

**IDENTIFICATION OF INTRAFUSAL MUSCLE
FIBRES ACTIVATED BY SINGLE FUSIMOTOR AXONS AND INJECTED
WITH FLUORESCENT DYE IN CAT TENUISSIMUS SPINDLES**

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SUMMARY

1. Intrafusal muscle fibres of cat tenuissimus spindles have been injected with the fluorescent dye Procion Yellow and identified histologically after recording their changes in membrane potential during 1/sec stimulation of single static or dynamic γ axons.

2. Thirteen intrafusal muscle fibres innervated by static γ axons were identified as eight bag₂ and five chain fibres. The fact that none proved to be a bag₁ fibre is not regarded as significant, for reasons given in the Discussion.

3. In one spindle Procion Yellow was injected into two intrafusal muscle fibres activated by the same static γ axon; they were identified as a bag₂ and a chain fibre.

4. Nine intrafusal muscle fibres innervated by dynamic γ axons were identified as seven bag₁ fibres, one bag₂ fibre, and one long chain fibre.

5. In one spindle two bag fibres were injected, one activated by a dynamic γ axon, the other by a static γ axon; the former proved to be a bag₁ fibre, the latter a bag₂ fibre.

6. Stimulation of static γ axons elicited junctional potentials in seven bag₂ fibres and one damaged chain fibre, and action potentials in one bag₂ and four chain fibres. In the whole sample of impaled intrafusal muscle fibres (identified and unidentified) activated by static axons, junctional potentials were recorded from twenty-three (62.2%), and action potentials from fourteen (37.8%). Stimulation of dynamic γ axons always elicited junctional potentials.

7. In a number of instances it was possible to examine the ultrastructure of motor endings belonging to the stimulated γ axon. The myoneural junctions of trail endings supplied by static γ axons to bag₂ and chain fibres were both smooth and folded; the deepest and most regular folding occurred on chain fibres. The terminals of p₂ plates supplied to bag₁ fibres by dynamic γ axons had smooth myoneural junctions.

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INTRODUCTION

Changes in the membrane potential of single intrafusal muscle fibres have been intracellularly recorded by Bessou & Pagès (1972) in cat tenuissimus spindles on stimulating single static or dynamic γ axons. They found that static γ axons evoked both action and junctional potentials, whereas dynamic γ axons evoked junctional potentials only. A natural extension of this study was to follow the intracellular recording with an electrophoretic injection of the fluorescent dye Procion Yellow (Stretton & Kravitz, 1968) so that the type of intrafusal muscle fibre activated could subsequently be determined by fluorescence and electron microscopy. In this way we hoped to obtain information about the distribution of static and dynamic γ axons and, possibly, the ultrastructure of their motor endings.

In the event the histological identification of muscle-fibre type and motor ending proved more difficult than we had anticipated. When our experiments began in 1971 there was increasing evidence to indicate the existence of two types of nuclear-bag fibre (Yellin, 1969; Barker & Stacey, 1970; Barker, Harker, Stacey & Smith, 1972*b*; Ovalle & Smith, 1972). However, their nature had not been established and there was some doubt as to how to classify them into two types, since the histochemical and ultrastructural observations reported by various workers had been made on separate preparations of different spindles. Their correlation was thus to some extent a matter for conjecture. The problem was finally resolved by devising a technique that allows for adjacent sections of the same spindle to be prepared for either histochemical or ultrastructural study (Banks, Barker, Harker & Stacey, 1975). Such studies revealed that the ultrastructure of bag fibres, and the histochemical profiles of both bag and chain fibres, undergo regional variation from equator to pole (Barker, Banks, Harker, Milburn & Stacey, 1976*a*; Banks, Harker & Stacey, 1977). Consequently it became obvious that the criteria we had been using for the identification of muscle-fibre type in the present investigation would have to be revised. In the final analysis we distinguished between two types of bag fibre, bag₁ and bag₂, on the basis of the criteria established by Banks *et al.* (1977) and on the distribution of associated elastic fibres (Gladden, 1976).

As the work progressed it also became obvious that the terminals of trail endings and p₂ plates could not reliably be distinguished in electron micrographs on the basis of the presence or absence of post-synaptic folding, as had been maintained by Adal & Barker (1967) and Barker, Stacey & Adal (1970). Identification and interpretation were ultimately facilitated by other collaborative work in which we were engaged (Banks, Barker, Bessou, Pagès & Stacey, 1976, and in preparation), and by observations on the ultrastructure of p₂ plates previously located and photographed in spindles stained with methylene blue (Barker *et al.* 1976*a*).

The time taken to resolve these difficulties has been mainly responsible for the delay in presenting a full account of this work following the publication of preliminary reports (Barker, Bessou, Jankowska, Pagès & Stacey, 1972*a*, 1975).

METHODS

Experimental procedures. Thirty-seven experiments were performed in Toulouse on adult cats anaesthetized with Nembutal (40 mg/kg given i.p., followed by small amounts injected i.v. as necessary). Each experiment was carried out on a spindle located in the distal half of a tenuissimus muscle, the neuromuscular preparation being the same as that described by Bessou & Pagès (1972). The spindle was located by electrical and mechanical stimulation of its primary ending under visual observation (Bessou & Laporte, 1965). The Ia primary axon, and from one to six single γ axons innervating the spindle, were prepared by splitting L7 and S1 dorsal and ventral roots. The static or dynamic function of the γ axons was determined by observing the effects produced by their repetitive 100/sec stimulation on the response of the primary ending to ramp stretch (Matthews, 1962).

During each experiment the spindle was observed through a binocular microscope at $\times 50$ magnification using dark-field illumination. A clear view was obtained by carefully removing the overlying extrafusal muscle fibres without damaging the innervation or blood supply of the intrafusal muscle bundle. Intrafusal contractions produced by repetitive 100/sec stimulation of a static γ axon innervating the spindle served to distinguish it during this dissection and to provide information regarding sites for subsequent micro-electrode impalement (see Bessou & Pagès, 1972). Impalements of activated intrafusal muscle fibres were attempted using micro-electrodes filled with a freshly prepared 5% aqueous solution of the fluorescent dye Procion Yellow (M4RS, Imperial Chemical Industries). The micro-electrodes usually had a tip broken to a diameter of $1.5 \mu\text{m}$ (range $0.9\text{--}1.9 \mu\text{m}$) and a resistance of $20\text{--}25 \text{M}\Omega$ (range $12\text{--}35 \text{M}\Omega$).

The procedure for impalement consisted of observing the micro-electrode tip under the binocular microscope as it slowly descended towards the intrafusal muscle fibres, whilst simultaneously applying 1/sec stimuli to each of the isolated single γ axons. Changes in potential recorded by the micro-electrode were observed on an oscilloscope. When an increase in amplitude of the extracellular potential indicated that the tip of the micro-electrode was in the vicinity of an activated intrafusal muscle fibre or motor terminal, stimulation was restricted to the single γ axon responsible. Successful impalement of the contracting muscle fibre was indicated when an intracellular potential was recorded after the tip of the micro-electrode had made a final minute descent. The fluorescent dye was then injected by passing a constant hyperpolarizing current of 20 nA through the micro-electrode. In most cases the duration of the electrophoretic injection depended on how long the micro-electrode could be maintained inside the muscle fibre, often less than 10 min. Histological results indicated an optimum period of 10–20 min, and this was often achieved. In order to ascertain whether the micro-electrode remained inside the muscle fibre, intrafusal muscle potentials were recorded throughout the period of dye injection. Their amplitude sometimes remained constant, but often gradually decreased as the injection proceeded. Injection continued as long as an intracellular potential was clearly visible. If impalement did not occur, the microelectrode was withdrawn to its original position above the surface of the intrafusal bundle and another attempt made, following either the same path of descent, or one slightly lateral or medial to it. Such attempts would sometimes fail to penetrate the activated fibre and impale another fibre near by. In some experiments only one activated fibre was impaled and, after injection, the spindle was fixed for subsequent histology, preferably while still impaled. In others, one or two further impalements and injections were made in other activated muscle fibres either in the same or in the opposite pole of the spindle. After injection a minimum interval of 1–2 min was allowed before fixation for the dye to diffuse within the fibre, though in experiments where one injection was followed by attempts to make others this interval could extend up to 60 min.

The site of each injection was recorded by measuring its distance from the point in the equatorial region where the spindle nerve crosses the periaxial space and reaches the intrafusal bundle. Injections were made within a distance of $0.9\text{--}3.3 \text{mm}$ from this reference point, the majority being located within $1.4\text{--}2.0 \text{mm}$. At the conclusion of each experiment, and before fixation, the reference point was marked for guidance during subsequent histology by stitching a fine thread at the same level around some adjacent extrafusal muscle fibres.

Histological procedures. The muscle was fixed in the experimental chamber (Bessou & Pagès, 1972) by pouring in a 5% solution of glutaraldehyde buffered at pH 7.2 with 0.1M -sodium cacodylate. After 5 min the portion of muscle containing the spindle was trimmed down to a

block measuring about 10×2 mm, polar orientation being maintained by cutting one corner off that end containing the proximal pole. Thus prepared, the specimen was returned to the fixative and despatched to Durham for histological analysis, fixation lasting 4–14 days depending on the vagaries of the postal service. On arrival the specimen was washed in the buffer solution, dehydrated through a graded series of ethanols, transferred to propylene oxide, and embedded in Epon. Post-fixation in osmium tetroxide was omitted since it degrades the fluorescent properties of Procion Yellow.

Before sectioning, a millimetre scale was scratched on the surface of the Epon block, taking the level of the reference-point stitch as zero. In this way the site(s) of injection in the embedded spindle could be marked with accuracy. Thick (approximately $2 \mu\text{m}$) serial transverse sections were then cut on an L.K.B. ultratome and stained with 1% toluidine blue in 1% borax. Sectioning began at the extremity of the injected pole and continued to within about 0.5 mm of the site of the injection. Section thickness was then increased to 10–15 μm and the sections mounted in Fluormount (E. Gurr) for the observation of fluorescence using a Zeiss fluorescence system with exciter filter II and barrier filters 53 and 44. A successfully injected intrafusal muscle fibre appeared as a bright yellow profile against a green autofluorescing background. Photographs were taken on Ilford FP4 film using an exposure time of 15–20 min.

With examination of the injection site completed, transverse sectioning of the pole was resumed at $2 \mu\text{m}$ thickness and continued to within about 0.2 mm of the equatorial reference point as indicated by the zero mark. The Epon block was then turned through an angle of 90° and longitudinal ultrathin sections cut for observation with an A.E.I. EM801 electron microscope after staining with uranyl acetate followed by lead citrate. Individual intrafusal muscle fibres in the longitudinal sections were recognized by reference to the positions they occupied in the last transverse section. When sectioning of the pole was completed it was thus possible to ascertain the following information about an injected fibre: its diameter and polar length; the nature of its nucleation and ultrastructure in the equatorial region; and its association with elastic fibres and with other members of the intrafusal bundle.

There were some departures from the sectioning procedure thus described. Skip-serial sectioning was often employed, every tenth $2 \mu\text{m}$ section being stained with toluidine blue. In some instances serial sectioning of the pole began close to the injection site, the polar extremity being discarded. In one instance fluorescence was examined in longitudinal sections of the injection site.

In the identification of muscle-fibre type, chain fibres were distinguished from bag fibres on the basis of their shorter length and smaller diameter, and their nucleation and ultrastructure (presence of a distinct M line) in the equatorial region. The two types of bag fibre were identified mainly on the basis of the abundance (bag_2) or scarcity (bag_1) of elastic fibres associated with them in the extracapsular polar region, and their association with (bag_2), or dissociation from (bag_1), chain fibres in the equatorial region. Length (bag_2 longer) was often helpful, but not ultrastructure, since in the equatorial area sectioned for electron microscopy this is the same for both types (M line absent, or present as a faint double line).

The presence of motor terminals could be detected in the $2 \mu\text{m}$ toluidine blue sections. In the case of nine successfully injected fibres ultrathin sections were cut whenever motor terminals were encountered during serial sectioning of the pole between the injection site and the equatorial region. Observations could thus be made on the ultrastructure of motor endings innervating the injected fibre, and in some cases (see Results) there was good evidence to identify a given ending as that which had activated the fibre during its impalement and injection.

RESULTS

Intrafusal muscle potentials

Membrane potential changes of single intrafusal muscle fibres were intracellularly recorded while applying 1/sec stimuli to thirty-seven static and fifteen dynamic γ axons. Successful impalements were made into thirty-seven intrafusal muscle fibres activated by static γ axons, and nineteen into those activated by dynamic γ axons. Stimulation of the static axons elicited junctional potentials in twenty-three fibres

(62.2%) and action potentials in fourteen (37.8%). Stimulation of the dynamic axons always elicited junctional potentials.

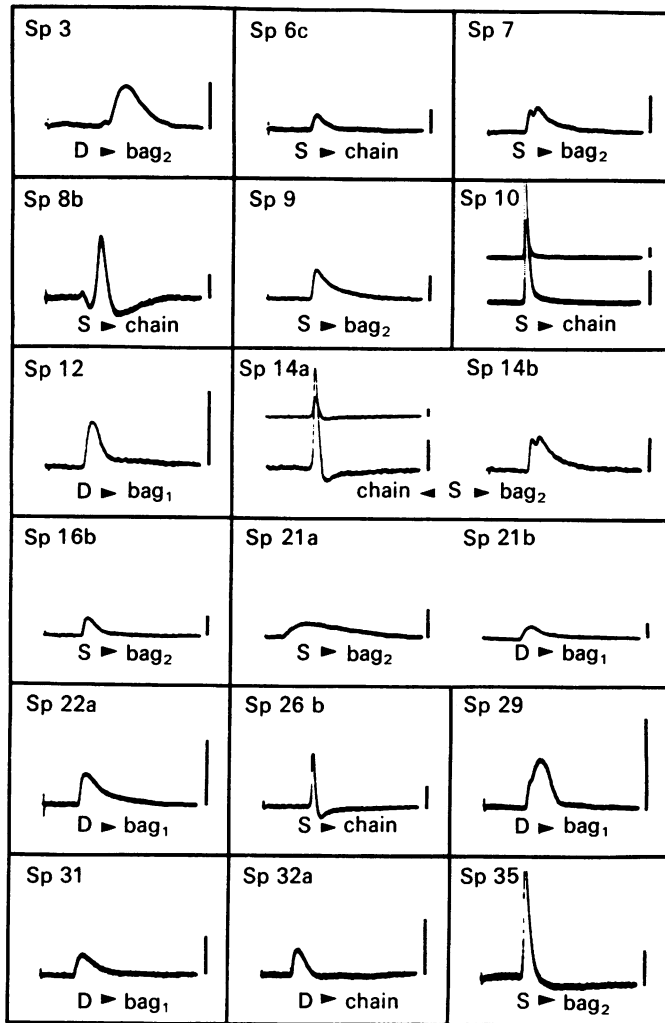
In interpreting the intracellular potentials recorded during these experiments the fact that relatively large micro-electrode tips were used (diameter 0.9–1.9 μm) to facilitate the injection of the fluorescent dye has to be borne in mind. Damage caused by penetration probably resulted in relatively low resting membrane potentials (20–60 mV) of the intrafusal muscle fibres impaled. While deterioration of the spike generation mechanism cannot be excluded, it is reassuring to note that our results compare well with those of Bessou & Pagès (1972), who used micro-electrodes with a smaller tip diameter (0.3–0.9 μm). In that study the ratio of action potentials to junctional potentials elicited on stimulating static γ axons was 1:2 as compared with 1:1.65 in our experiments. In both studies all the potentials elicited on stimulating dynamic γ axons were junctional.

Text-fig. 1 shows the intracellular potentials recorded from seventeen intrafusal muscle fibres that were successfully injected with Procion Yellow, and from one fibre (in spindle 3) in which the injection failed but nevertheless caused selective damage such that the fibre could subsequently be identified. This Text-fig. should be compared with Table 1, which provides a correlation between the results of intracellular recording and the histological identification of muscle-fibre type. Both the membrane potential values of the muscle fibres, and the amplitude of the potentials elicited by fusimotor axon stimulation, were generally smaller than those recorded by Bessou & Pagès (1972). The amplitudes of the action potentials ranged from 35 to 60 mV and the respective resting membrane potentials from 30 to 50 mV. The junctional potentials were of 6–15 mV amplitude and 5–40 msec duration, and were built on resting potentials of 20–40 mV. Their shape varied from fibre to fibre. Some showed a shoulder or notch (compound junctional potentials; see spindles 7, 14b, 29) probably resulting from superimposed local potentials elicited by the activation of several synaptic contacts supplied by the stimulated axon to the impaled muscle fibre. Some of the action potentials were preceded by a prepotential (see spindles 10, 35) indicating that the micro-electrode was close to the activated motor ending. In some instances a small diphasic wave (presynaptic spike potential) preceded the intrafusal muscle potential (see spindles 3, 8b). We interpret this as the intracellular recording of an action potential invading a terminal of the stimulated axon located close to the impalement site (see Hubbard & Schmidt, 1963; Jankowska & Roberts, 1972; Bessou & Pagès, 1972).

Despite the small amplitude of the potential recorded from the chain fibre in spindle 8b, and the absence of overshoot above the zero membrane potential, we regard it as an action potential since the extent of the depolarization (24 mV) is much greater than that of a junctional potential. Moreover, the presence of a positive afterpotential indicates that the spike generation mechanism has been impaired owing to membrane damage. Such damage is probable since the resting membrane potential was low (30 mV), and it so happened in this experiment that one of our thickest micro-electrode tips (1.5 μm) was used to impale the thinnest type of intrafusal muscle fibre.

Examination of Text-fig. 1 and Table 1 shows that, whether elicited by stimulating static or dynamic γ axons, all the potentials recorded from bag fibres were junctional,

except that recorded from the bag₂ fibre in spindle 35, which was an action potential. It is also apparent that all the potentials recorded from chain fibres activated by static γ axons were action potentials except that in spindle 6c. However, it is possible that in this instance the chain fibre recorded from was damaged, for prior to recording a non-propagated potential two muscle-fibre impalements (6a and 6b) had been made with the same micro-electrode in neighbouring areas while stimulating the same static γ axon. Recordings made during the first impalement (6a) revealed a



Text-fig. 1. Intrafusal muscle potentials elicited on stimulation of single static or dynamic γ axons. The spindle (Sp) number is indicated at the top left of each recording, successive impalements in the same spindle being indicated by letters (a, b, c). The functional type of γ axon stimulated (D, dynamic; S, static), and the type of intrafusal muscle fibre, histologically determined, providing the recorded potential, are shown below each recording. The vertical bar on the right in each case represents 10 mV. Sweep duration 20 msec in all but spindle 3 (10 msec) and spindle 21a (50 msec). In spindle 10 and spindle 14a the same potential was recorded at low and high amplification.

resting membrane potential of 50 mV and an action potential, but membrane damage was suspected since 30 sec after impalement the resting membrane potential had decreased to 15 mV and the action potential had become an abortive spike of 10 mV amplitude. It is possible that the second and third impalements (6b and 6c) were made into the same muscle fibre as the first, since the resting membrane potential recorded was low (15 mV) and the responses elicited were not spikes but local potentials of low amplitude (10 and 7 mV, respectively).

TABLE 1. Identification of intrafusal muscle fibres in cat tenuissimus spindles activated by static or dynamic γ axons following recording of intracellular potentials and injection with Procion Yellow

Criteria used for identifying fibre types denoted by letters as follows. Bag fibres: *a*, nuclear bag observed. Bag₁ fibres: *b*, associated with few elastic fibres in extracapsular polar region; *c*, dissociated from chain fibres equatorially; *d*, shortest bag fibre present. Bag₂ fibres: *e*, associated with many elastic fibres in extracapsular polar region; *f*, associated with chain fibres equatorially; *g*, longest bag fibre present. Chain fibres: *h*, nuclear chain observed; *i*, M line present equatorially; *j*, small diameter; *k*, shorter than bag fibres; *l*, associated with bag₂ fibre equatorially. Asterisk indicates fibre marked by selective damage caused by injection failure. c.v.: conduction velocity; eq.ref.pt.: equatorial reference point

Expt and spindle no.	Axon c.v. (m/sec)	Distance (mm) of impalement from eq.ref.pt.	Intracellular potential		Fibre type injected			Identification criteria satisfied
			junc-tional	action	bag ₁	bag ₂	chain	
Static axons								
2	25.4	2.0	+	.	.	+*	.	<i>e</i>
4b	34.5	2.3	+	.	.	+	.	<i>e</i>
6c	29.7	1.3	+	? (see text)	.	.	+	<i>j, k</i>
7	37.0	2.0	+	.	.	+	.	<i>e, f</i>
8b	29.0	1.2	.	+	.	.	+	<i>i, j, k</i>
9	32.5	1.4	+	.	.	+	.	<i>e</i>
10	38.0	1.6	.	+	.	.	+	<i>h, i, j, k, l</i>
14a	} same axon	2.0	.	+	.	.	+	<i>h, i, j, k, l</i>
b		32.2	1.4	+	.	.	.	<i>a, e, f, g</i>
16b	35.0	1.8	+	.	.	+	.	<i>a, e, f, g</i>
21a	32.4	3.3	+	.	.	+	.	<i>a, e, f, g</i>
26b	42.1	2.0	.	+	.	.	+	<i>h, i, j, k, l</i>
35	26.1	1.7	.	+	.	+	.	<i>a, e, f, g</i>
Dynamic axons								
3	37.1	1.8	+	.	.	+*	.	<i>a, f</i>
12	37.0	1.5	+	.	+	.	.	<i>a, b, c</i>
21b	36.3	2.6	+	.	+	.	.	<i>a, b, c, d</i>
22a	} same axon	2.3	+	.	+	} same fibre	.	<i>a, b, d</i>
b		41.0	1.3	+	.		+	.
23	38.7	2.4	+	.	+*	.	.	<i>b, d</i>
25	37.1	2.1	+	.	+	.	.	<i>b, d</i>
29	32.3	1.4	+	.	+	.	.	<i>a, b, d</i>
31	36.3	1.0	+	.	+	.	.	<i>a, b, c, d</i>
32a	} same axon	2.5	+	.	.	.	} same fibre	<i>a, b, c, d</i>
b		41.0	2.0	+	.	.		+

Injection success

Of fifty-two injections attempted, twenty-one were successful in that subsequent sectioning revealed the presence of fluorescing Procion Yellow inside the muscle fibre in the region of micro-electrode impalement. In twenty-six injections the micro-electrode tip slipped out prematurely after 1–8 min; seven of these (duration 4–7 min) nevertheless proved successful. Of the remainder, eleven of nineteen injections were successful after passage of current for 10–18 min, and three of seven after 25–30 min. The success rate was thus highest (58%) for injections lasting 10–18 min, and the main factor responsible for failure was probably insufficient injection time. In the case of some failures it is evident that damage to the fibre led to the dye leaking out. The length of time allowed for the dye to diffuse inside the fibre after injection appeared to be of no consequence, fluorescence being obtained after diffusion times varying from 1 to 60 min.

Identification of intrafusal muscle fibres activated by static γ axons

Twelve of the thirty-three injections into intrafusal fibres activated by static γ axons marked them successfully for fluorescence microscopy. One of the injections that failed nevertheless selectively damaged the activated fibre so that it could be identified. Thirteen fibres were thus marked for identification of which eight proved to be bag₂ fibres and five chain fibres. Action potentials were recorded from one bag₂ and four chain fibres; in the rest of the fibres the potentials were junctional. The results are summarized in Table 1 and examples of fluorescence in injected fibres are illustrated in Pl. 1, figs. 1–3, 6.

In one spindle two fibres activated by the same static axon were successfully injected in the distal pole (see Table 1, spindle 14a, b). Impalement of the first fibre was achieved at the first attempt 2.0 mm from the equatorial reference point. After propagated action potentials had been recorded (see Text-fig. 1, spindle 14a) current was passed and Procion Yellow injected until, after 4 min, the micro-electrode slipped out. Two further attempts to impale fibres at distances of 1.7 mm and 1.5 mm from the reference point failed, but a third succeeded at 1.4 mm. On this occasion a current was passed for an injection time of 25 min. During the passage of current, spontaneous miniature potentials were observed, suggesting that the micro-electrode might be close to activated motor terminals. Subsequent histological examination revealed that the first injection had been made into a chain fibre, the second into a bag₂ fibre. Both fibres had been impaled in the intracapsular part of the pole, the injection site in the chain fibre lying only 300 μ m from its point of insertion. Sections were fluorescent over a length of about 0.5 mm in both fibres, the injected regions overlapping so that over a short stretch sections showed fluorescence in both fibres (Pl. 1, fig. 3). The indications from intracellular recording of the presence of a motor ending close to the impalement point in the second fibre were substantiated by finding motor terminals in sections of the bag₂ fibre cut 100 μ m proximal from this point. The terminals spread over a length of 90 μ m and were connected to an axon which could be traced in serial section to branch and supply two endings to the injected chain fibre, one with terminals occupying a length of 16 μ m, the other 32 μ m. Since these were the only motor terminals innervating the chain fibre it may be assumed that

they, together with their partners on the bag₂ fibre, were the terminals supplied by the static axon which activated the two fibres injected in this experiment. Branches of the static axon were also traced to terminals on two other chain fibres. The ultrastructure of motor endings belonging to γ axons activating injected fibres in these experiments is described in a later section (see pp. 159–160).

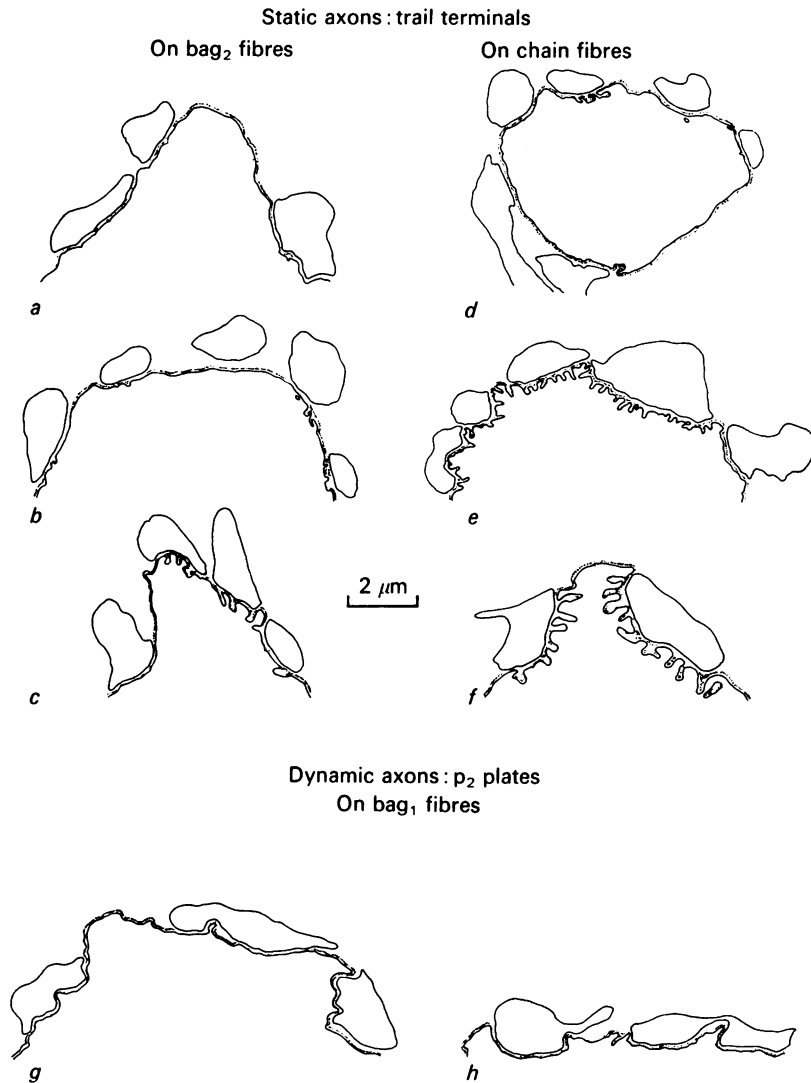
Recordings of intracellular potentials indicated the proximity of the micro-electrode tip to activated motor terminals in four other experiments, namely, those on spindles 7, 8b, 10 and 35. The terminals were sectioned for ultrastructural observation in two of these spindles, 8b and 10. In spindle 8b the action potential recorded prior to injection was preceded by a small presynaptic spike potential, and in spindle 10 the recorded action potential was preceded by a prepotential (Text-fig. 1, spindles 8b, 10). In each case the injection produced fluorescence in a chain fibre, that in spindle 8b being the longest of five present in the proximal pole. A motor ending lay close to the impalement point in both fibres, in one (8b) 140 μm proximal to it and 100 μm long, in the other (10) 170 μm proximal and 60 μm long. In spindles 16b and 21a the nearest motor endings found innervating the injected bag₂ fibres lay, respectively, 370 μm and 2070 μm distal to impalement; both were connected to further terminals lying 530 μm (16b) and 2160 μm (21a) distal. The junctional potentials recorded had given no hint of their presence, but the endings were presumably those that activated the fibres, since they were the only motor terminals supplied to them in the injected poles. The axon that supplied the terminals to the bag₂ fibre in spindle 21a was traced in serial sections and found also to supply terminals to four chain fibres.

Identification of intrafusal muscle fibres activated by dynamic γ axons

Injections into seven of nineteen intrafusal fibres activated by dynamic γ axons were successful. In two experiments the same fibre was marked by two injections. Of the injections that failed, two nevertheless damaged the activated fibre selectively so that it could be identified. Nine fibres were thus marked for identification of which seven were bag₁ fibres, one a bag₂ fibre, and one a long chain fibre. All the intracellular potentials recorded from these fibres were junctional (see Text-fig. 1). The results are summarized in Table 1, and examples of fluorescence in injected fibres are illustrated in Pl. 1, figs. 4–5.

One of the successfully injected bag₁ fibres belonged to the proximal pole of the same spindle in which a bag₂ fibre activated by a static γ axon had previously been successfully injected (see Table 1, spindle 21a, b and Pl. 1, fig. 5). Both fibres were impaled at the first attempt at distances of 3.3 mm (bag₂) and 2.6 mm (bag₁) from the equatorial reference point, and were injected for 4 min and 25 min, respectively. Two motor endings were located on the bag₁ fibre, one 25 μm long, lying 710 μm distal to impalement, the other 15 μm long, lying 1000 μm distal. These were the only motor endings innervating the proximal pole of this bag₁ fibre so it may be assumed that one or both belonged to the dynamic γ axon stimulated. The endings were evidently not close enough to impalement to modify the potentials recorded before injection. This was also the case in spindle 12 where a 22 μm long ending was located on the injected bag₁ fibre 460 μm distal to impalement; serial sectioning established that it was the only motor ending supplied to the fibre in the injected pole.

In spindle 3 the extracellular recordings showed a notch in the declining phase of the junctional potential suggesting that at least two synaptic contacts were located on the impaled part of the fibre, and the intracellular recordings indicated the proximity of motor terminals since the junctional potentials were preceded by clear presynaptic spike potentials (see Text-fig. 1, spindle 3). Histological examination confirmed that



Text-fig. 2. Outline tracings made from electron micrographs of transverse sections through the myoneural junctions of static (*a-f*) and dynamic (*g, h*) γ axons. The terminals shown were all located on injected intrafusal muscle fibres except those in *f*, which were supplied by a branch of the stimulated static axon to a chain fibre that was not injected. The junctions of the static (trail) terminals are seen to be both smooth and folded, folding being most pronounced on chain fibres; those of dynamic (p_2 plate) terminals are smooth. The sections were obtained from the following experiments: *a*, 16b (see also Pl. 3, fig. 1); *b*, 14b; *c*, 21a; *d*, 10 (see also Pl. 2, fig. 1); *e*, 14a (see also Pl. 2, fig. 2); *f*, 21a; *g*, 21b (see also Pl. 3, fig. 2); *h*, 12.

motor terminals were indeed present at the impalement site. They were supplied by two axon branches to a bag₂ fibre and extended over a length of 50 μm . Unfortunately the injection failed and damage to the fibre was such that it was constricted and hollow at the site of impalement. An underlying chain fibre was damaged, but the adjacent bag₁ fibre escaped intact. The positional relationships of the bag and chain fibres in the equatorial region leave no doubt that the type of bag fibre activated in this experiment was bag₂.

Ultrastructure of the motor endings of static and dynamic γ axons

Static axons. As has already been noted (pp. 156–157) there were several instances when it proved possible to examine the ultrastructure of a motor ending which almost certainly belonged to the static γ axon stimulated during the experiment. We may identify these endings as belonging to trail endings, since single static γ axons have been observed to supply trail endings to tenuissimus spindles in which all other motor axons had degenerated (Barker, Emonet-Dénand, Laporte, Proske & Stacey, 1973). Ultrathin sections of spindles 8b, 10, 14a, b, 16b and 21a provided electron micrographs of the axon terminals of nine trail-ending ramifications that activated injected fibres, five supplied to bag₂ fibres and four to chain fibres. Terminals belonging to a further six trail-ending ramifications were supplied by branches of the stimulated static axon to chain fibres that were not injected. The length of the ramifications, 16–100 μm , compares with a range of 10–210 μm measured in teased, silver preparations (Barker *et al.* 1970). Representative myoneural junctions are shown as outline tracings in Text-fig. 2*a–f* and are illustrated in Pl. 2, figs. 1, 2 and Pl. 3, fig. 1.

The only characteristic presynaptic feature of trail endings evident in electron micrographs is the frequent presence of preterminal axons running in between muscle fibres. Post-synaptically, smooth and folded junctions occur on both bag₂ and chain fibres. The deepest and most regular folding occurs on chain fibres (compare Text-fig. 2*a–c* with *d–f*); thus eight of the deepest folds on bag₂ fibres had a mean depth of 250 nm (range 200–350 nm), whereas the mean depth of thirteen deep folds on chain fibres was 450 nm (range 250–650 nm). Terminals with smooth junctions may be present in the same section as those with folded junctions (see Text-fig. 2*c* and *e*; Pl. 2, fig. 1). Probably each trail-ending ramification shows some degree of junctional folding amongst its synaptic contacts. We were unable to make detailed observations on this point since our ultrathin sections were not cut serially but sampled. There appeared to be no ultrastructural difference between trail-ending ramifications located at different polar levels, or between those that generated spikes rather than junctional potentials.

Dynamic axons. There were three experiments (3, 12 and 21b) in which it proved possible to examine the ultrastructure of terminals that almost certainly belonged to the stimulated dynamic γ axon. These terminals may be regarded as p₂ plates, since the ultrastructure of plates found at the site of observed local contractions produced by stimulating a dynamic γ axon is very similar to that of p₂ plates previously located and photographed in spindles stained with methylene blue (Barker *et al.* 1976*a*; Banks *et al.* 1976). The myoneural junctions of such plates are smooth.

The four plates sectioned in these experiments had lengths of 15, 22, 25 and 50 μm ; Barker *et al.* (1970) reported an average length of 72.9 μm (range 27–120 μm) for p_2 plates in teased, silver preparations. The plate in spindle 3 was located at the site of impalement (see p. 158) and was too damaged to yield any reliable post-synaptic information. Representative myoneural junctions belonging to the plates from spindles 12 and 21b are shown as outline tracings in Text-fig. 2*g, h* and are illustrated in Pl. 3, fig. 2.

All the sections sampled from these p_2 plates show smooth myoneural junctions. Such sections could equally well have been cut through part of a trail-ending ramification in which all the junctions happened to be smooth (compare Text-fig. 2*a* and *g*). Extensive sectioning of a γ ending is essential if the presence or absence of post-synaptic folds is to be relied on as a criterion for distinguishing between trail and p_2 terminals.

DISCUSSION

At the time our experiments were being carried out in 1971–3 there were many (e.g. Boyd, 1971; Matthews, 1972) who considered that the mammalian muscle spindle was composed of two types of intrafusal muscle fibre, bag and chain, and that these were to a large extent selectively innervated by dynamic and static γ axons, respectively. Such was the attraction of this morphological and functional duality that there was a tendency to minimize histological evidence that indicated non-selective fusimotor innervation (e.g. Barker & Ip, 1965; Corvaja, Marinozzi & Pompeiano, 1969; Barker *et al.* 1970; Kennedy, 1970) and the existence of more than two types of intrafusal fibre (e.g. Ogata & Mori, 1962, 1964; Yellin, 1969; Barker & Stacey, 1970). This situation was altered by the findings of a number of investigations in which experimental procedures were combined, as in the present study, with histological or cinematographic analysis (Barker *et al.* 1973; Bessou & Pagès, 1975; Boyd & Ward, 1975; Brown & Butler, 1973, 1975). These studies demonstrated that whereas dynamic γ axons are almost exclusively distributed to bag fibres, static γ axons do not exclusively supply chain fibres, but are frequently distributed to both bag and chain fibres. The results of our own 1971 experiments (Barker *et al.* 1972*a*) showed that of ten intrafusal fibres activated by single static γ axons and injected with Procion Yellow six were bag fibres (in the final analysis the bag/chain ratio is 8/5; see Table 1). Moreover, one of these experiments (14*a, b* in Table 1) demonstrated the activation of a bag and a chain fibre by the same static γ axon (see Pl. 1, fig. 3).

With the recognition of two types of bag fibre firmly established (Banks *et al.* 1975, 1977; Gladden, 1976) it is now generally agreed that the mammalian spindle is composed of three types of intrafusal fibre, namely, bag₁ fibres responsible for dynamic actions, and bag₂ and chain fibres responsible for static actions. This has become evident from the cinematographic analysis of intrafusal contractions elicited by the stimulation of single γ axons (Bessou & Pagès, 1975; Boyd & Ward, 1975; Boyd, Gladden, McWilliam & Ward, 1977), and the mapping of zones of glycogen depletion produced in intrafusal muscle fibres by prolonged tetanic stimulation of single γ axons (Brown & Butler, 1973, 1975; Barker, Emonet-Dénand, Harker, Jami & Laporte, 1974, 1976*b*). In comparing the results of these studies with our own it is important to note that whereas the glycogen-depletion method reveals the distribu-

tion of the stimulated axon amongst, at best, all the spindles it innervates, the other techniques are concerned with its distribution within one particular spindle, injection with Procion Yellow being restricted to only one, or at the most two (as in spindle 14), of the activated intrafusal fibres in one pole.

In the nine experiments in which single dynamic γ axons were stimulated, the activated intrafusal fibre that was injected with Procion Yellow, or selectively damaged by injection failure, proved to be a bag₁ fibre in seven spindles, a bag₂ fibre in one spindle, and a long chain fibre in another (see Table 1). These results agree well with those from experiments in which glycogen depletion is produced in intrafusal fibres on stimulating single dynamic γ axons. Barker *et al.* (1976*b*) found that bag₁ fibres were depleted in each of seventeen spindles activated by four dynamic γ axons; in three of them other fibre types were also depleted, in one a bag₂ fibre, in two a long chain fibre. Taken together the two investigations indicate that in addition to bag₁ fibres dynamic γ axons activate bag₂ or longchain fibres in about one in every four or five spindles. Such activation should result in a static modification of the dynamic action and, since most spindles are innervated by one dynamic γ axon (Boyd *et al.* 1977), it is significant that of forty-four single γ axons producing a predominantly dynamic action Emonet-Dénand, Laporte, Matthews & Petit (1977) found that ten produced an action with suspected static modification. We may note, however, that those who have observed and filmed contractions in living spindles (Bessou & Pagès, 1975; Boyd & Ward, 1975; Boyd *et al.* 1977) have not seen them to occur in chain fibres on stimulating dynamic γ axons. Nor have Boyd *et al.* (1977) found bag₂ fibres to respond to such stimulation, but in a recent experiment we (P.B. and B.P.) have observed a dynamic γ axon to elicit contractions in two bag fibres present in a spindle, and these were subsequently established histologically to be one bag₁ and one bag₂ fibre (R. W. Banks, D. Barker, P. Bessou, B. Pagès & M. J. Stacey, in preparation).

Whereas it is generally agreed that static γ axons activate bag₂ and chain fibres, opinions differ as to whether they also ever activate bag₁ fibres. Observations on living spindles suggest that they do not, since a bag fibre activated by a dynamic γ axon has never been seen to be activated also by a static γ axon (Bessou & Pagès, 1975; Boyd & Ward, 1975; Boyd *et al.* 1977). On the other hand, glycogen-depletion studies indicate that static γ axons activate both types of bag fibre about equally (Barker *et al.* 1976*b*), and Emonet-Dénand *et al.* (1977) provide strong indirect evidence (if their interpretation is correct) of the participation of bag₁ fibres in about one third of predominantly static primary-ending responses. The results of our own experiments with static γ axons show that among the intrafusal fibres they activated in twelve spindles there were eight bag₂ and five chain fibres identified with the aid of Procion Yellow injection or selective damage caused by injection failure (see Table 1). In one experiment in which the same spindle (21a, b) was separately activated by single dynamic and static γ axons the injected fibres were identified as bag₁ and bag₂, respectively (see Pl. 1, fig. 5).

Is it significant that our static sample does not include a bag₁ fibre? Apart from the small number of observations involved, we think not for two reasons. (*i*) The nature of the technique is such that we have no information about other intrafusal fibres that may have been activated, either in the pole containing the injected fibre

or in the other pole. Data from the glycogen-depletion experiments of Barker *et al.* (1976*b*) for spindles innervated by static γ axons conducting over a range of velocities (28–45 m/sec) comparable to our own (25.4–42.1 m/sec) show that among eighteen whole and five half-spindles bag₁ (b₁), bag₂ (b₂), and chain (c) fibres were depleted in thirty poles in the following combinations and percentages: b₂c, 33.3; c, 20.0; b₁b₂c, 13.3; b₂, 10.0; b₁c, 10.0; b₁, 6.7; b₁b₂, 6.7. Thus in this sample the chances of impaling a pole in which bag₂ and/or chain fibres were activated would have been about 6 in 10, as against a 4 in 10 chance of impaling a pole in which a bag₁ fibre was being activated alone or in addition to bag₂ and/or chain fibres. Had the spindles in our static sample been activated by a considerable proportion of slow-conducting axons the chances of impaling a pole in which a bag₁ fibre was being activated might have been much higher, since Barker *et al.* (1976*b*) found glycogen depletion among bag fibres to be restricted to the bag₁ type in spindles innervated by static γ axons with conduction velocities of 19 and 23 /sec. (ii) Most of the impalements were intracapsular and made within 2.0 mm of the equatorial reference point. For most of this region the ultrastructure of the bag₁ fibre differs from that of the bag₂ and chain fibres (Barker *et al.* 1976*a*; Banks *et al.* 1977). It is conceivable that some factor associated with this difference introduces a bias in favour of impaling bag₂ or chain fibres in this region in poles where there is also bag₁ fibre activation. Such a bias could also be regarded as operating in experiments 3 and 32 in our small dynamic sample (see Table 1).

Stimulation of nine dynamic γ axons at 1/sec in our experiments always elicited junctional potentials, whether recorded from bag₁ (7), bag₂ (1), or chain (1) fibres. Bessou & Pagès (1972) stimulated at frequencies of up to 150/sec and also always recorded junctional potentials. It must be noted, however, that none of the recordings from bag₁ fibres was made from sites of impalement located in the extracapsular region, where their ultrastructure corresponds with that of bag₂ and chain fibres. Until recordings from bag₁ fibres are made in this region, well clear of the capsule limits, the possibility that they are capable of propagating action potentials cannot be excluded.

Our ultrastructural findings show that the myoneural junctions of trail endings are both smooth and folded on bag₂ and chain fibres, the deepest and most regular folding occurring on chains. They also show that the junctions of p₂ plate terminals located on bag₁ fibres are smooth. Whether folding in such junctions occurs on bag₂ and chain fibres, and whether there are any post-synaptic differences between p₂ plates located in the contrasting ultrastructural regions of bag₁ fibres, has yet to be established.

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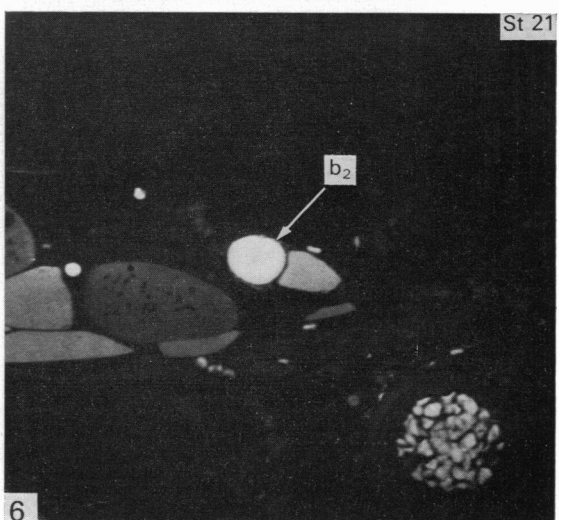
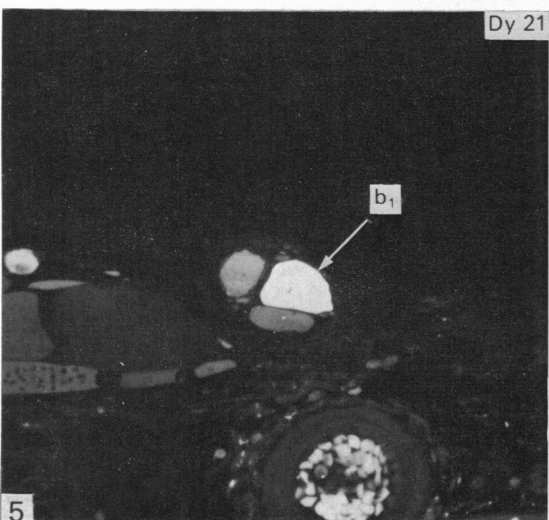
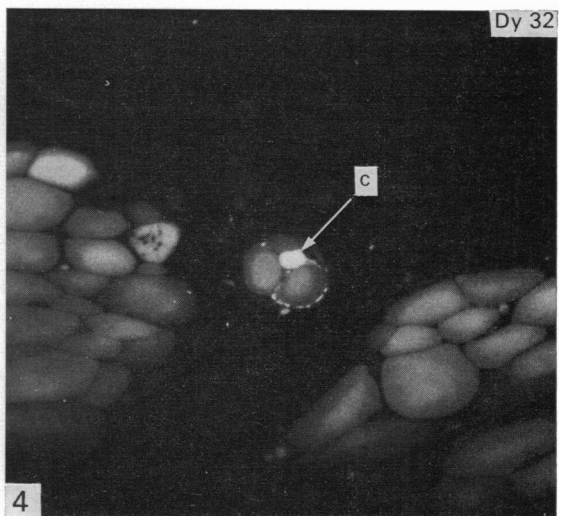
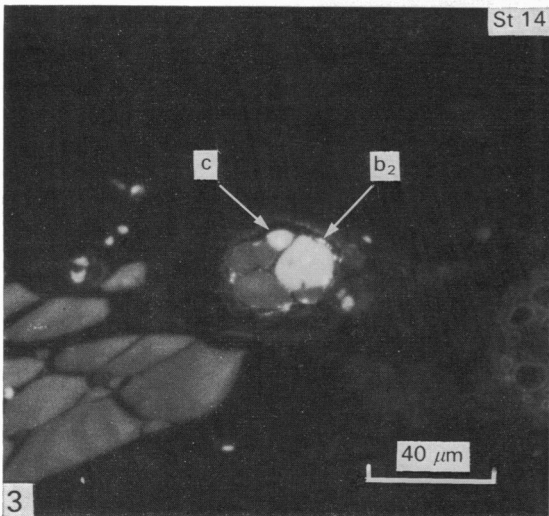
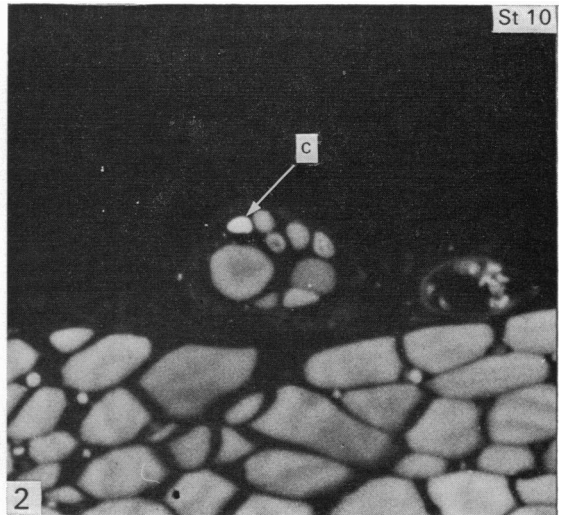
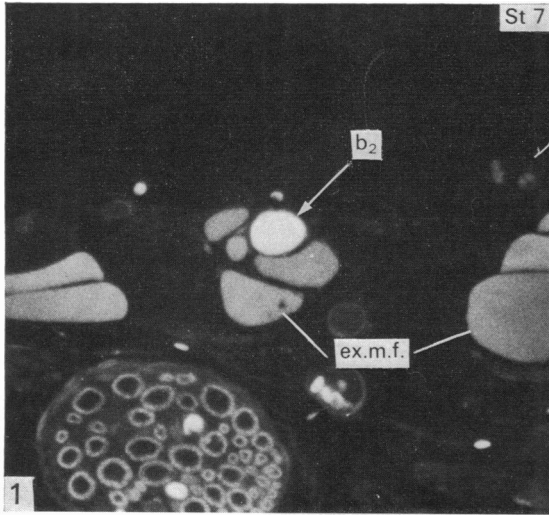
EXPLANATION OF PLATES

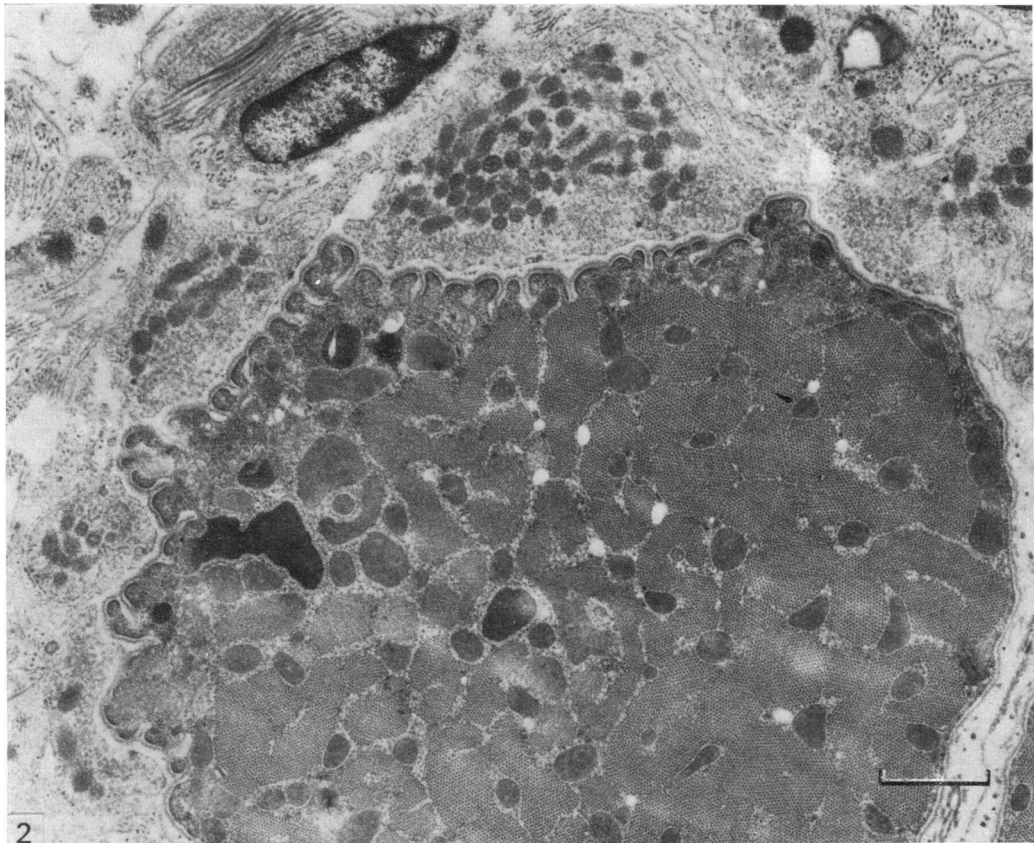
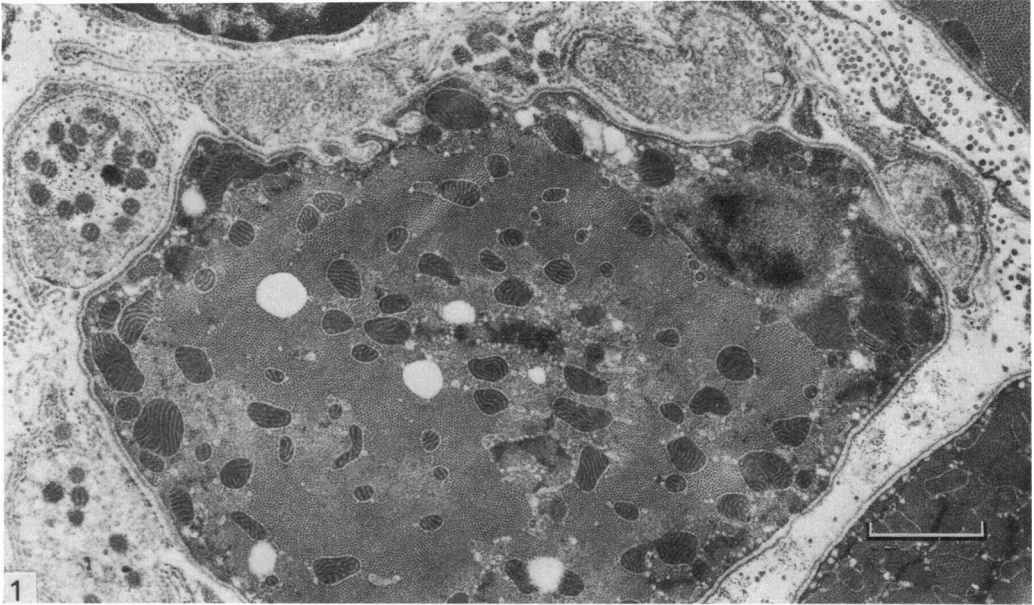
PLATE 1

Transverse sections of cat tenuissimus spindles showing fluorescence of muscle fibres (arrowed) impaled and injected with Procion Yellow following recording of their membrane potentials on activation by single static (St; figs. 1-3, 6) or dynamic (Dy; figs. 4, 5) γ axons. Experiment and spindle number is placed in top right-hand corner of each figure. All photographs to scale shown in fig. 3. The sections also include extrafusal muscle fibres (ex.m.f.); an intramuscular nerve trunk (fig. 1, bottom left); a venule (fig. 2, right of spindle); an arteriole (figs. 5, 6, bottom right). In fig. 4 note bright autofluorescence of elastic fibres around the periphery of the muscle fibre below the fluorescing chain fibre; this is a bag₂ fibre. b₁, bag₁ fibre; b₂, bag₂ fibre; c, chain fibre.

PLATES 2 AND 3

Electron micrographs of transverse sections through terminals belonging to the single γ axon stimulated in the experiment concerned. Since post-fixation in osmium tetroxide degrades the fluorescent properties of Procion Yellow, it had to be omitted from the preparation of the material; hence no cell membranes are visible in the sections. The presence of holes among the myofibrils represents injection damage; all the sections illustrated were cut within a few hundred microns of the site of impalement and injection of the muscle fibre. Bar line on each micrograph measures 1 μ m.





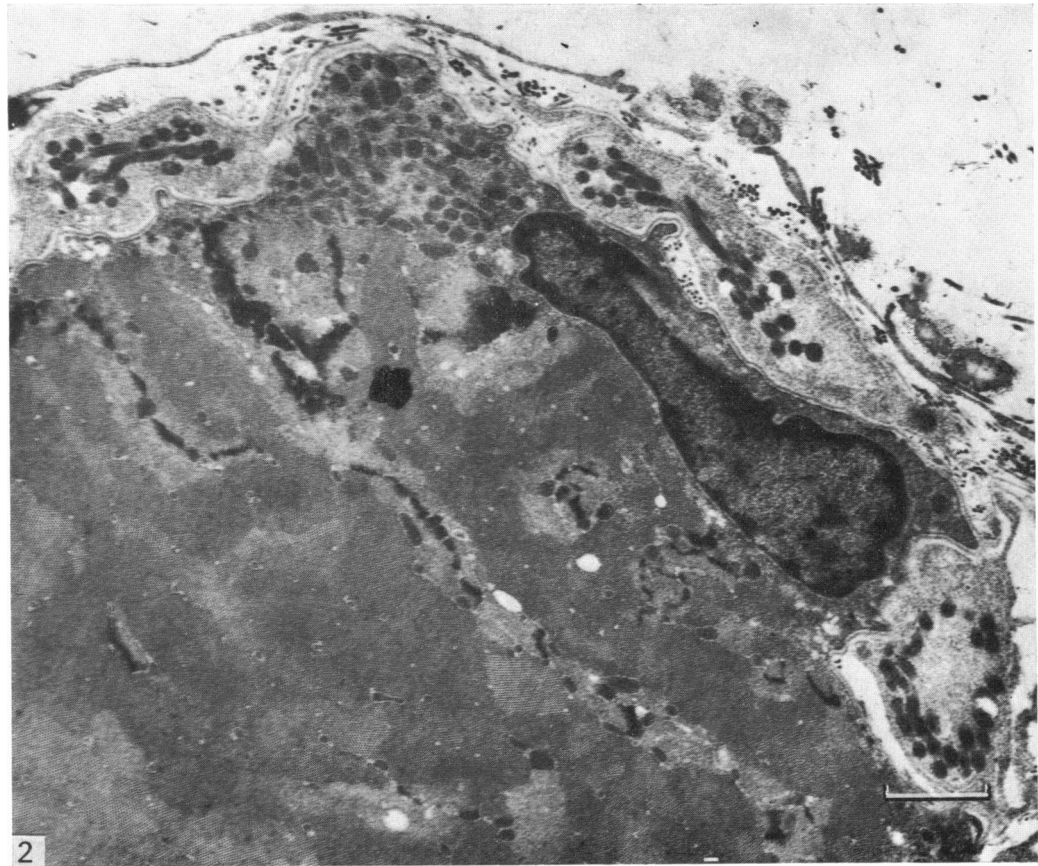
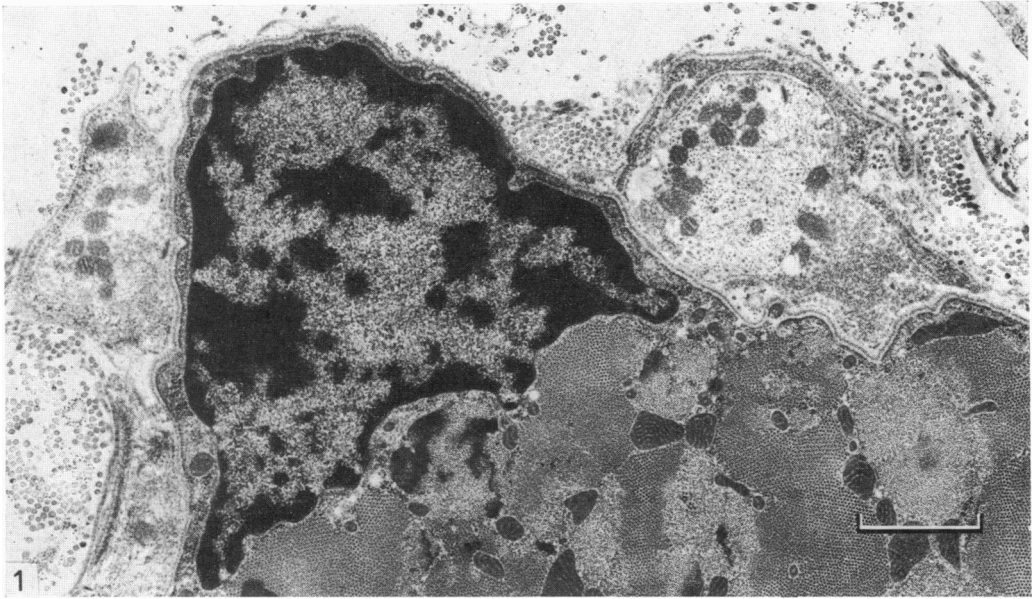


PLATE 2

Fig. 1. Experiment 10; static (trail) terminals on chain fibre 200 μm from site of injection. Post-synaptic folds present in one myoneural junction, absent from the others (cf. Text-fig. 2*d*).

Fig. 2. Experiment 14a; static (trail) terminals on chain fibre 800 μm from site of injection. Post-synaptic folding present in all myoneural junctions except one (cf. Text-fig. 2*e*).

PLATE 3

Fig. 1. Experiment 16b; static (trail) terminals with smooth myoneural junctions on bag₂ fibre 544 μm from site of injection (cf. Text-fig. 2*a*).

Fig. 2. Experiment 21b; dynamic (p₂ plate) terminals with smooth myoneural junctions on bag₁ fibre 720 μm from site of injection (cf. Text-fig. 2*g*).