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OBSERVATIONS ON THE ELIMINATION OF POLYNEURONAL INNERVATION IN DEVELOPING MAMMALIAN SKELETAL MUSCLE

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SUMMARY

1. The mechanism responsible for the elimination of polyneuronal innervation in developing rat soleus muscles was studied electrophysiologically and histologically.

2. Initially all the axons contacting a single end-plate have simple bulbous terminals. As elimination proceeds one axon develops terminal branches while the other terminals remain bulbous and may be seen in contact with, or a short distance away from, the end-plate. It is suggested that the branched terminal remains in contact with the muscle fibre while the other terminals withdraw.

3. At a time when polyneuronal innervation can no longer be detected electrophysiologically, the histological technique still shows the presence of end-plates contacted by more than one nerve terminal.

4. The effect of activity on the disappearance of polyneuronal innervation was examined. Activity was increased by electrical stimulation of the right sciatic nerve. This procedure also produced reflex activity in the contralateral limb. In both cases polyneuronal innervation was eliminated more rapidly in the active muscles.

5. The finding that proteolytic enzymes are released from muscles treated with acetylcholine (ACh), and the observation of the more rapid elimination of supernumerary terminals at the end-plates of active muscles, lead to the suggestion that superfluous nerve-muscle contacts are removed by the proteolytic enzymes in response to neuromuscular activity. The selective stabilization of only one of the terminals is discussed in the light of these results.

INTRODUCTION

The developing nervous system makes many superfluous synaptic connexions and, with maturation, the inappropriate terminals are eliminated. Such a process is illustrated at the neuromuscular junction, where electrophysiological and histological studies have shown that the skeletal muscle fibres of new-born animals are innervated by several axons at the same end-plate and that this polyneuronal innervation is lost during the first few weeks of post-natal development (Redfern, 1970; Bagust, Lewis & Westerman, 1973; Bennett & Pettigrew, 1974; Brown, Jansen & Van Essen, 1976; Riley, 1977). Since morphological examination of the neuromuscular junctions of these developing muscles reveals no signs of nerve degeneration

it has been suggested that the supernumerary axons lose contact with the muscle by withdrawal (Korneliussen & Jansen, 1976; Riley, 1977).

The mechanism involved in the elimination of polyneuronal innervation is unknown, but the observation that it coincides with the general increase in the activity of the animal (Redfern, 1970) suggests that neuromuscular activity may have an important influence. This is supported by the discovery that the disappearance of polyneuronal innervation in the rat soleus is delayed when muscle activity is reduced by tenotomy (Benoit & Changeux, 1975). Increasing the activity would therefore be expected to accelerate the elimination process, and the results of the present investigation show that this is indeed the case.

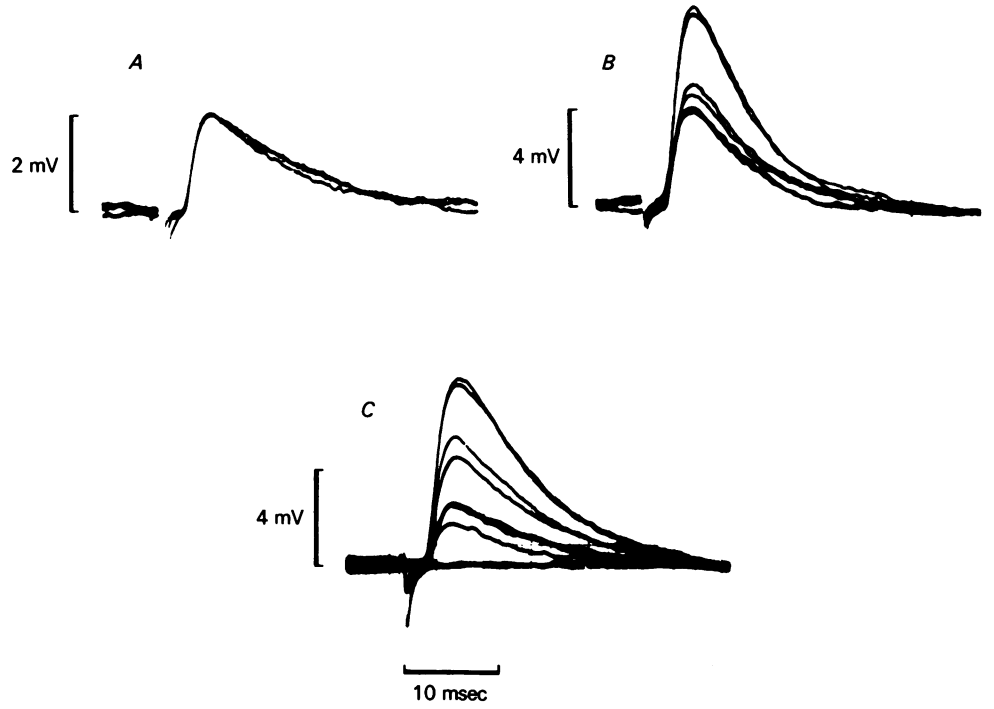
It is known that two or more nerve endings can form permanent synapses on neonatal and adult soleus muscle fibres only if the end-plates are at least 1 mm apart (Brown, Jansen & van Essen, 1976; Frank, Jansen, Lømo & Westgaard, 1975). This, together with our findings that increased muscle activity leads to an earlier elimination of polyneuronal innervation (reported here and by O'Brien, Purves & Vrbová, 1977) suggests as one possibility that neuromuscular activity might stimulate the release of a diffusible substance at the end-plate region. This substance could attack the nerve terminals within its range of influence and cause the withdrawal of all but the most resistant terminal. Poberai, Sávay & Csillik (1972) and Poberai & Sávay (1976) demonstrated the release of proteolytic enzymes at the end-plates of stimulated muscles, and we have investigated the release of these enzymes from whole muscles in response to ACh. A model is described to show how we think nerve activity and the release of proteolytic enzymes could lead to the elimination of all but one terminal from the end-plate during post-natal development.

METHODS

Implantation and stimulation. Electrodes made from Teflon-coated stainless-steel wire (Clark Electromedical Instruments) were implanted under aseptic conditions in Wistar rats anaesthetized with ether. The rats were 6, 7 or 8 days of age and of either sex. In the right leg the bared ends of two electrodes were secured to the muscles on either side of the sciatic nerve with silk suture, so that the wires did not touch the nerve. The other ends of the electrodes were led subcutaneously to the back of the neck and secured so that about 5 cm of wire protruded from the skin for connexion to the stimulator leads. Stimulation was at 8 Hz, with a pulse width of 0·2–0·4 msec and a voltage just sufficient to produce visible contraction of the muscles (between 2 and 9 V). The animals suffered no apparent discomfort from the stimulation, the duration of which was limited to between 4 and 6 hr a day to reduce disruption of their feeding habits. After 2–4 days' stimulation both soleus muscles were removed under anaesthetic and either prepared for histology or tested electrophysiologically for polyneuronal innervation. Histological examination was also made of muscles from 13 day old rats that had been stimulated from 7 to 11 days of age. Litter-mates of the stimulated rats were used as control animals, and these were either unoperated rats or implanted but unstimulated rats.

Electrophysiology. Muscles were tested for polyneuronal innervation with the method of Redfern (1970). The muscle was mounted in a shallow bath perfused at room temperature with oxygenated Krebs solution containing 5 mM-Ca. The nerve was stimulated via a suction electrode and (+)-tubocurarine (0·5–1·5 μg/ml.) was added to the perfusion medium until muscle contractions could no longer be elicited. E.p.p.s were measured with intracellular glass microelectrodes filled with 3 M-KCl, having resistances of 15–30 MΩ. The nerve was stimulated at 1 Hz and, when a muscle fibre was impaled, the stimulus amplitude was gradually increased from zero until an e.p.p. was observed. If the size of the e.p.p. remained constant when the stimulus strength was raised further (Text-fig. 1*A*) it was concluded that the muscle fibre was innervated

by one axon, but if the size of the e.p.p. increased (Text-fig. 1 *B*) this indicated that the stimulus threshold had been reached for a second axon innervating the same muscle fibre, producing a large compound e.p.p. Raising the stimulus strength even further often produced additional increases in the size of the compound e.p.p. (Text-fig. 1 *C*), especially in the younger rats, indicating that the muscle fibres were innervated by several axons. To ensure that the microelectrode was near the end-plate region, recordings were not made from fibres in which the rise-time of the first e.p.p. was more than about 4 msec. At least twenty fibres were impaled in each muscle and any fibre that produced a compound e.p.p. was defined as being polyneuronally innervated, as described by Brown *et al.* (1976).



Text-fig. 1. Examples of e.p.p.s recorded from soleus muscle fibres of young rats. In each case the intensity of the nerve stimulation was increased during the recording. In *A* no increase in e.p.p. size was recorded, indicating that the fibre was functionally innervated by one axon. In *B* and *C* stepwise increases in the e.p.p. were observed, indicating that the fibres were innervated by two and three axons respectively. The rise times of the compound e.p.p.s show that polyneuronally innervated fibres are innervated at one end-plate.

Histology. For the simultaneous examination of nerve terminals and muscle end-plates the combined cholinesterase and silver method of Namba, Nakamura & Grob (1967) was used, with the following modifications.

Slightly stretched muscles were fixed for 2 hr at 4 °C in 0·1 M-phosphate buffer (pH 7·4) containing 4 % formaldehyde, 0·005 % $CaCl_2$, 5 % sucrose and 1 % dimethylsulphoxide (DMSO). The tissues were then washed for 1 hr at 4 °C in the same solution, but without the formaldehyde, before 50 μm frozen sections were taken. The sections were incubated in maleate-buffered acetyl thiocholine (Karnovsky, 1964) for 1 hr at 4 °C, washed in distilled water, toned in 0·25 % KCN for 10 min, washed in distilled water for 10 min, defatted in absolute ethanol for 1 hr, washed in distilled water and then incubated for 20 min at 37 °C in the silver solution (0·1 g $CaCO_3$, 0·05 g $CuSO_4$ and 10 g $AgNO_3$ in 100 ml. distilled water). They were then rinsed in distilled water and developed in a reducer solution (10 g Na_2SO_3 and 1 g quinol in 100 ml. distilled water).

The sections were then washed well in distilled water and mounted on gelatinized slides with DPX (Searle). This method stains the end-plates with a brown reaction product and the nerves with a black silver deposit. The muscle fibres and nuclei are stained pale green.

For quantitative analysis about 800 end-plates were examined from each muscle. End-plates that were contacted by two or more axons were defined as being polyneuronally innervated.

It was considered unwise to make the histological examinations on muscles used for electrophysiology, since artefacts might be created by the curare, the time taken for the experiment, etc. In the unoperated rats one soleus muscle was taken for electrophysiology, the other for histology. For the experiments on implanted rats, litter-mates were used for histology and electrophysiology whenever possible.

Proteolytic activity. The secretion of protease by the soleus and extensor digitorum longus (EDL) muscles of normal 8 day and 28 day old rats was studied using a modification of the method of Fratello (1968). After removal from the animal the muscles were washed for 10 min in oxygenated Krebs solution, dried on a filter paper and placed on strips of Agfachrome 50 L colour reversal film which had been processed but not exposed (thus having a dark background). The film had been preincubated for 2 hr at 37 °C in 150 mM-Na acetate buffer, pH 5·0. A drop of Krebs solution or a solution of ACh in Krebs was put on each muscle and the excess fluid removed with a tissue. Drops of Krebs or ACh alone were also put on the film as controls. The films were then incubated in moisture chambers for 1·5 hr at 37 °C, after which the muscles were removed and the films rinsed in distilled water. The change in colour of the film and the area of film that was digested gave a relative estimate of proteolytic activity.

Since some batches of film gave no reaction it was necessary to use as a control a tissue that is known to be a good source of proteolytic enzymes. In all experiments, therefore, in addition to the muscles a slice of liver was placed on each film. Unless the liver produced a reaction the experiment was discarded.

RESULTS

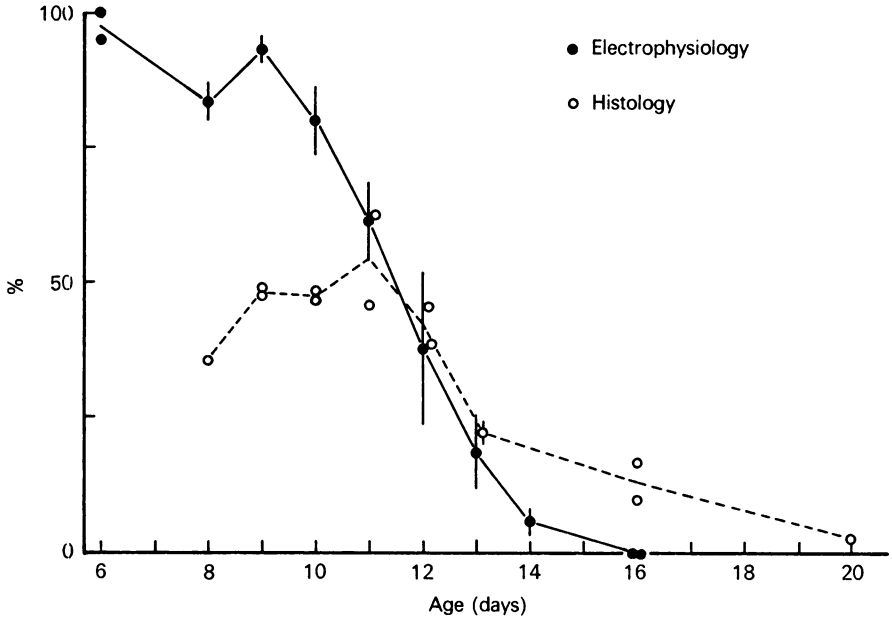
Histological appearance of end-plates during normal post-natal development

The morphological observations show that in animals up to 11 or 12 days of age many soleus end-plates were innervated by two or more axons. In muscles from 9 and 10 day old rats many end-plates had several fine axons (Pl. 1*A*) often with simple bulbous terminals (Pl. 2*A*). At some end-plates one of these axons was thicker than the others and had several prominent terminal branches overlying the end-plate (Pl. 2*B*), while the thinner axons had a bulbous enlargement in their preterminal region and terminated in very fine branches or in a simple bulb (Pl. 2*B*, 2*C*). The proportion of end-plates displaying this feature increased from 30% at 12 days to 65% at 16 days. During this period many end-plates were seen in which a single innervating axon had a second, thinner axon lying alongside it and ending in a bulbous terminal a short distance from the end-plate (Pl. 2*D*). These 'bulbs' correspond to those described by Riley (1977), and we believe that the sequence of morphological changes follows the pattern illustrated in Pl. 2 and described in the Discussion, and that the 'bulbs' are a feature of the retracting supernumary axons.

Comparison of physiologically and morphologically detected polyneuronal innervation

The proportion of end-plates that showed the morphological signs of polyneuronal innervation is compared with the electrophysiological results in Text-fig. 2. In rats aged 6–10 days, 70 to 100% of end-plates were functionally innervated by two or more axons, as shown by the electrophysiological results. This conflicts with the morphological data, which show that only about 50% of the fibres were poly-

neuronally innervated during this period. This discrepancy could be due to a lack of impregnation of the finest axons by the silver stain, or to an inherent bias in the electrophysiological technique. Since neither technique is free from criticism when applied to early development, when the nerve and muscle fibres are small and immature, it is not possible to determine if the onset of the elimination of polyneuronal innervation really occurs sooner when tested physiologically than when



Text-fig. 2. Normal rat soleus muscles: extent of polyneuronal innervation estimated electrophysiologically (continuous line) and histologically (broken line). Ordinate: number of polyneuronally innervated fibres expressed as a percentage of the total number of fibres examined. Abscissa: age of rat. Vertical bars: ± 1 S.E. of mean. Where the number of observations is less than 3, the individual values are plotted.

examined histologically, as might be implied by Text-fig. 2. The results can be considered more reliable as the muscles develop; in the 16 day rats polyneuronal innervation was detected histologically although the functional elimination of supernumary contacts was complete, indicating that some of the terminals seen to be in contact with the end-plates in these muscles were no longer functional.

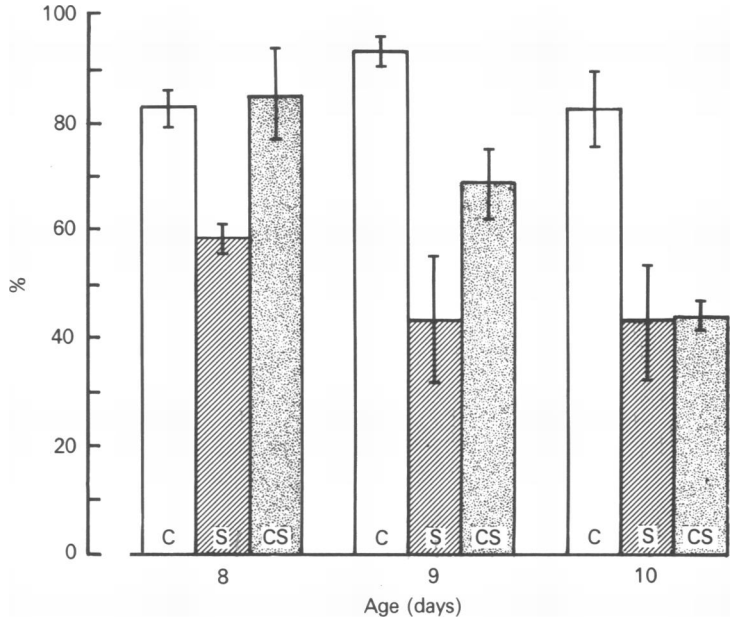
Effect of activity on the elimination of polyneuronal innervation

Electrophysiology. In a group of rats aged 6 or 7 days electrodes were implanted so as to activate the soleus muscles of one leg. Rats that were implanted but not stimulated were used as controls; they were separated from their mothers for the same daily periods as the stimulated rats.

The muscles were examined 2 or 3 days after implantation, when the rats were 8, 9 or 10 days old. The proportion of fibres that were polyneuronally innervated in normal animals between 8 and 10 days of age was $86{\cdot}9 \pm 4{\cdot}2\,\%$ (mean ± S.E. of mean, $n = 11$), and that of implanted control animals at the same ages was $84{\cdot}0 \pm 3{\cdot}3\,\%$

(n = 6), so it is clear that the operation itself had no effect on the extent of polyneuronal innervation in these muscles.

The extent of polyneuronal innervation in the stimulated muscles is compared with that in normal muscles in Text-fig. 3. The stimulated muscles from the 8 day rats had received a total of only 8 hr of stimulation, yet they underwent a significant reduction of polyneuronal innervation compared with the normal muscles ($P <$ 0·025; Wilcoxon test). Polyneuronal innervation was not reduced much further at 9 and 10 days, probably because of the limited duration of the daily periods of stimulation.



Text-fig. 3. Extent of polyneuronal innervation measured electrophysiologically in control animals, in stimulated muscles, and in muscles contralateral to the stimulated side. Axes as in Text-fig. 2. Values are mean ± S.E. of mean. Number of rats used at 8, 9 and 10 days respectively; control (C): 6, 5, 6: stimulated (S): 3, 4, 3: contralateral (CS): 3, 4, 4.

In the experimental animals there was frequently a noticeable extension of the contralateral limb during the stimulation period, presumably because of the crossed-extensor reflex. The contralateral soleus muscles were therefore also examined for polyneuronal innervation, and it was indeed found that in these muscles polyneuronal innervation was also reduced (see Text-fig. 3). However, in muscles that were reflexly activated, polyneuronal innervation became reduced later than in muscles stimulated via the implanted electrodes. This delay is not surprising, for the amount of activity imparted to a muscle by reflex movement is less than, and the pattern different from, activity induced by continuous artificial stimulation of all motor units in a muscle.

Histology. The faster disappearance of polyneuronal innervation in active muscles revealed by the electrophysiological observations could not be detected using morpho-

logical techniques; the proportion of polyneuronally innervated end-plates in the stimulated and contralateral muscles did not differ significantly from that of the normal litter-mates at 9, 10, 11, and 13 days of age.

Release of proteolytic enzymes from muscles treated with ACh

Since the electrophysiological evidence suggests that increased nerve activity speeds up the elimination of superfluous nerve-muscle connexions, we considered the possibility that ACh released at the neuromuscular junction during activity might itself cause the release from the muscle of some agent that acted back to affect the nerve terminal. We therefore examined the effect of ACh on the release of acid proteinases from whole muscles of young rats.

Normal soleus and extensor digitorum longus muscles from 8 to 10 day old normal rats were placed on strips of processed colour reversal film (see Methods) that had been soaked in buffer at pH 5, and drops of Krebs solution or a solution of ACh in Krebs were placed on the muscles. Some of the muscles were pre-soaked for 5 min in a solution of (+)-tubocurarine or atropine at a concentration equimolar with the dose of ACh to be used, and the drug was included in the ACh solution applied to these muscles. The area of the gel that was digested, relative to the area of muscle in contact with the gel, and the colour produced in the emulsion of the film were used as an indication of the extent of the proteolytic enzyme activity; the results are shown in Table 1. Little or no proteolytic activity was observed when the muscles were treated with Krebs solution, but the addition of ACh at concentrations of 0·1 mg/ml. and above caused digestion of the gel layers. A similar response was seen in muscles from a 28 day old rat. Direct application of Krebs or ACh solution to the film did not cause digestion of the gel. High doses of atropine blocked the ACh-induced release of the enzymes from soleus and EDL, though, like the untreated muscles, the atropine-treated muscles contracted on the films when the ACh was placed on them. Too few experiments were performed on atropine- or curare-treated muscles to allow any firm conclusion, but this result suggests that muscle contraction was not itself responsible for digestion of the gels.

In each of three rats aged 28 days, one sciatic nerve was cut under aseptic contions, and the soleus and EDL muscles were tested for proteolytic activity 3 days later. The muscles were placed on the films in the usual way and exposed to ACh 1 mg/ml. The gel layers were substantially digested beneath the muscles, implying that proteolytic enzymes are released from muscle and not from the nerve terminals.

DISCUSSION

The present results show that while electrophysiological methods reveal that almost all end-plates of the soleus muscle of 8–10 day old rats (70–100 %) are innervated by more than one axon, histological findings reveal only 50 % of the end-plates to be polyneuronally innervated, while the rest are contacted by only one axon. Riley (1977) reports that in his histological material 73 % of end-plates were supplied by more than one axon in 11 day old rats. The discrepancy between the present results and those of Riley may be due to the different method used for identifying the end-plates in the two sets of experiments. Riley used as an indication of an end-

TABLE 1. Proteolytic activity from soleus and EDL muscles of young rats

ACh g/ml.	Control		Curare		Atropine	
	Sol	Edl	Sol	Edl	Sol	Edl
0	+	−				
	−*	−*				
10^{-6}	−	−				
10^{-5}	−	−				
10^{-4}	+	+ + +				
	−	+ +	+ +	+ +		
	+	−	+	+ +		
	+	+ +			−	−
	+	+ + +			−	−
10^{-3}	+ + +	−				
	+ + +	+ + + +				
	+ +	+	+ + + +	−		
	+ + +	−	+ + +	−		
	+	+ + +			−	+
	−	+ +			−	−
	+ + +	+			+	−
10^{-2}	+ + + +	+ + + +				
2×10^{-1}	+ +	+ + +				
	+ +	−				
	+ + + +	+ + + +				
	+ + + +*	+ + +*				

Release of proteolytic enzymes from muscles, assessed by the colour changes seen on a photographic film (Fratello, 1968), and the area over which the changes occurred. The area was measured with a microscope fitted with a graticule, and the results expressed in 5 degrees of magnitude: −, no reaction; +, faint reaction (a red colour in the photographic emulsion); + +, strong (blue or white) reaction over a small area; + + +, strong reaction over a larger area; + + + +, strong reaction over most of the muscle surface. The muscles were from 8–10 day old rats except those marked with an asterisk, which were from a 28 day old rat. Each row represents an individual experiment; curare and atropine were used in equimolar concentrations with the ACh.

plate the accumulation of nuclei that characterizes end-plates of adult muscle, but is often difficult to discern in muscles of immature animals, while we identified end-plates using a cholinesterase stain, which ensures a much more precise localization. Moreover, Riley only counted approximately 100 end-plates in each muscle, and he does not comment how he selected them, while in our experiments a minimum of 800 end-plates were counted in each muscle. As indicated in the results, the differences between the physiological and histological results may be due to inherent biases in either technique. These biases are likely to diminish as the nerves and muscles mature.

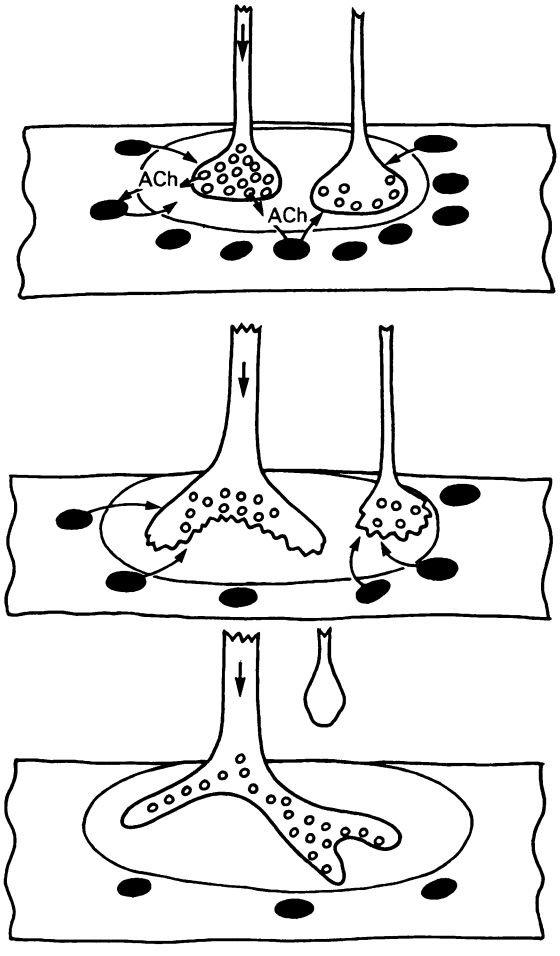
In the 16 and 20 day old rats, the histology revealed the presence of a substantial number of polyneuronally innervated muscle fibres while the electrophysiology showed no polyneuronal innervation. Furthermore, stimulation of the muscles speeded up the elimination of polyneuronal innervation measured physiologically while no change

was detected by the histology. This suggests that, during maturation, the end-plates may be contacted by non-functional nerve terminals which presumably are later removed from the end-plate. The mechanism involved in their removal is poorly understood.

Korneliussen & Jansen (1976) found no signs of axonal degeneration at soleus end-plates during the elimination of polyneuronal innervation. The present results show that, in the early stages of post-natal development, the axons innervating each end-plate have simple terminals, without well developed terminal arborizations. As development progresses one of the terminals produces complex terminal branches, while the other terminals remain simple. During the elimination process these simple terminals appear to withdraw gradually from the end-plate; it is as if the axons with the simpler terminals were being separated from the end-plate while the axon that had formed branches at the end-plate followed the growth of the muscle and so maintained its connexion with the end-plate. The terminals that had lost contact with the muscle withdrew further from the end-plate until they could no longer be seen when examined histologically.

To explain why only one nerve terminal remains at the end-plate we propose that the elimination of supernumary terminals is brought about by the action of proteolytic enzymes that are released at the end-plate region in response to the transmitter, ACh. The sequence of events at the developing end-plate is imagined as follows.

Early in development an end-plate can be innervated by several axons, each being relatively inactive except for the release of an occasional packet of ACh to produce the low m.e.p.p. frequency observed by Diamond & Miledi (1962). As the animal becomes more active, motoneurone activity increases and more ACh is released at the end-plate. The ACh causes proteolytic enzymes to be released at the end-plate region, either from the muscle fibre itself or from other cells near the end-plate. These enzymes diffuse across the end-plate and into the surrounding interstitial space. Where their concentration is optimal (at the end-plate and within about 1 mm of the end-plate) the enzymes affect the nerve terminal membrane or the connexion of the terminal with the end-plate. The terminals have to be replaced in order to maintain contact with the muscle fibre, but the neurone only has the capacity to support a limited number of terminals. Thus the terminals with the poorest supply of the material necessary for their replacement lose contact with the end-plate. A schematic illustration of this is shown in Text-fig. 4. If the surviving terminal is also digested faster than it can replace itself the end-plate will receive less ACh and the enzyme output will be reduced, allowing the nerve terminal to grow back. This could provide the basis for a feed-back mechanism that maintains the axon at the end-plate. The elimination of a particular terminal enables the parent neurone to increase the supply of material to its other branches and thus to support the surviving terminals at other end-plates. Ultimately, the number of end-plates innervated by a neurone depends on the activity of the neurone and on its ability to support its terminals; smaller neurones innervate fewer muscle fibres because they have a capacity to synthesize and transport membrane-replacing materials to only a relatively small number of terminals, and more active neurones also innervate fewer muscle fibres because they induce a greater proteolytic activity at each end-plate. This would explain why slow motor units, which are innervated by small, tonically



Text-fig. 4. Schematic diagram representing hypothesized involvement of proteolytic enzymes in the elimination of polyneuronal innervation. Top: two nerve terminals with simple bulbous endings at a single end-plate in a young animal. The activity of one or both of the nerves causes the release of ACh which, in turn, stimulates the release of proteolytic enzymes into the synaptic clefts. Middle: the enzymes digest the nerve terminals, but the larger axon is more resistant because it has a better supply of material for the replacement of the terminal. Bottom: the larger axon matures and produces terminal branches, while the smaller, less resistant axon withdraws from the end-plate.

active neurones, comprise fewer muscle fibres than fast motor units (Burke, 1967). The pattern of innervation may also be influenced by other factors, such as the metabolism of the muscle and its blood flow.

Although this model is hypothetical, it is supported by several observations. The involvement of a diffusible factor is suggested by the observation of Brown *et al.* (1976), that developing soleus muscle fibres can accept innervation at more than one end-plate as long as the end-plates are separated by a distance of more than 1 mm. The present experiments have shown that increasing the activity of the motor nerve accelerates the elimination of polyneuronal innervation, and Benoit & Changeux (1975) have found that reduced muscle activity delays the elimination process. Our

experiments also indicate that isolated muscles produce proteolytic activity when treated with ACh, and it has been shown by Poberai and her colleagues that indirect muscle stimulation *in vivo* (either by stimulating the nerve or by injecting neostigmine) causes a marked increase in the proteolytic enzyme activity at the neuromuscular junctions of adult rats (Poberai *et al.* 1972; Poberai & Sávay, 1976).

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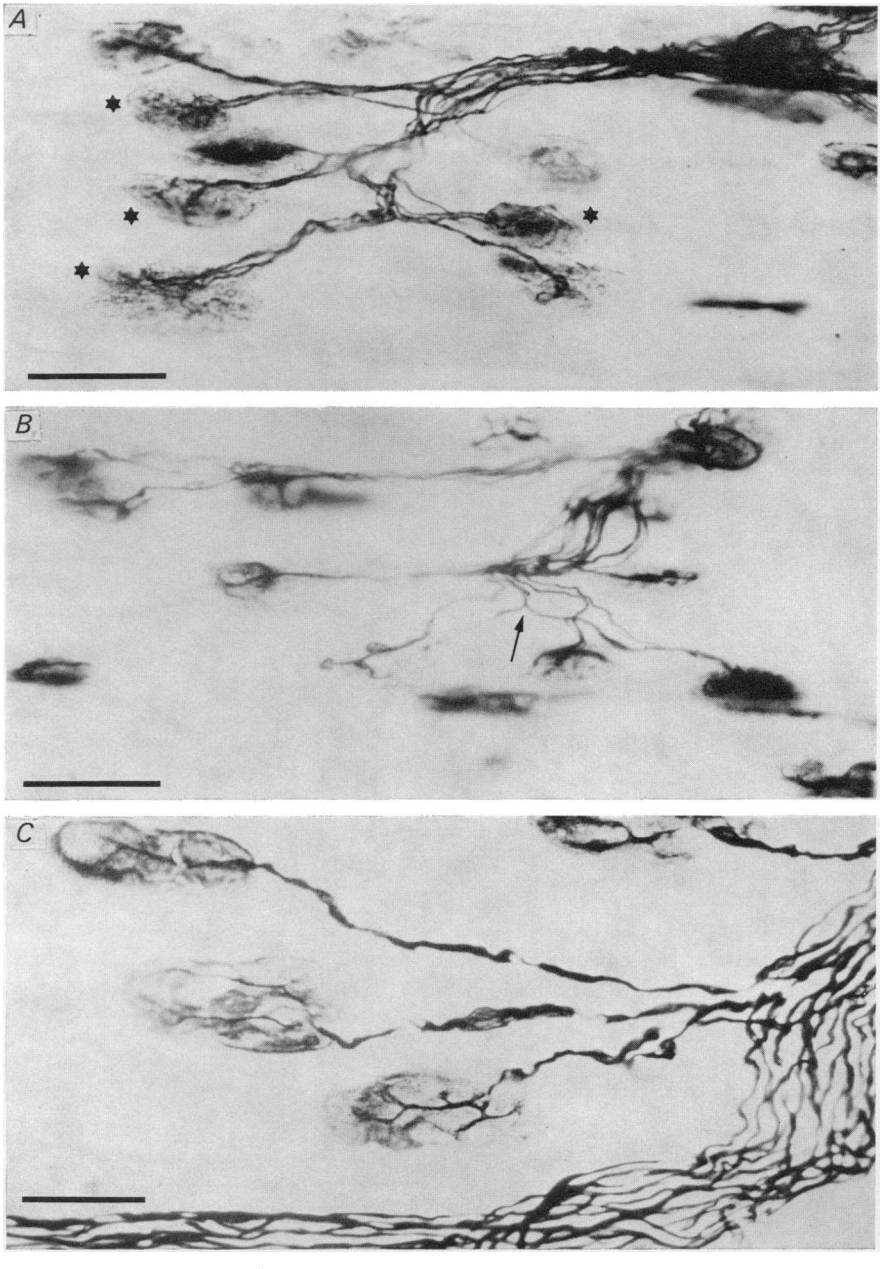
EXPLANATION OF PLATES

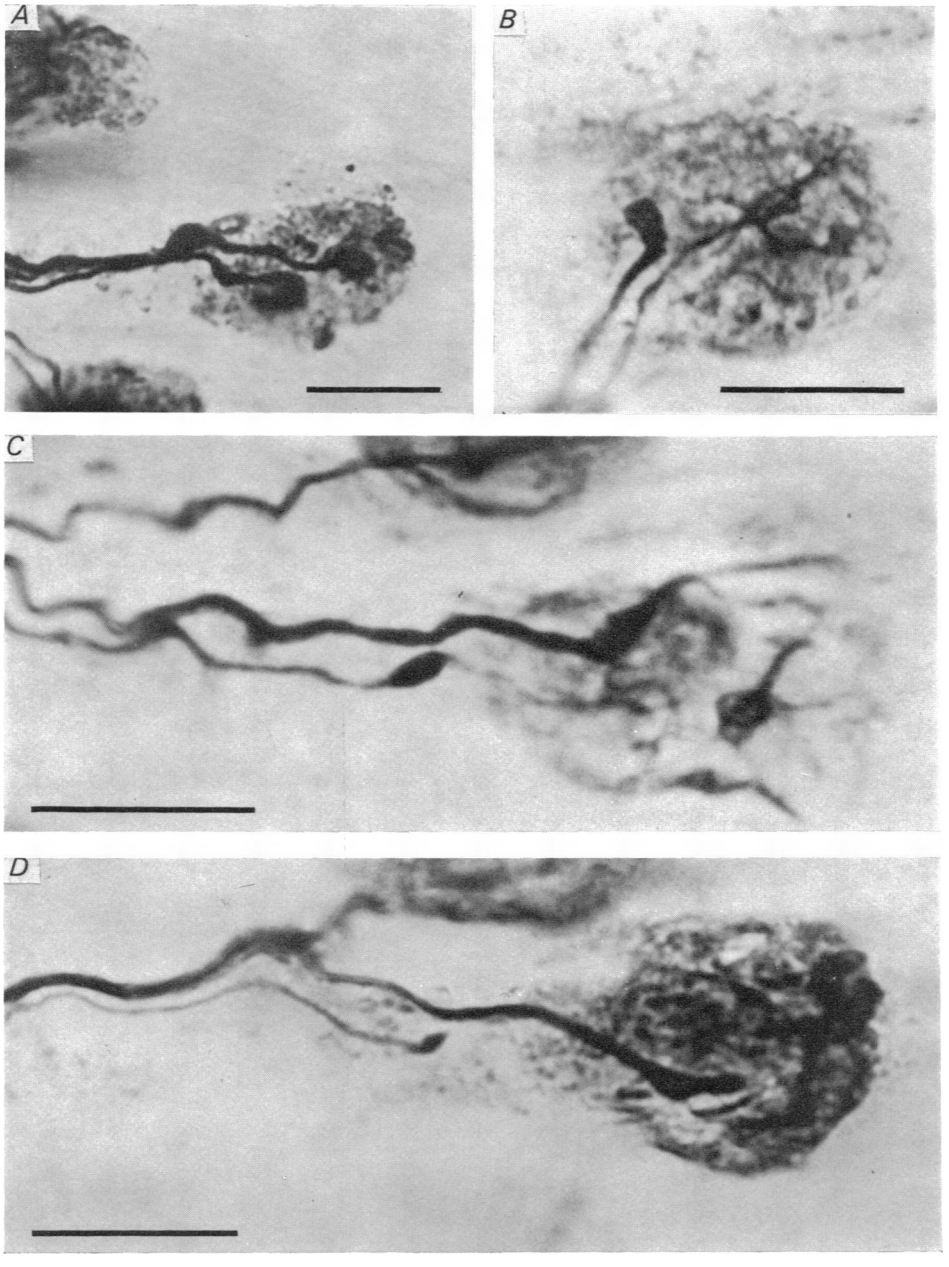
PLATE 1

Combined cholinesterase-silver stained sections of soleus muscles from young rats. *A*, polyneuronally innervated end-plates in a muscle from a 10 day old rat. Four end-plates (*) are contacted by two or more axons. *B*, end-plates in a muscle from a 12 day old rat. One axon (arrow) has a thick branch to one end-plate and a thin branch to another; the thin branch ends in a bulb on the edge of an end-plate already occupied by a thick axon with a branched terminal. *C*, end-plates in an adult muscle. Each end-plate is contacted by a single axon with many terminal branches. Calibration bars are 25 μm.

PLATE 2

High power micrographs of axon terminals in soleus muscles from 11–13 day old rats. *A*, end-plate contacted by two axons, both ending in simple bulbous expansions. *B*, one of the two axons innervating this end-plate is branched while the other terminates in a 'bulb'. *C*, the thinner of the two has a bulbous expansion about 10 μm from the end-plate and is connected by a thread to its fine terminal branches. *D*, the thinner axon ends in a 'bulb' about 10 μm from the end-plate. Calibration bars are 10 μm.


A
B
C


A
B
C
D