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MOTOR UNITS IN THE RAT DIAPHRAGM

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The electrical 'units' recorded in electromyographic studies are commonly assumed to represent the activity of motor units (Smith, 1934; Seyffarth, 1940; Denny-Brown, 1949; Gordon & Holbourn, 1949; Bigland & Lippold, 1954; Li, Shy & Wells, 1957). This would seem to imply that the muscle fibres innervated by a single motor nerve fibre are grouped in compact bundles. It is known, however that efferent fibres supply the widely separated end-plates of muscle fibres arranged end to end in the cat tenuissimus (Adrian, 1925) and sartorius muscles (Cooper, 1929). The possibility clearly exists that, in all muscles, fibres of one motor unit may be much dispersed.

Evidence for the manner in which fibres are actually distributed is both scanty and indirect. The scatter of atrophied patches in disease of the motoneurone (Wohlfart, 1949) and after experimental section of spinal roots (Van Harreveld, 1947) suggests that the motor unit does not form a single compact bundle. The spatial spread of potentials recorded by electromyography also points to a substantial overlap of active units (Jasper & Ballem, 1949; Buchthal, Guld & Rosenfalck, 1957). According to the last authors, in the human biceps the motor unit extends over a region with a diameter of about 5 mm. A direct anatomical analysis of the ramifications of a single motor nerve fibre is made exceedingly difficult by the profuse branching and interlacing of fibres in the muscle. Nevertheless, Tergast (1873) and Feindel (1954) have claimed that under special conditions they could follow the branches of single fibres, in cat and rabbit muscles respectively. Their descriptions show clear overlap of motor units; muscle fibres of the same motor unit may be separated by as many as eight unrelated fibres according to Feindel.

The distribution of a motor unit in a muscle can be shown by isolating and then stimulating a single motor nerve fibre and analysing the corresponding muscle action potentials. This approach has not hitherto been attempted with unqualified success (Denslow & Gutensohn, 1950, 1951). By such a method we

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have found that in the rat diaphragm the muscle fibres activated by one single motor nerve fibre are irregularly scattered over a relatively wide region.

METHODS

Dissection. Albino rats (Wistar origin) weighing 100–200 g were anaesthetized with ether. The two halves of the diaphragm were removed, together with the phrenic nerves, and left for some minutes in a Ringer–Locke solution aerated with 5% CO_2 in O_2, at room temperature. A single motor nerve fibre was isolated from the phrenic nerve at a point about 2–3 mm away from the corresponding hemidiaphragm and all the remaining fibres were cut. For confirmation, all preparations were examined either at the beginning or the end of an experiment with a microscope ($\times 60$). The hemidiaphragm was trimmed so as to preserve only that portion which contained the active motor unit.

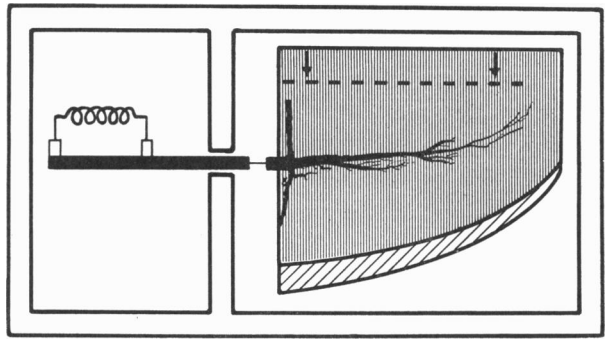

Fig. 1. Diagram showing the recording chamber, with the phrenic nerve on stimulating electrodes on the left, and a portion of the diaphragm on the right. The broken line indicates the approximate path of the surface electrodes which recorded the potentials illustrated in Fig. 2; the two arrows mark the area of distribution of that motor unit.

Recording chamber (Fig. 1). The preparation was fixed in a Perspex chamber with two compartments separated by a sliding partition. On one side the muscle and the distal part of the phrenic nerve, including the isolated fibre, were bathed in Ringer–Locke solution identical with that described by Liley (1956) except for a greater glucose content in some experiments (5 g/l. instead of 2 g/l.). The solution was very effectively stirred by constant vigorous bubbling of the aerating mixture of CO_2 and O_2. It was kept at room temperature (22–24° C), and it was not changed except by the addition of small amounts of fresh solution when, at intervals of some hours, surface froth and scum had to be removed by suction. The proximal part of the phrenic nerve extended through the partition into the adjacent compartment, where it rested on a pair of platinum stimulating electrodes submerged in a pool of liquid paraffin.

Recording technique

The solution in the muscle compartment was earthed through an Ag–AgCl coil in series with a resistance of 50 Ω (across which were applied calibrating and balancing potentials).

A. *External recording.* The electrodes were fine platinum wires (diameter 175 μ) fused in glass, separated by a distance of 0·19 mm. They were connected to the input of an R-C differential preamplifier, and were attached to a micromanipulator (Eccles, Fatt, Landgren & Winsbury, 1954) which made possible a detailed survey of the surface of the muscle. Further amplification was by a conventional R-C amplifier, with a flat frequency response up to about 20,000 c/s.

B. *Intracellular recording.* Glass micropipettes were made in the device described by Winsbury (1954), and then filled with 3 M-KCl by boiling under reduced pressure. They had an electrical

resistance of 7–20 MΩ. They were connected by a saline-agar bridge, and then an AgCl–Ag link to the grid of a cathode follower, whose output was fed into an R.C. amplifier and a millivoltmeter.

Stimulation. The stimulator output consisted of condenser discharges (with a time constant of 15 μsec), slightly modified by an isolating transformer. Care was taken to have the intensity of the stimuli always at least three times the threshold intensity.

Analysis of records. The oscilloscope tracings were photographed with a Grass camera, and then analysed after suitable magnification (× 8–10).

Histological techniques

Glycogen stain. The diaphragm was fixed in 10% (v/v) formol in ethanol saturated with picric acid, dehydrated in absolute ethanol and embedded in paraffin. The 10 μ sections were floated on 70% ethanol and then covered on the slides with a film of celloidin. The sections were stained in haemalum and Best's Carmine. Contact with water was avoided at all stages. Control sections of rat liver were treated in the same manner, and the specificity of the glycogen reaction shown by preliminary spitting on some of these sections.

Phrenic nerve. The nerve was stretched on a rectangular glass frame and fixed in Fleming's fluid. 10 μ paraffin sections were stained in haemalum and counter-stained in aqueous eosin.

RESULTS

The isolated single-fibre preparation is exceedingly fragile, but once set in the recording chamber it will survive under the conditions already described for a period of time more than adequate for the purposes of the experiment. In two cases, single fibres conducted impulses to the diaphragm for 12 hr; during most of this time the fibre was stimulated at frequencies varying between 2 and 25/sec, and, although at the end of this period there was a diminution in contraction, the isolated fibre had not obviously deteriorated.

The results are based upon eight successful experiments. In four cases the preparations had only one nerve fibre. In the four remaining cases two fibres were actually left for technical reasons: in three of these preparations, there was only one active motor fibre. This was shown by exhaustive tests for differences in threshold, particularly with two stimuli and during a tetanus, since even a minute difference is usually revealed clearly during the recovery period. Close examination with the microscope was also of help in showing injured parts of one fibre. In one case we had two active motor fibres but the two motor units could be distinguished by a small difference in thresholds. The results obtained with these two-fibre preparations are less obviously significant, but they are included since they confirmed in every respect what was found with single-fibre preparations.

Visual inspection

When a single motor nerve fibre is stimulated it is immediately and very strikingly clear to the naked eye that the active muscle fibres are dispersed over a relatively wide area. Instead of a localized contraction, one sees surprisingly diffuse movement, differing from the normal twitch quantitatively rather than qualitatively. Although we have been able to record electrically

from only eight preparations, we have seen another five single-fibre preparations which did not survive further manipulation. In no case did we observe sharply circumscribed contractions. The region involved differed in various preparations; the largest was as much as half the total area supplied by the phrenic nerve.

With a dissecting microscope (× 10–25), one can discriminate to some extent between different groups of fibres; yet it is almost impossible to identify single contracting muscle fibres. But if the latter are stimulated tetanically, they can be localized approximately, because adjacent resting fibres are usually drawn into a characteristic zigzag pattern. When a single stimulus to the nerve is near threshold, the visible contraction behaves in a perfectly all-or-none manner. However, with repetitive stimulation at higher frequencies (> 10/sec), the distribution and the amount of contraction fluctuate and diminish substantially.

A. *External recording*

The diaphragm is a thin, flat, but comparatively wide muscle which can be surveyed very conveniently with a pair of electrodes probing over its surface. It is also short enough for its fibres to extend from origin to insertion. Accordingly, Coërs (1953) found only a single, narrow innervation band in the rat diaphragm. In confirmation, while recording with intracellular electrodes from widely separated parts of the same muscle fibre, or when stimulating fibres directly after curarization (Krnjević & Miledi, 1958), we have never seen any indication of a series of fibres arranged end to end.

The amplitude of the signals depended of course on the height of the electrodes above the muscle. This was kept approximately constant in any one series of explorations. Care was always taken to avoid dragging electrodes along the surface.

The electrical potentials recorded near the surface of a hemidiaphragm while stimulating a single nerve fibre (2/sec) are shown in Fig. 2. The electrodes moved across the muscle, roughly parallel with the innervation band, but 2–3 mm away from it. Active fibres were spread over an area of the posterior half of the muscle 8·15 mm wide (see Fig. 1). The sequence of variable peaks, troughs and inactive gaps corresponds to the activity of widely dispersed fibres. External recording in a volume conductor with relatively large electrodes gives compound potentials from fibres which are comparatively close together. Better discrimination was obtained with the two electrodes set in line with, rather than at a right angle to, the fibres. Clearly the shape and amplitude of the potentials recorded by such electrodes is determined by their distance from the active fibres, both in a horizontal and in a vertical plane. Although single potentials are not obvious except where one active fibre is relatively isolated (e.g. at 9·15 mm in Fig. 2) in most cases the basic units can be identified because they may differ in, (*a*) the point at which their maximum

amplitude is recorded, (*b*) their latencies, (*c*) their refractory periods, and (*d*) their ability to respond to tetanic stimulation. This laborious counting procedure was only performed in full detail in three cases. The counts were 7, 13 and 17 definite fibres respectively. The fibres were not distributed according to any obvious plan, as can be seen in Fig. 3, which shows diagrammatically the relative position of the active fibres in the muscle irrespective of depth. The

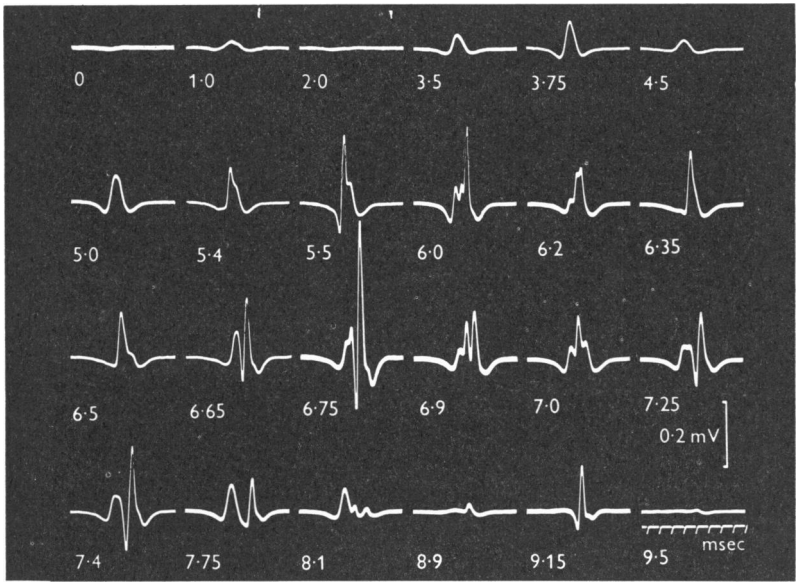

Fig. 2. Distribution of potentials evoked by the activity of a motor unit. Single nerve fibre preparation, stimulated at 2/sec. The records were obtained by displacing a pair of electrodes across the surface of the muscle in a direction perpendicular to that of the muscle fibres and parallel to the branch of the phrenic nerve (about 3 mm distant) that innervates this part of the muscle. The electrodes were oriented in the same direction as the muscle fibres. The numbers beneath each record indicate the distance of the electrodes (mm) from the level at which the phrenic nerve enters the muscle.

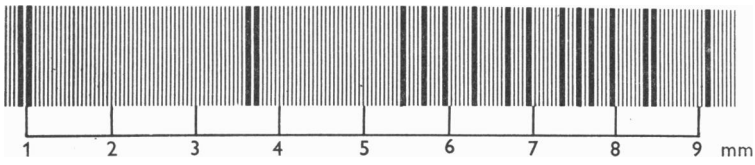

Fig. 3. Schematic representation of the distribution irrespective of depth of muscle fibres in a motor unit. Diagram constructed from data partly shown in Fig. 2. The columns represent fibres of 50μ average diameter. The fibres of the motor unit are illustrated by the solid columns. They are placed according to the position at which the potential of each fibre reached its maximum. Individual fibres were identified by their latency and refractory period.

vertical lines represent very approximately muscle fibres, assuming that their average diameter is 50μ. In fact, the actual density of fibres would have been even greater; we found in histological sections that most fibre diameters were between 25 and 50μ, in agreement with Muscholl (1957).

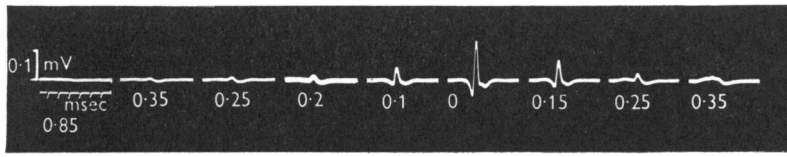

Fig. 4. Potentials recorded at different distances from the muscle fibre shown in Fig. 2 at 9·15 mm. The pair of differential electrodes was oriented in line with the direction of the muscle fibre and moved perpendicular to it. The figure in each record denotes the distance of the electrodes measured in mm from the point at which the potential is maximal. The record at the extreme right shows some complication due to the potential contributed by other fibres of the unit.

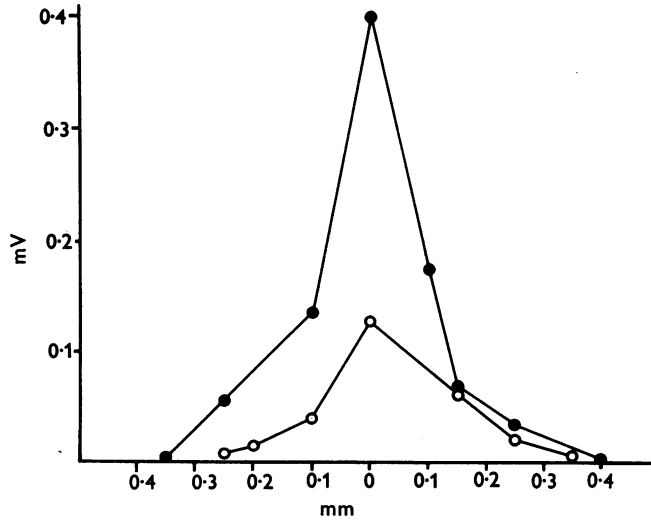

Fig. 5. Relation between distance from an active muscle fibre and amplitude of the potential recorded by a pair of electrodes moving at a right angle to the direction of the fibre. ● and ○ show the relation for the fibres whose maximal potentials are illustrated at 6·75 mm and 9·15 mm respectively in Fig. 2. The point at which the amplitude is maximal is taken as zero distance: i.e. no allowance has been made for the depths at which the fibres were located.

The manner in which the potential from a single muscle fibre varies in shape and amplitude as the differential electrodes approach and then recede, along the surface, is shown in detail in Figs. 4 and 5. Similar changes were seen in an experiment in which the electrodes were moved in a vertical plane, in line with the active fibre. The depths of the active fibres were not measured exactly, but

they were known to within about 0·1 mm. It is clear that with this method of recording the amplitude of the potential from a single fibre is reduced to 10μV or less within a distance of about 0·5 mm.

B. *Intracellular recording*

Intracellular recording has, of course, the great advantage of identifying single active fibres with certainty. However, it is by no means convenient for mapping out the total distribution of a motor unit. The individual fibres are scattered over a volume of muscle which is very large compared to that of one fibre. Localization of contracting areas by inspection gives only a very rough indication; the process of searching for an active fibre with fine micro-electrodes is somewhat like looking for a needle in a haystack.

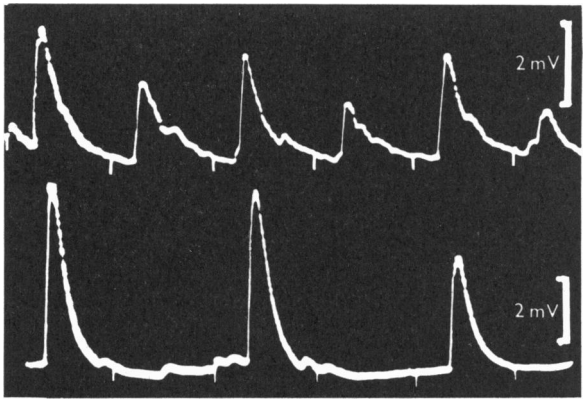

Fig. 6. Intracellular potentials recorded simultaneously from two end-plates of a motor unit. Single nerve fibre preparation stimulated at 64/sec. End-plate potentials are seen superimposed on a background of spontaneous miniature potentials. The distance between the electrodes was approximately 3 mm.

Several conclusions based upon the observations with surface electrodes were confirmed with intracellular electrodes recording from fibres belonging to single motor units.

(*a*) Single active fibres are found over a wide region of muscle.

(*b*) Active fibres may be quite superficial or more or less deep within the diaphragm. It is possible to insert an electrode into fibres showing the characteristic action potential in the 1st and the 8th layer from the surface.

(*c*) The number of fibres identified with intracellular electrodes varied between five and ten in different motor units.

The wide separation between two active end-plates can be shown directly with two micro-electrodes about 3 mm apart (Fig. 6). We have not seen activity in contiguous fibres; the electrode always passed through several silent fibres before it reached another active one.

C. *Behaviour of fibres belonging to a motor unit*

It became clear at an early stage in the course of these experiments that the activity of individual members of the motor unit had certain distinguishing features. We observed definite differences in the latency of arrival of the action potential, in the refractory period, and in the ability of different fibres to respond to repetitive stimulation.

(*a*) Differences in latency are obvious in the records of Fig. 2. The interval between the stimulus artifact and the apex of the potential was measured for eleven separate fibres in one motor unit; the range of values was 5·2–7·6 msec. Only in two cases were the latencies identical. There was a general but by no means regular tendency for the intervals to increase as the electrode moved from the proximal towards the distal edge of the mucle (in a direction roughly parallel with the corresponding intramuscular branch of the phrenic nerve, as shown in Fig. 1).

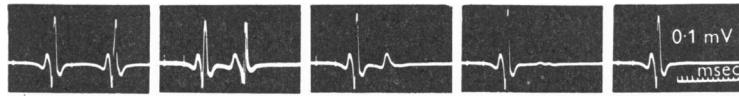

Fig. 7. Effect of decreasing interval between two stimuli on the responses recorded at 7·3 mm in the motor unit shown in Fig. 2. The pairs of stimuli were applied at 2/sec. It can be seen that the four fibres contributing to the potential at this point reach their refractory period at different intervals.

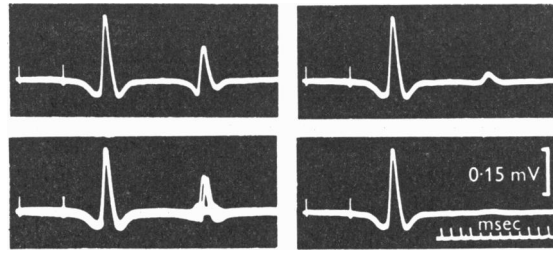

Fig. 8. Responses obtained during the refractory period at 3·7 mm in the motor unit illustrated in Fig. 2. Pairs of stimuli applied at 2/sec, with a constant interval of 4·5 msec. Although the refractory period for neuromuscular propagation is similar in the two muscle fibres that make up the potential recorded from this point, independent small fluctuations cause the fibres to fail asynchronously. Each record is composed of 1–5 superimposed traces.

(*b*) In the wake of a first impulse, differences in the duration of the refractory period for neuromuscular propagation cause certain fibres to become excitable before others (Fig. 7). The refractory period of nine fibres was measured by applying pairs of stimuli at a frequency of 2/sec. The durations ranged from 3·1 to 4·6 msec. Only two pairs of fibres gave identical values. However, even where the refractory periods are similar, individual fluctuations are independent; this causes potentials of the different fibres to fail asynchronously (Fig. 8).

(*c*) Sooner or later all muscle fibres begin to fail to respond when tetanic stimulation (direct or indirect) is above a certain frequency (Krnjević & Miledi, 1958). The time and frequency at which failure occurs, and also the mode of failure, is apparently characteristic of each fibre and not of the motor unit as a whole. This can be shown with extracellular or intracellular electrodes. In Fig. 9 recognizable single components of the externally recorded compound potential are beginning to fail in an all-or-none manner while others maintain their activity. In Fig. 6 end-plate potentials from two separate fibres are seen simultaneously with two intracellular electrodes. Intermittent failure of propagation occurs in only one fibre. More or less regular variations in the amplitude of end-plate potentials are very commonly seen during tetanic stimulation (see also Krnjević & Miledi, 1958).

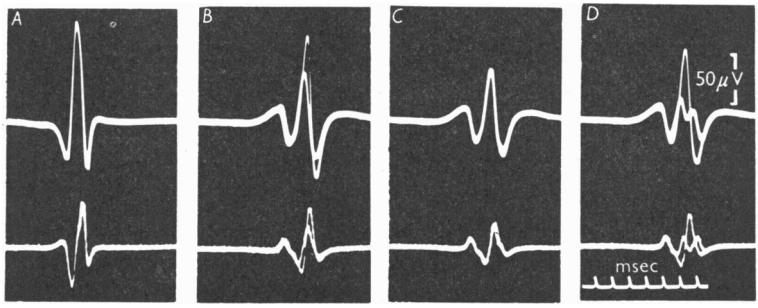

Fig. 9. Asynchronous failure of activation of muscle fibres in a motor unit caused by tetanic stimulation of the nerve fibre at 20/sec. 45 sec after beginning the stimulation the potential (*A*) is similar to the one recorded before the tetanus. 30 sec later (*B–D*) the fibres show intermittent failure. Each record is composed of two to three superimposed traces. The lower traces are the electrically differentiated records of the potential shown in the upper traces.

Histological investigations

Identification of a motor unit. An attempt was made to obtain a permanent record of the distribution of the active fibres in a single motor unit preparation by staining for glycogen in histological sections. However, there was too little glycogen in the muscles to allow one to distinguish between fibres which had and those which had not been contracting.

Certain measurements were made in these sections with the following results relevant to the present study:

Thickness of the diaphragm, 0·31–0·78 mm, with an average of 0·5 mm.
Fibre layers in the diaphragm, 12–24, with an average of about 17.
Diameter of fibres, mostly between 25 and 50μ; the largest diameter observed was 80μ.

Innervation ratio. The total number of fibres counted in microscopic sections of the rat phrenic nerve was about 400. From a width of some 25 mm we

estimated that the total number of muscle fibres in the rat diaphragm was about 10,000. The innervation ratio would therefore be 25:1. This is only a very approximate ratio, which makes no allowance for the presence of sensory fibres in the phrenic nerve. However, these are not likely to exceed 10% of the total, well within the margin of error of the estimate of fibres in the diaphragm (probably ±20%).

DISCUSSION

Evidence has been presented that in the rat diaphragm each motor unit extends over a relatively large area of muscle. The reason for this is not a high innervation ratio but a wide scatter of individual fibres. The three counts of fibres were actually somewhat lower than might be expected. Even when allowance is made for afferent nerve fibres, innervation ratios of muscles, other than those controlling movements of the eyes and the face, are usually 100 or more (Clark, 1931; Feinstein, Lindegard, Nyman & Wohlfart, 1955). In the rat diaphragm, however, the over-all ratio is apparently only about 25. We do not think that the numbers we have counted are gross underestimates of the active fibres, for the following reasons:

(1) The muscles had an average thickness of 0·5 mm, as determined in histological sections (Creese, 1954, gives an average of 0·6 mm in 150–200 g rats). Judging by the change in amplitude of potentials from single fibres with increasing distance (Fig. 4), the surface electrodes should detect all active fibres, except possibly those from the very deepest layers. (2) Even when deep potentials are masked by overlying activity, it is unlikely that they cannot be revealed by close inspection during tetanic stimulation, or when pairs of stimuli are employed. Some active fibres perhaps do escape detection, but their number would probably not change our counts very substantially. A more serious cause of error in estimating the size of the motor unit might be failure of propagation in a substantial fraction of the fibres. Partial block of transmission in isolated preparations is well known (Adrian & Lucas, 1912; Brown & Harvey, 1938) and we have observed the absence of activity in some fibres, right at the beginning of an experiment, both with the rat diaphragm *in vivo* and gracilis *in situ*. However, when the rat diaphragm is kept at room temperature, less than 5% of the fibres fail to respond to nerve stimulation at 2/sec even after a period of 3 hr (Miledi, unpublished observation). Muscholl (1957) found action potentials in 252 out of 254 impaled fibres in the rat diaphragm. This source of error can never be totally discounted, but it can be very much reduced by having a fresh preparation at room temperature, with plenty of O_2, glucose, etc. In order to minimize the effects of injury we made an initial count of the active fibres with recording by surface electrodes.

Several of the observations lead to conclusions which are of some relevance to the interpretation of electromyography in general. (1) It should be recognized that the term 'motor unit', as commonly used in electromyography, does

not correspond to Sherrington's original description (Sherrington, 1925). Potentials should not be ascribed to entire motor units without direct evidence that the whole unit is really compact. In a recent paper, Buchthal *et al.* (1956) described very sharp electrical 'units' recorded with micro-electrodes in the human biceps, which seem to be very much like spikes from single fibres. The authors' conclusion that they are probably fromgroups of, on the average, nineteen fibres is based upon rather indirect considerations. It seems more likely that the spikes do represent not more than one or two fibres, and that the authors have either underestimated the volume of distribution of the motor units, or overestimated the appropriate innervation ratio (or both).

(2) Although the motor unit is a functional unit, it cannot be assumed that the individual muscle fibres of which it is made up all behave in exactly the same manner and have the same characteristics. There may be substantial differences in latency and in refractory period, and in the respective rates of discharge at different frequencies of stimulation. It is possible to record several millimetres apart potentials quite dissimilar in amplitude, shape, latency and refractory period, and even firing at different frequencies, yet both belong to the same motor unit. In fact, if a muscle were stimulated artificially through its nerve, one could not ascribe definitely two such potentials to the same motor unit. In voluntary contractions, however, motoneurones are not likely to fire exactly synchronously. Even if two muscle fibres which belong to the same motor unit have distinct rates of discharge (Figs. 6 and 9), their interconnexion ought to become evident upon close examination of the relative timing of their respective impulses.

According to Lorente de Nó (1947) a simple relationship cannot be expected between the amplitude of a nerve potential recorded in a volume conductor and the distance from the active source. However, Buchthal *et al.* (1956) have claimed that the amplitude is a simple logarithmic function of the distance. The logarithmic index which they calculate from their electromyographic data depends very much upon the assumed least distance between their multiple electrode and the active fibre. Since the exact distance was also not determined in our experiments, it did not seem profitable to derive, using an arbitrarily assumed zero distance, what is in any case only an approximate index. It is worthy of note that the signal recorded from an active muscle fibre with two differential electrodes diminishes to 10μV or less within a distance of about 0·5 mm.

The wide scatter and apparent irregularity of innervation by a single motor nerve fibre raise some interesting questions in relation to the manner in which the connexions between nerve and muscle originally take place. Weiss (1955, p. 360) suggested that the repeated peripheral branching of motor fibres occurs after, and as a result of, the consecutive divisions of young muscle fibres. With such a scheme, the most likely result would seem to be motor units in compact

bundles. Wide scatter of innervation, on the other hand, would occur if effective collateral branching became possible as soon as further progress ceased at the growing tip, i.e. when contact was established with a muscle fibre. This hypothesis is in keeping with the general belief (Weiss, 1955) that the rate of axonal growth and branching is limited by the available flow of neuroplasm from the cell body.

SUMMARY

1. The distribution of single motor units in the rat phrenic-hemidiaphragm preparation has been studied by isolating and stimulating single motor nerve fibres and recording the electrical potentials of active muscle fibres with extracellular and intracellular electrodes.

2. The potentials were scattered irregularly over areas several millimetres in width, up to nearly half the total area of muscle available. They were not confined to any particular depth within the muscle. There was considerable overlap of motor units.

3. In those motor units in which detailed counts were made, the numbers of fibres which could be distinguished with certainty were 7, 13 and 17 respectively.

4. Potentials recorded from active muscle fibres belonging to the same motor unit may differ in latency, refractory period and even frequency of discharge; with extracellular recording, there may also be large differences in shape and amplitude.

5. The signal recorded from a single active muscle fibre with differential electrodes is reduced to $10\mu V$ or less within a distance of about 0·5 mm.

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