific depletion by some process such

as spreading hypoxia during the depletion regime, we carried out the following control experiment on a separate group of muscles from rats of the same age range. The twitch or tetanic tension development of the whole muscle (stimulating the nerve) and of the unit was recorded before and after repetitively stimulating the unit to exhaustion.

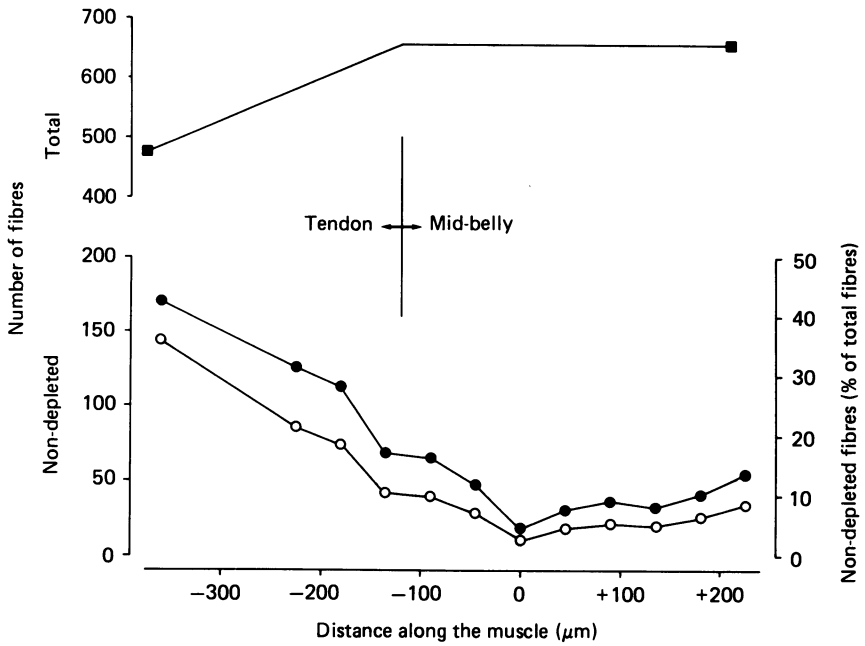

Fig. 3. The number of non-depleted fibres in sections along the length of a lumbrical muscle (4 day). The whole-muscle nerve was stimulated under the regime for glycogen depletion until the muscle was fully fatigued (see Methods). It was then frozen and sections cut and stained for glycogen. The number of non-depleted fibres in selected sections was counted (●). The same data are presented as approximate percentages of fibres in the section (○). The percentages were calculated from the line at the top representing estimates of the total number of fibres in each section. It is based on total counts in two sections (■) and is drawn with the following assumptions: that the number of fibres in the mid-belly region of the muscle does not change with length; and that the reduction in fibre numbers is linear with length once the tendon has begun to appear at one side of the section. The point at which the tendon appears is shown by the vertical line. The point at zero distance corresponds with the section shown in Pl. 1 *A*.

After stimulation the unit developed no tension, so one would expect the whole-muscle tension to have declined by the initial tension of the motor unit, if no non-specific exhaustion (which, presumably, would be associated with non-specific glycogen depletion) had taken place. However, if fibres other than those in the unit had been affected the decline in whole-muscle tension should have been more than by the initial tension developed by the motor unit.

The results are shown in Fig. 2, and for tetani were unexpected, since the decline in whole-muscle tension was less than expected in the absence of non-specific effects, for all but one motor unit (this unit is the smallest, and is unit 12 of Table 1). For

twitches the observed decline in whole-muscle tension was in all cases close to that expected. We conclude from these data that there is little or no non-specific exhaustion of fibres during the glycogen depletion regime, and it would seem likely therefore that there is little or no non-specific glycogen depletion. However, we are

TABLE 1. Number of S and F muscle fibres in motor units and whole muscle

	Fibre counts					
	S fibres		F fibres		% S fibres	
Unit	Unit	w.m.	Unit	w.m.	Unit	w.m.
1	48 (45)	87	286 (233)	604	16·2	12·6
2*	20 (18)	93	235 (181)	612	9·0	13·2
3*	18 (16)	85	140 (93)	524	14·7	14·0
4	40 (38)	70	294 (240)	524	13·7	11·8
5	15 (13)	89	205 (140)	635	8·5	12·3
6	47 (46)	88	256 (205)	676	18·3	11·5
7*	42 (40)	88	278 (225)	592	15·1	12·9
8*	20 (18)	80	183 (123)	656	12·8	10·9
9	23 (22)	83	338 (270)	676	7·5	10·9
10	42 (39)	103	223 (168)	607	18·8	14·5
11	18 (17)	83	219 (175)	641	8·9	11·5
12	20 (19)	96	93 (41)	658	31·7	12·7

Whole muscle, w.m. Values corrected for control counts are given in parentheses.
*Experiments with good-looking but uncountable controls; corrections have been made using mean control values (see text).

left with the finding that for tetani the decline in whole-muscle tension after unit exhaustion is less than expected.

One possible explanation for this is that during repetitive stimulation of the unit the safety margin for conduction in many of the muscle fibres is reduced, so that conduction block develops near, but not immediately at, the end-plate. This would spare the ends of these fibres from glycogen depletion, although their mid-belly portions proceed to depletion. On stimulating the whole-muscle nerve the much larger end-plate potential arising from the additional synaptic inputs (see Figs. 5 and 6 of Jones & Ridge, 1987) leads to activation of some or all of these fibres beyond the blocked portion of membrane. This would lead to partial restoration of tension development in these fibres and consequently less reduction in whole muscle tension than predicted from the loss of the full unit tension. If this explanation is correct we would expect to find proportionally fewer fibres depleted in a unit towards the ends of the muscle than in the mid-belly region. There is evidence that Na^+ channel density reduces away from the end-plate and towards the tendon in adult rat and snake muscle fibres (Caldwell, Campbell & Beam, 1986), which could contribute to conduction block.

Variations in depletion along the muscle length

On examining serial sections from the units it was a common observation that the apparent proportion of depleted to non-depleted fibres fell as the distance of the section from the mid-belly increased. This was also obvious in a muscle in which the

complete muscle nerve had been subjected to the stimulus regime used for glycogen depletion, and we counted the number of depleted and undepleted fibres in twelve sections at various positions along the length of this muscle. The mid-belly section

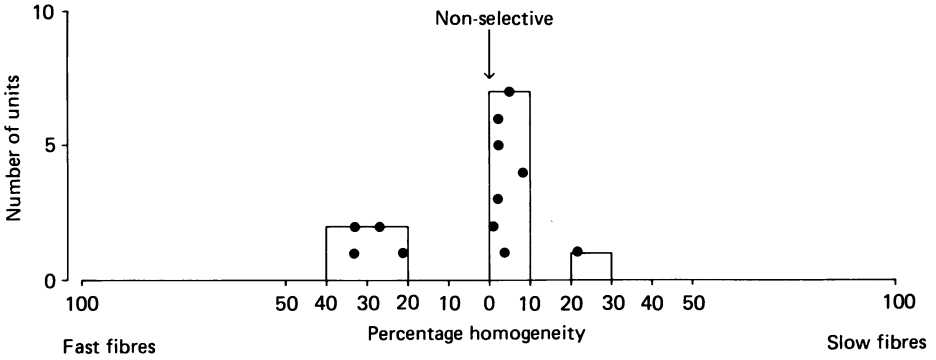

Fig. 4. Distribution of selective innervation (as percentage homogeneity) of motor units. The percentage homogeneity of the y axis is derived in the following way: the percentage of fibres that are S in the whole muscle is subtracted from the percentage of fibres in the unit that are S, to give a difference in percentages. This is positive if the proportion of S fibres is higher in the unit (right of zero), negative if the proportion of S fibres is lower in the unit (left of zero), and zero if the two proportions are the same. This difference is then multiplied by one of two scaling factors, depending on its sign. This is because a pure S unit would give a percentage difference of +87·6 % (100–12·4 %) and a pure F unit would give a percentage difference of −12·4 % (0−12·4 %), which is the average percentage of S fibres in the whole muscles. Thus the scaling factors are 8·065 (100/12·4) to the left of zero and 1·142 (100/87·6) to the right of zero. The appearance of four units with selective innervation of F muscle fibrers could be artifactual, since these units may not have been completely depleted of their glycogen (see text). The mean value of % S in the whole muscle for all the muscles was used. Using values for individual muscles does not materially alter the distribution. Filled circles show individual values.

is shown in Pl. 1 *A*. The fibre counts are given in Fig. 3; details of how the counts and estimates were made are given in the Figure legend.

Two points emerge from these data. First, the proportion of depleted fibres declines towards one end of the muscle, and this is particularly clear beyond the point at which the muscle tendon appears in the sections, and the total number of fibre profiles begins to reduce. Secondly, for about 210 μm the total number of non-depleted fibres varies between about 20 and 40, so that provided sections are cut within this mid-belly length the underestimate in motor units will be between 3–6 %.

Counts of the numbers of S and F muscle fibres

In whole muscles. The numbers of S and F muscle fibres counted in each whole muscle containing a depleted motor unit are given in Table 1. In this age range the total number of fibres varies between 594 and 764 (mean ± S.D. = 704·2 ± 54·7). Of these 70–103 are S fibres (mean 87·1 ± 8·3). Thus at 3–5 days 10·9–14·5 % of the fibres are S type (mean 12·4 ± 1·1; column 7 of Table 1).

In motor units. The numbers of muscle fibres in the units varies widely (from 60

to 292: corrected counts), as one would expect from the wide range in motor-unit sizes assessed from measurements of tension development (Betz, Caldwell & Ribchester, 1979; Jones & Ridge, 1987). The proportion of these that are S fibres also varies more widely in the motor units (7·5–31·7 %) than in the whole muscles. The values for each unit are given in column 6 of Table 1 (percentages for units are calculated from corrected counts).

The differences between the percentages of S fibres in the unit and in the muscle give an indication of how much selectivity of innervation has taken place in each unit by this age. However, because of the large difference in the numbers of S and F fibres in the whole muscle, homogeneous S and F units would give very different percentage differences between the whole muscle and the unit (there are, on average, 12·4 % S fibres in the whole muscle; therefore the percentage difference would be +87·6 % for a pure S unit and −12·4 % for a pure F unit). To take account of this the data are presented in a different way in Fig. 4. In this histogram the y axis is scaled differently and appropriately on either side of the mid-point zero. Zero represents no selective innervation (see legend to Figure for further details). One interpretation of Fig. 4 is as follows: seven units are as yet non-selectively innervated (< 10 % homogeneous), four are selectively innervated (> 20 % homogeneous) and on their way to becoming F motor units, and one unit is selectively innervated in favour of S fibres, and is on its way to becoming a 'slow' motor unit.

However, one should be cautious about this interpretation in relation to the four units apparently innervated in favour of F fibres. All four (units 2, 3, 5 and 8) had greater motor-unit sizes as measured by tetanic tension than they had as measured by summed fibre cross-sectional areas (Table 2 and Fig. 5). It is therefore possible that they were not fully depleted of glycogen. If at this age S fibres become glycogen-depleted less readily than F fibres possibly the number of S fibres in these units is underestimated and they were really non-selectively innervated.

It is interesting to speculate a little further about the nature of the one unit selectively innervated in favour of S muscle fibres. In the adult rat about 9 % of fibres in the fourth deep lumbrical muscle are S type (there are more muscle fibres in adult muscles but those generated post-natally are all F: Jones *et al.* 1987), and on average there are eleven motor units (Betz *et al.* 1979), so there could be only one slow-twitch motor unit of average size. To find one future slow-twitch unit in twelve would be entirely consistent with this. Of course there may be two or more smaller slow-twitch units in adults, and it is possible that a proportion of the as yet non-selective units is destined to become the slow-twitch type. The unit selectively innervated in favour of S fibres (unit 12 of Tables 1–3) stands apart from the other units in that it is by far the smallest (see Table 2); also its physiological properties are somewhat different (along with some other small units), as described in the preceding paper (Jones & Ridge, 1987) in which it is separately identified.

Motor-unit sizes

The size of each motor unit, expressed as a percentage of the whole muscle, and calculated in a number of ways, is given in Table 2.

Unit size was obtained from the histological sections as fibre counts (uncorrected and corrected) and as summed fibre cross-sectional areas (uncorrected and corrected).

In all units unit size was somewhat smaller for summed fibre cross-sectional areas than for fibre counts. This implies that small fibres are included in more motor units (and therefore have higher levels of polyneuronal innervation) than large ones (mean fibre areas are described separately below).

TABLE 2. Motor-unit size, calculated in different ways

Unit	No of fibres (% of w.m.)		Fibre area (% of w.m.)		Unit size (% of w.m.) 25 °C, 2 mM-Ca^{2+} tet.t	25 °C, 2 mM-Ca^{2+} tw.t.	30 °C, 5 mM-Ca^{2+} tet.t.	30 °C, 5 mM-Ca^{2+} tw.t.
1	48·3	(40·2)	39·3	(35·8)	30·2	12·9		
2*	36·2	(28·2)	21·7	(19·1)	31·7			
3*	25·9	(17·9)	18·7	(16·1)	37·3	17·7		
4	56·2	(46·8)	44·9	(42·3)	24·4	12·4		
5	30·4	(21·1)	21·1	(18·5)	35·3	17·6	37·0	19·4
6	39·7	(32·9)	33·1	(30·9)	15·3	9·5	14·4	13·1
7*	47·1	(39·0)	36·4	(33·8)	31·9	19·5	27·0	21·5
8*	27·6	(19·2)	19·7	(17·0)	38·3	16·7	32·7	21·5
9	47·6	(38·5)	37·6	(34·3)	27·8	20·5	26·0	21·4
10	37·3	(29·2)	29·4	(26·4)	32·9	22·9	29·3	21·3
11	32·7	(26·5)	22·5	(20·6)	21·7	12·9	17·9	15·3
12	15·0	(8·0)	7·5	(5·1)	3·9	3·1	3·5	3·6

Abbreviations: w.m., whole muscle; tet.t., tetanic tension; tw.t., twitch tension. Values after correcting for control values are given in parentheses.

*Experiments with good-looking but uncountable controls; corrections have been made using mean control values.

The counts and cross-sectional areas were corrected by subtraction of values obtained from controls, as described above. These controls showed that 6·2–9·4 % of apparently depleted fibres could have been glycogen-free before depletion. When the two fibre types were examined separately it was found that 6·9–10·4 % of F fibres were glycogen-free in controls, but only 1·1–4·0 % of S fibres (1–4 fibres per muscle) were glycogen-free in controls. It is quite possible that all the S fibres without glycogen were in fact intrafusal fibres (occasionally these could be identified by the presence of the spindle capsule, in which case they were not counted), and in all but two of the control muscles their cross-sectional areas were much less than the glycogen-containing S fibres. The glycogen-free F fibres were also much smaller than glycogen-containing F fibres (ranges were 14·0–22·9 μm^2 and 41·9–60·1 μm^2 respectively), which makes it likely that they were immature. Some may not have been muscle fibres, since all cell profiles were counted. Motor-unit size was also assessed from both twitch and tetanic tension measurements at 25 °C in 2 mM-Ca^{2+}, and, for eight units, at 30 °C in 5 mM-Ca^{2+} (the conditions of glycogen depletion). Motor-unit sizes for twitches are smaller than those for tetani. This finding has been explained in terms of low junctional efficacy in a proportion of the neuromuscular junctions of single motor units (Jones & Ridge, 1987). The increase in motor-unit sizes that is found for twitches in 5 mM-Ca^{2+} at 30 °C is less than that expected on raising $[Ca^{2+}]_o$ at 25 °C (Jones & Ridge, 1987) for most units, which implies that raising temperature alone depresses unit size found from twitches. The effect of raising temperature on unit sizes from

tetani was to reduce motor-unit size a little in seven of the eight units. We do not know which measure of unit size from tension measurements is the most appropriate for comparisons with the histological data, so we have chosen the measure that most

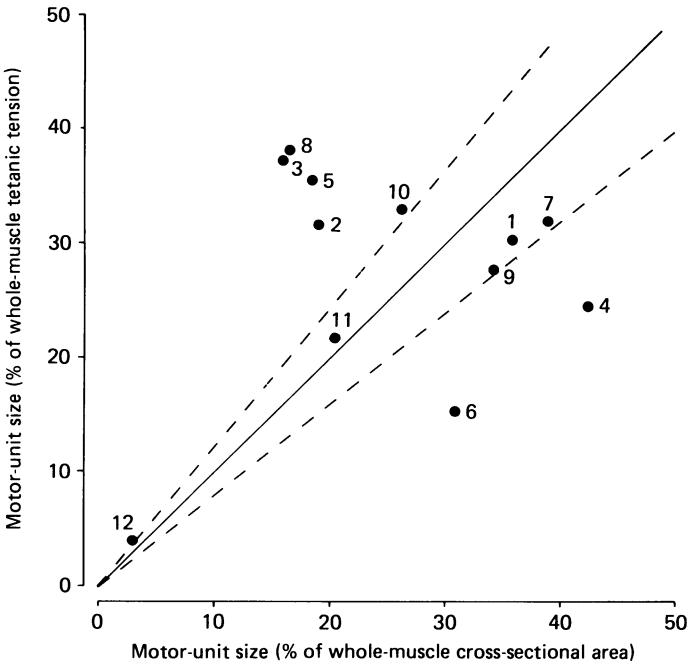

Fig. 5. Comparison of single motor-unit sizes obtained by physiological and histological measurement. For each unit the size derived from tetanic tension development, expressed as a percentage of whole-muscle tetanic tension, is plotted against the summed cross-sectional areas of all the muscle fibres in the unit as seen in a mid-belly section of the muscle. The numbers refer to the individual units in Tables 1–3, and the dashed lines indicate $\pm 20\%$ divergence from the line of equality.

often gave the largest unit size i.e. tetani at 25 °C. This is compared with unit size from summed cross-sectional area measurements, since these would be expected to be most closely related to tension.

Such a comparison is made in Fig. 5. Six units fall within or close to $\pm$ 20% of the line of equality. In four units the unit size as measured by tetani is considerably greater than that measured by summed fibre cross-sectional areas. The simplest explanation for this is that the units were incompletely depleted of their glycogen. This could not account for the fact that they contained both F and S fibres, of course, but it might affect artificially the ratio of F to S fibre numbers. In fact these four units are those that in Fig. 4 are biased in favour of F fibres, and consequently this should be interpreted with caution, as previously noted. Two units contain a much larger depleted cross-sectional area than one would predict from tetanic-tension measurements (units 4 and 6). We do not know why this should be, and the possibility that some non-selective depletion did take place cannot be categorically discounted, which would render useless the data from these units.

It should be pointed out that various unquantifiable factors enter into the relationship between unit tensions and cross-sectional areas and there is no reason to expect a linear or near-linear relationship between tension and fibre cross-sectional area over the whole range of fibre sizes. Immature fibres may not yet extend to the

TABLE 3. Mean fibre areas ±S.D. (n) in 3–5-day-old muscles

		Fibre area (μm^2)					
Unit		Slow fibres		Fast fibres		Total fibres	
1	Whole muscle	99·7±21·2	(87)	66·0±32·4	(604)	70·2±32·9	(691)
	Unit	97·3±27·7	(48)	50·3±26·6	(286)	57·0±31·0	(334)
2	Whole muscle	112·5±36·6	(93)	59·8±37·1	(612)	66·8±40·9	(705)
	Unit	90·3±53·3	(20)	35·7±23·9	(235)	39·9±30·4	(255)
3	Whole muscle	87·5±17·3	(85)	52·1±30·5	(524)	57·1±31·3	(609)
	Unit	81·1±23·0	(18)	35·9±26·9	(140)	41·1±29·4	(158)
4	Whole muscle	75·5±22·9	(70)	45·8±26·5	(524)	49·3±27·6	(594)
	Unit	69·3±26·6	(40)	35·3±23·7	(294)	39·3±26·2	(334)
5	Whole muscle	85·2±23·4	(89)	48·5±28·5	(635)	53·0±30·3	(724)
	Unit	77·4±30·6	(15)	33·8±23·0	(205)	36·8±25·4	(220)
6	Whole muscle	57·6±13·7	(88)	35·4±19·5	(676)	38·0±20·1	(764)
	Unit	56·1±14·0	(47)	27·2±14·7	(256)	31·7±17·7	(303)
7	Whole muscle	61·3±11·6	(88)	35·5±21·9	(592)	38·9±22·5	(680)
	Unit	63·9±14·3	(42)	24·9±17·9	(278)	30·2±21·3	(320)
8	Whole muscle	95·9±19·0	(80)	60·3±29·0	(656)	64·1±30·0	(736)
	Unit	100·8±16·1	(20)	39·7±22·6	(183)	45·7±27·7	(203)
9	Whole muscle	97·1±17·8	(83)	58·6±30·1	(676)	62·8±31·2	(759)
	Unit	90·3±29·8	(23)	46·9±28·4	(338)	49·7±30·0	(361)
10	Whole muscle	60·0±11·0	(103)	35·4±20·6	(607)	39·0±21·2	(710)
	Unit	60·7±14·4	(42)	25·0±16·4	(223)	30·7±20·4	(265)
11	Whole muscle	66·5±14·1	(83)	43·9±25·4	(641)	46·5±25·3	(724)
	Unit	71·3±14·5	(18)	28·7±18·4	(219)	31·9±20·8	(237)
12	Whole muscle	54·1±13·2	(96)	36·6±22·6	(658)	38·8±22·3	(754)
	Unit	47·8±19·1	(20)	13·2±9·6	(93)	19·3±17·2	(113)

tendons at both ends (Ontell, 1977), in which case they will contribute little or no tension. Specific tensions (tension per unit cross-sectional area) may depend on maturity, and are unlikely to be the same for S and F fibres. However, units 4 and 6 do not stand out either in their mean fibre cross-sectional areas or in the proportions of S and F fibres.

Mean fibre cross-sectional areas

Mean fibre cross-sectional areas (uncorrected) for all the 3–5 day units and the whole muscles that contained them are given in Table 3. They lead to the following conclusions about fibre areas.

(a) In every muscle S fibres are on average larger than F fibres.

(b) In every unit F fibres are on average smaller than they are in the corresponding whole muscle. Presumably this is because each smaller, less mature F fibre receives, at this age, more motoneuronal terminals than the average F fibre in the whole muscle.

(c) In contrast, S fibres are about the same size in units as in whole muscles.

(d) In unit 12, which is biased towards S fibres, the difference in size between F fibres in the unit and in the whole muscle is more marked than in the other units. Perhaps in this unit these immature F fibres are emitting a strong signal causing *innervation* by the motoneurone which, in these fibres, is successfully opposing the process of *withdrawal* by the motoneurone from F fibres.

DISCUSSION

The experiments described show that, at an age when the level of polyneuronal innervation is high, motor units in a future fast muscle in neonatal rat contain both F and S muscle fibres. Thompson, Sutton & Riley (1984) found the same thing in rat soleus muscle, a predominantly slow muscle in the adult. In the adult muscle motor units are either fast or slow, but not mixed. Therefore there must be some process whereby the neonatal condition gives rise to the adult. This could be either by fibre conversion mediated by motoneuronal influences (as is well known in adults: e.g. Jolesz & Sreter, 1981) or it could be by selective withdrawal of mismatched connexions during synapse elimination. In the paper following (Jones *et al.* 1987) we give evidence that suggests that fibres are not converted during this period, and so we favour the second alternative; this is discussed further by Jones *et al.* (1987).

We have found that in many of our units there is no selective innervation on the basis of fibre types, the units containing S and F fibres in similar ratio to that applying to the whole muscle. This contrasts with the findings of Thompson *et al.* (1984) in soleus muscle who found all the units heavily biased in favour of one or other fibre type. However, the younger of the two groups of animals studied by Thompson *et al.* was 8 days old, and by this age one would expect that considerable synapse elimination would have already taken place, with the possible corollary that many mismatched contacts would have been withdrawn. At 8 days virtually all the fibres in soleus muscle are still polyneuronally innervated (Fig. 4 of Brown, Jansen & Van Essen, 1976), but possibly by then the average number of inputs could be close to two per fibre (by 10 days some are not polyneuronally innervated). We calculate that on average there were 3·1–3·5 synaptic inputs onto the fibres in our muscles. Also we should note that in the adult rat the fourth deep lumbrical is a fast muscle which contains a small proportion of slow fibres (which is the commonest combination in limb muscles), whereas the adult rat soleus is a slow muscle with a small proportion of fast fibres.

The number of inputs per fibre is calculated in the following way: twelve units (our sample) innervate 331 S fibres (the total number of S fibres in the units: see Table 1). Therefore eleven units (the average number of units per muscle) would provide 303 endings on S fibres. The mean number of S fibres per muscle is 87 fibres, so on average there must be 3·5 inputs per fibre. Considering F fibres, the twelve units contained a total of 2094 F fibres, so eleven units provide 1919 endings on F fibres. There are on average 617 F fibres in 3–5 day muscle, so there are on average 3·1 inputs per fibre. This compares well with the estimate in the previous paper (Jones & Ridge, 1987), obtained by different reasoning, of 3·3 inputs per fibre.

The smallest unit in our sample (unit 12; also see Jones & Ridge, 1986, for physiological characteristics) differs from the others by being markedly selective for

S fibres. It would seem, therefore, that the motor units destined to be S in the adult are already differentiating at this age. One should not rely too heavily on the results from one small unit; however, in the preceding paper (Jones & Ridge, 1987) there were other small units with relatively long contraction and relaxation times that may well have contained a preponderance of S fibres.

Recently Gordon & Van Essen (1985) have reported a wider range of motor-unit contraction times in post-natal (1·5–4 day) rabbit soleus muscle than in embryonic (4–6 days before birth) muscle. Using ATPase histochemistry they found fibre differentiation in the post-natal muscles but not in the embryonic, and conclude that this and the contraction-time data together are evidence of selective innervation of muscle fibres by motoneurones at these young post-natal ages. Our data and that of Thompson *et al.* (1984) indicate that such selectivity in the rat is certainly not complete. It is also likely that some degree of fibre specialization in terms of myosin type had occurred in the embryonic rabbit muscle but was not detectable by ATPase histochemistry, as we find to be the case in 3–5 day lumbrical muscles of the rat. Unfortunately it would be difficult to investigate this at younger ages in the rat because of the added complication of electrical coupling between muscle fibres (which may well occur in embryonic rabbit muscle also).

In both the experiments in the preceding paper (Jones & Ridge, 1987) and in the present paper the ratio of small slower units to others is close to one in eleven (0·09 and 0·08 respectively), which is what one might expect if there is one slow-twitch unit in the adult muscle and if the sampling is unbiased. Of course, with small samples, such close agreement could be fortuitous. It is important to know how many slow-twitch units there are in the adult muscle. Unit 12 contains one-fifth (19 out of 96 = 0·20) of the S muscle fibres in the muscle. If this is typical of the units that are destined to become slow-twitch units then either there must be several of them in each muscle, or during subsequent development there must be growth of axonal terminals through the muscle in an active process of seeking out and contacting S muscle fibres.

Our main conclusions from the data described in this paper are that motor units at 3–5 days are of mixed-fibre composition, and that in at least some of these motor units there is no selective innervation of F or S fibres. Therefore there must be some sorting out process occurring between 3–5 days and adulthood. The nature of this process is one of the subjects of the paper following (Jones *et al.* 1987).

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EXPLANATION OF PLATES

PLATE 1

Mid-belly sections of frozen fourth deep lumbrical muscles from 4 day rats. Section thickness 15 μm. *A*, control muscle where the muscle nerve was stimulated with the glycogen depletion regime. All but a few fibres were depleted (unstained) PAS. *B*, control muscle frozen when freshly dissected (unstimulated). All but a few cell profiles stain for glycogen with PAS (dark). Bar = 100 μm.

PLATE 2

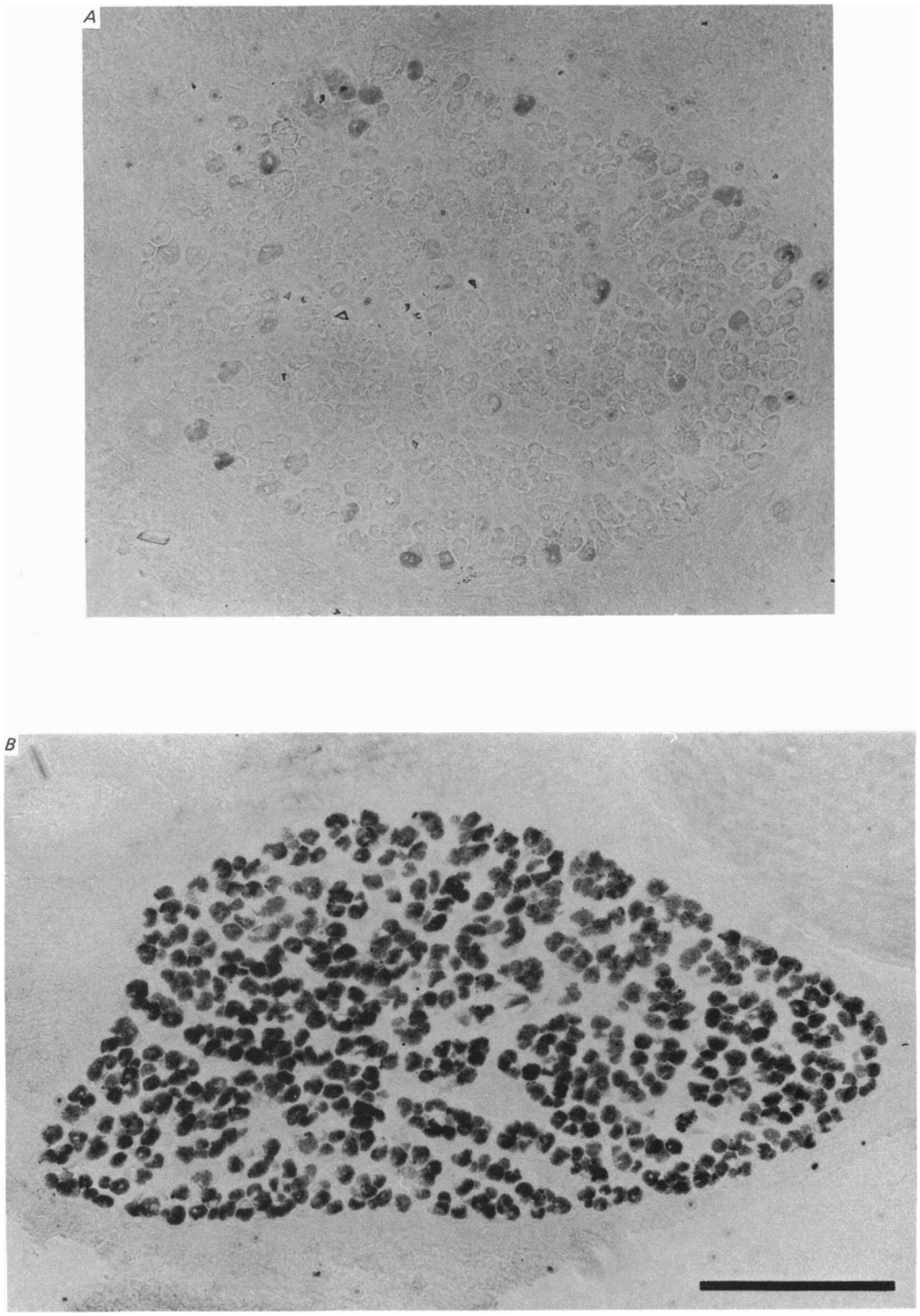
Sections through a composite block of 4 day fourth deep lumbrical and adult extensor digitorum longus muscle. Both muscles contain positive-reacting fibres to slow myosin-specific antibody. These fibres are distributed fairly evenly throughout the neonatal muscle. Anti-slow-myosin antibody; peroxidase method. Bar = 100 μm.

PLATE 3

Adjacent sections from a 4 day lumbrical muscle in which a single motor unit had been depleted of glycogen. *A*, PAS. *B*, anti-slow-myosin antibody. (Unit 10, Table 1.) Bar = 100 μm.

PLATE 4

Adjacent sections at higher magnification from a 4 day lumbrical muscle in which a single motor unit had been depleted of glycogen (different unit to that shown in Pl. 3). *A*, PAS. *B*, anti-slow-myosin antibody. (Unit 2, Table 1.) Examples of a depleted F fibre (one arrow) and S fibre (two arrows). Bar = 20 μm.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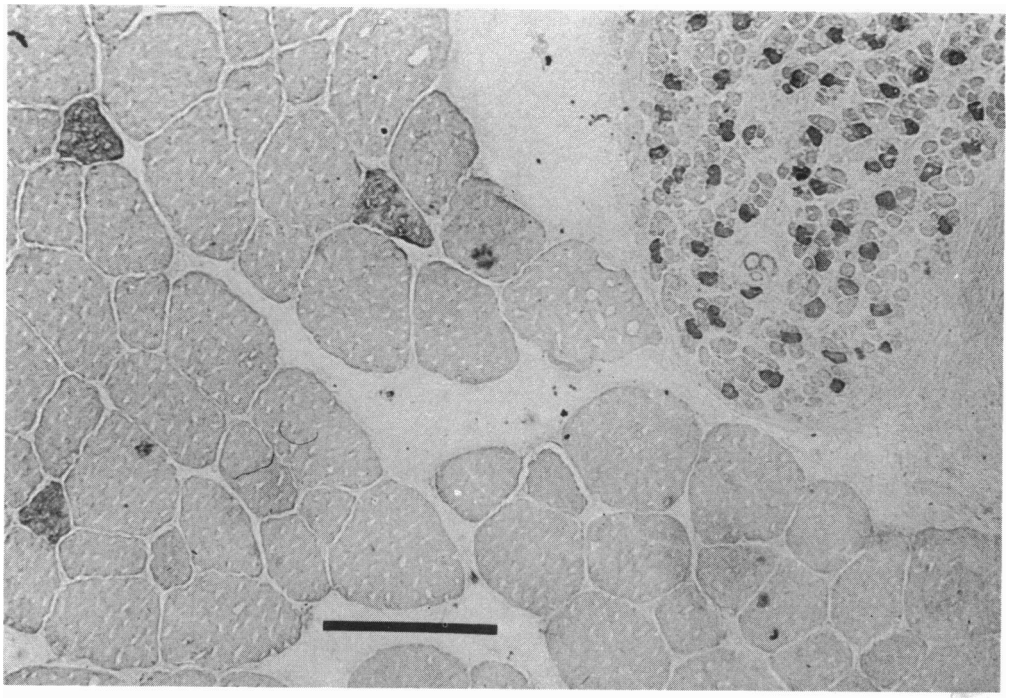

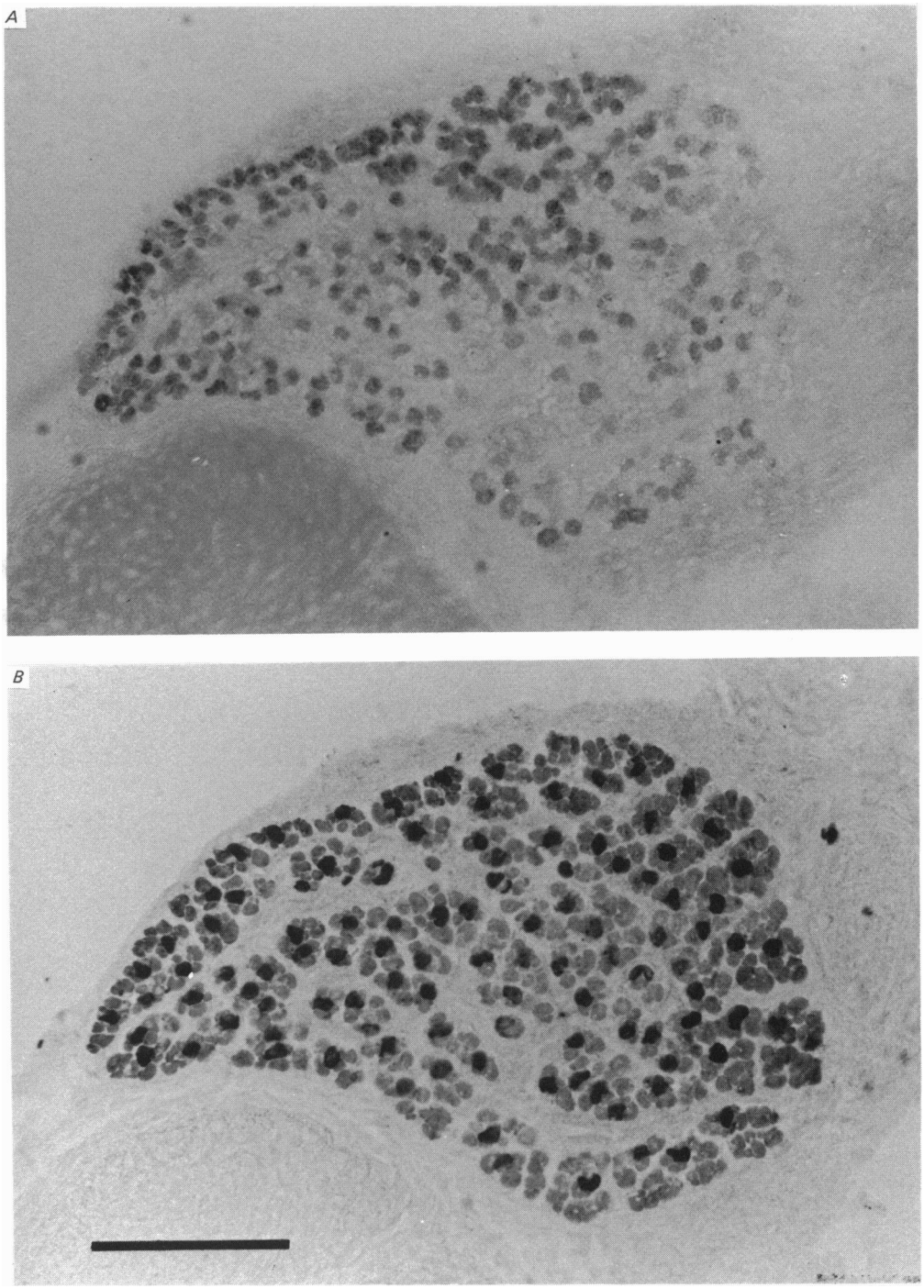
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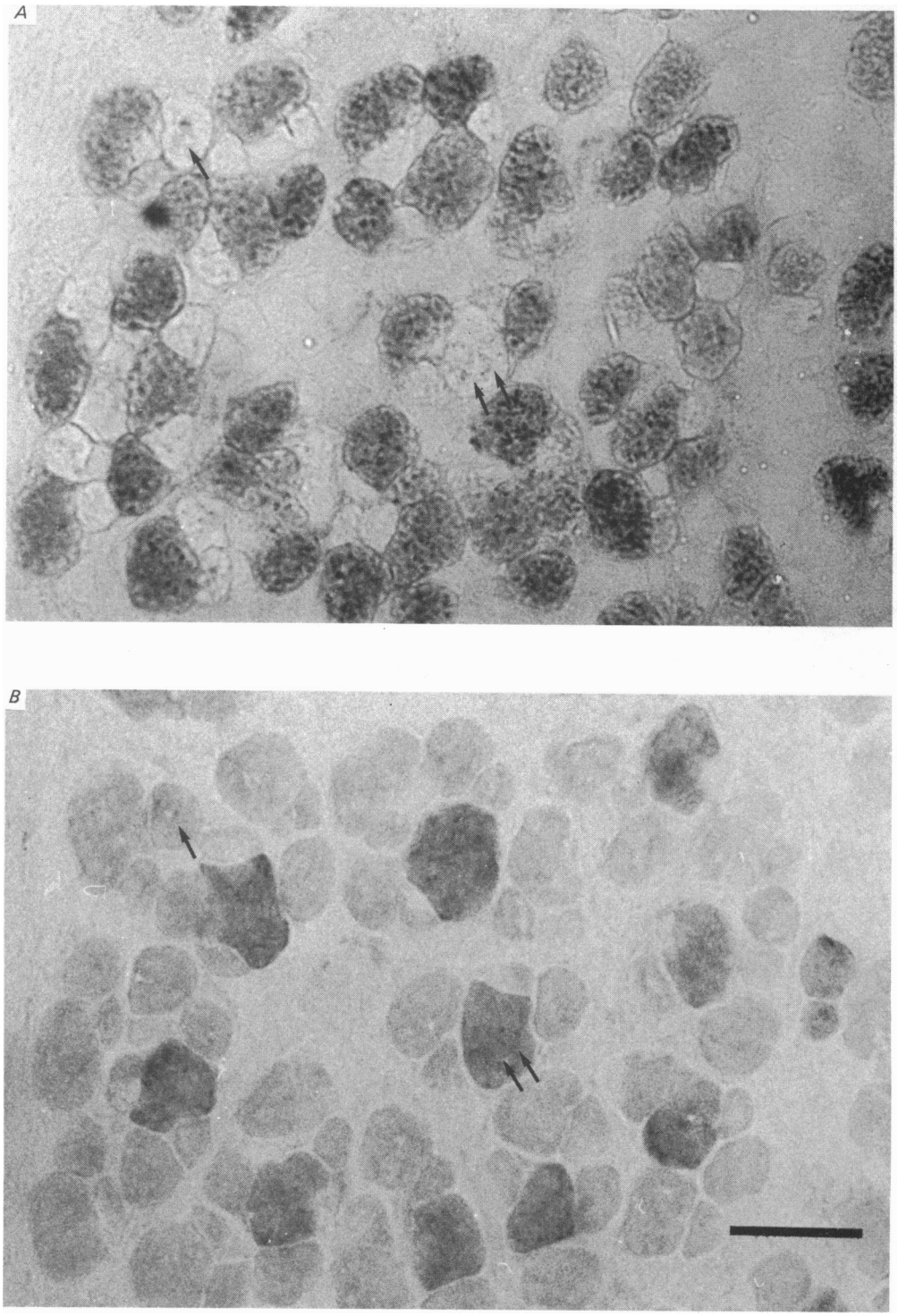
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