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The localization of end-plates in unstained muscle

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INTRODUCTION

The end-plate region of a muscle fibre may be identified either by staining the terminal fibres of the motor nerve (Kühne, 1864; Cohnheim, 1865), or by histochemical demonstration of the cholinesterase in the junctional membrane (Gomori, 1948; Koelle & Friedenwald, 1949). The present paper describes a method for localizing end-plates in fresh unstained muscles frozen on solid carbon dioxide.

This method has been used to study depolarizing drugs such as decamethonium. In these experiments muscles exposed to the radioactive drug were frozen and cut into slices as described by Taylor, Creese, Nedergaard & Case (1965). The slice which contained the end-plates was easily identified, and the drug uptake in this slice could then be measured and compared with the uptake in other regions of the muscle.

METHODS

Frozen muscles. With flat muscles such as the rat diaphragm, a suitable cooling rate can be achieved by placing the muscles on a thin brass plate whose upper surface is covered with two thicknesses of Sellotape, and whose lower surface is in contact with a block of solid carbon dioxide. A Perspex cover is placed over the block of solid carbon dioxide to reduce 'frost' formation on the muscle surface. To prevent 'frosting' for longer periods, the muscles may be immersed under chilled ether, which has a suitable refractive index and a low freezing point.

Relationship of white areas in frozen muscle to end-plates. Latissimus dorsi muscles were dissected from Wistar rats weighing 100–120 g. The bony attachments of the muscles were preserved, and used to secure the muscles in position. The muscles were frozen, as described above, and the white areas were photographed with the muscles under chilled ether. The muscles were then thawed, fixed and stained by the azo-dye method for cholinesterase (Barka & Anderson, 1963). Finally another photograph was taken to compare with that of the frozen muscle.

Cooling curves. The rate of cooling of these preparations was studied by placing a small constantan/copper thermocouple underneath the muscle. The reference thermocouple was placed in iced water, and the potential developed was recorded with an S.E. 2005 ultra violet recorder (S.E. Laboratories, London). The thermocouple gave a linear response over the range +20 to −20 °C, and when the couple was rapidly chilled in isopentane at −160 °C a temperature fall from +20 to −20 °C was recorded in 40 ms.

Dehydration experiments. For these experiments diaphragm muscles were dissected from mice weighing 20–30 g. These preparations are very thin sheets of muscle, and consequently dehydration is facilitated. Twenty-four mouse diaphragms were frozen on brass plates, and the white end-plate areas became visible (Fig. 11). The muscles were then transferred to sealed tubes in a refrigerator at −30 °C. Twelve of the tubes contained phosphorus pentoxide to dehydrate the muscles, and the other twelve tubes contained crushed ice, and served as controls. After 2 weeks the crust on the surface of the phosphorus pentoxide was broken, and the muscles left a further 2 weeks. All of the muscles were then inspected.

To estimate the efficiency of the dehydration procedure, the water content was measured on six of the dehydrated muscles, six of the controls and six freshly dissected mouse diaphragms. The water content was taken as the loss in weight after heating for 18 h at 105 °C, and was expressed as a percentage of the initial weight.

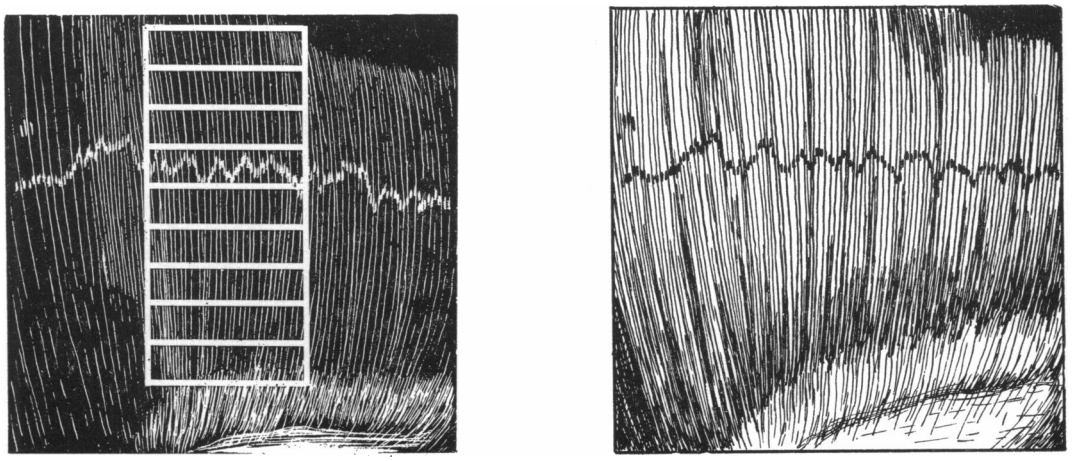

Fig. 1. Diagram showing method of slicing rat diaphragm. Left: frozen muscle, with rib at the top, and central tendon at bottom. The white end-plate areas are indicated, and the grid shows how the slices are cut. Right: drawing of diaphragm after staining for cholinesterase. The stained end-plates resemble the white areas.

Relationship of decamethonium uptake to position of end-plates. Wistar rats, weighing 100–120 g, were anaesthetized with chloralose, 80 mg/kg, and sodium pentobarbitone, 24 mg/kg, injected into the tail vein. The jugular vein was cannulated, and decamethonium −(^{3}H–methyl) dichloride, specific activity 0·84 mC/mg (276 mC/mM), obtained from the Radiochemical Centre, Amersham, was injected at a dose of 1·64 mg/kg. One hour later the diaphragm, latissimus dorsi and epitrochlearis muscles were removed, and frozen as described. In these rat muscles the end-plates are aligned in adjacent fibres, forming a band running across the muscle. The end-plates may be irregularly placed but it is always possible to select an area of muscle where the end-plates form a linear band. This area of muscle was then sliced at 1 mm intervals with a razor blade, cutting the slices parallel to the direction of the line of end-plates and noting which strip contained the end-plate region.

Figure 1 illustrates the application of this technique to the rat diaphragm. Each

muscle strip was weighed, and the samples were prepared for liquid scintillation counting as described by Creese & Taylor (1967). The samples were counted at room temperature in an I.D.L. 2022 Counter (Isotope Developments Ltd, Reading), using optimal allocation of counting time (England & Miller, 1969). The counter efficiency was 6·6% and the quenching less than 1%. The uptake of drug was expressed as counts/min/mg of tissue.

RESULTS

Relationship of white areas in frozen muscle to end-plates

Figure 6 shows a rat latissimus dorsi muscle which was photographed frozen (left), and then re-photographed after staining for cholinesterase (right). It can be seen that the thawing, fixation and staining have somewhat distorted the tissue, but the white areas in the frozen muscle still clearly correspond to the stained end-plates.

When examined under higher magnification, as shown in Fig. 7, the white areas (left) cannot be clearly resolved, but they seem to be composed of white ovals about 200 μm long, which are much larger than the stained end-plates (right).

The white end-plate areas have also been seen in other frozen rat muscles such as sternocostalis and internal intercostal (Fig. 8), epitrochlearis (Fig. 9) and diaphragm (Fig. 10). In all of these, and in mouse diaphragm (Fig. 11), the white areas in the frozen muscle were shown to correspond with the distribution of stained end-plates.

The white end-plate areas can be seen particularly well in frozen rat sternocostalis (Fig. 8). White areas corresponding to the end-plates can also be seen in each of the internal intercostal muscles, and it is possible to see small branches of the intercostal nerve which pass to the end-plates.

The branches of the motor nerve can, however, be readily distinguished from the white end-plate area. This is shown in Fig. 12, which is a photomicrograph of a frozen mouse diaphragm. The white end-plate area, seen on the left, is composed of small ovals as in the rat latissimus dorsi (Fig. 7). These ovals are clearly distinguishable from the nerve fibres which are seen in the upper right of the plate.

Properties of the white areas in frozen muscle

Study of cooling curve. Cooling curves obtained with rat diaphragm preparations are shown in Fig. 2. Line A represents rapid chilling, when a muscle, pinned on cork, was plunged into isopentane at -160 °C. Under these circumstances the entire muscle takes on a white appearance, and the end-plate area cannot be distinguished (six experiments). Line B represents the cooling of a muscle placed on a brass plate whose under surface was in contact with solid carbon dioxide. Under these circumstances the white areas appear in the tissue, but the contrast with the surrounding muscle is enhanced if the rate of cooling is slightly slowed by two thicknesses of Sellotape between the muscle and the brass plate (line C) (six experiments in each case).

In curves B and C it can be seen that the rate of cooling remains relatively constant until the muscle temperature falls to just below 0 °C, and then the muscle begins to cool much more slowly. This is probably because ice crystals start to separate after the temperature reaches the freezing point depression, and this process releases latent heat (Glasstone, 1951). It is interesting to observe that the white end-plate

areas begin to appear when the temperature falls to about −1 °C, which is shortly after the rate of cooling decreases. This suggests that the white areas might be related to ice crystallization in the tissue.

The white areas become more clearly visible as the tissue continues to cool to approximately −8 °C. The appearance of the white areas is then unaltered by further cooling, either on solid carbon dioxide or in isopentane at −160 °C.

Dehydration experiments. After 4 weeks over phosphorus pentoxide at −30 °C no white areas were visible in the mouse diaphragm preparations. The muscles had become transparent, and their water content was reduced to 34·8%. The white areas were still clearly visible in the control muscles after 4 weeks over ice at −30 °C, and their water content was 83·8%, that of fresh mouse diaphragms being 86·6% (mean of six determinations in each case).

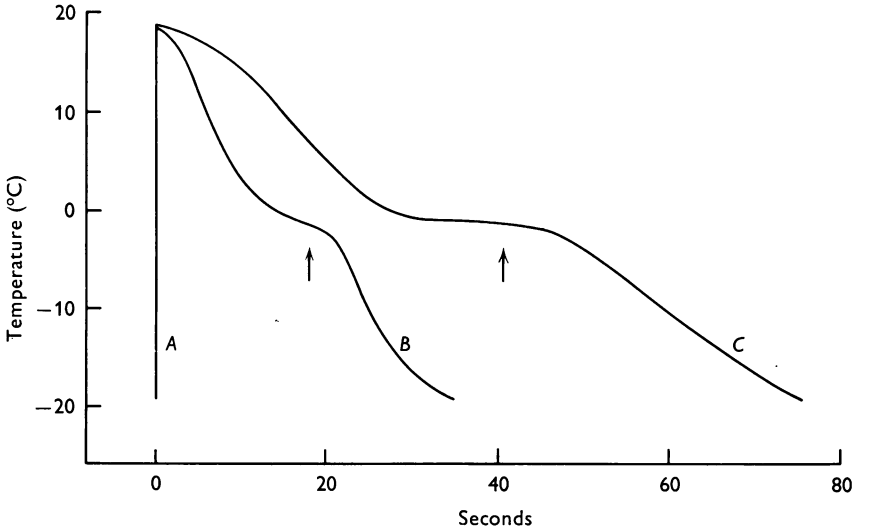

Fig. 2. Cooling curves of rat diaphragm muscle. A. muscle pinned on cork, and plunged into isopentane at −160 °C. B. muscle placed on brass plate whose under surface was in contact with solid carbon dioxide. C. similar to B, but two thicknesses of Sellotape between muscle and brass plate. The arrow indicates the time at which the white end-plate areas begin to appear.

Relationship of decamethonium uptake to position of end-plates

Figures 3–5 show the uptake of radioactive drug by rat muscles 1 h after intravenous injection. Latissimus dorsi, epitrochlearis and diaphragm muscles were used, and the uptake was measured in slices cut at 1 mm intervals. The slice containing the white end-plate region is indicated by an arrow. In each of the muscles the highest uptake of drug is seen to be in the region of the end-plates.

In latissimus dorsi the uptake of drug is low near the humeral insertion, and reaches a peak value in the region of the V-shaped array of end-plates seen in Fig. 6. Moving towards the costal origin, the uptake declines somewhat, and then increases slightly again near the scattered end-plates which may also be seen in Fig. 6.

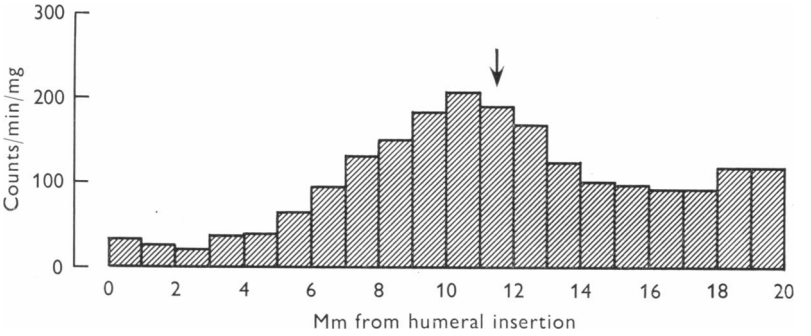

Fig. 3. Uptake of radioactive decamethonium by rat latissimus dorsi. The muscle was cut into slices, and the arrow indicates the slice which contains the white end-plate areas.

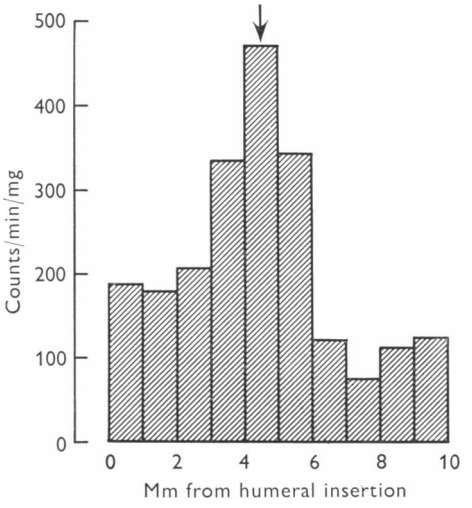

Fig. 4

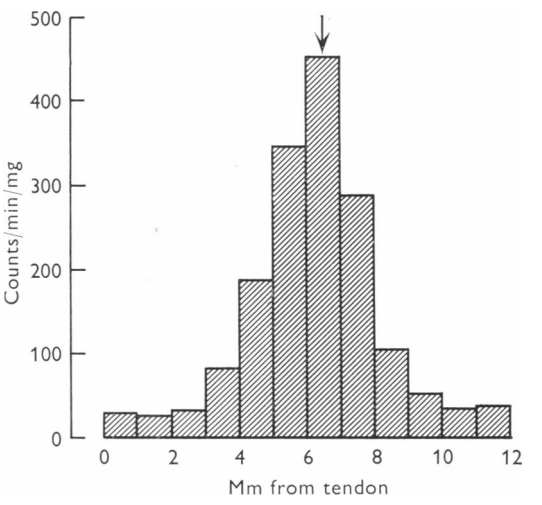

Fig. 5

Fig. 4. Uptake of radioactive decamethonium by rat epitrochlearis. Arrow indicates position of end-plates.

Fig. 5. Uptake of radioactive decamethonium by rat diaphragm. Arrow indicates position of end-plates.

DISCUSSION

The white areas which appeared when rat muscles were frozen slowly have been shown to correspond to the positions of the stained end-plates (Fig. 6). In the frozen internal intercostal muscle it was also possible to find small branches of the motor nerve which pass to the region occupied by the white end-plate areas (Fig. 8). Under the microscope these white areas were seen to be composed of oval structures (Figs. 7, 9), which could be distinguished from the intramuscular nerve fibres (Fig. 9). The white ovals were, themselves, often difficult to resolve with the light microscope, but one could still see that they were much larger than the stained end-plates.

In an attempt to understand why white ovals should be formed in relation to the

end-plates, a study was made of the temperature at which the white areas became visible to the naked eye (Fig. 2B, C). It was found that they began to appear at around $-1{\cdot}0$ °C, and that their appearance was, for all practical purposes, completed by -8 °C. The white areas were then not noticeably affected by further chilling. The cooling curves (Fig. 2B, C) showed that at around $-1{\cdot}0$ °C the rate of cooling

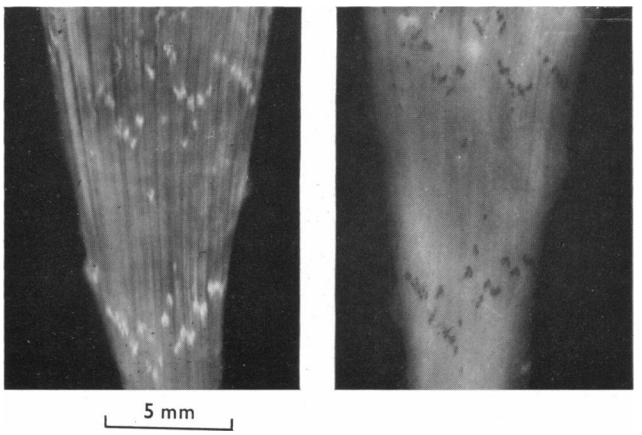

Fig. 6. Rat latissimus dorsi muscle, humeral end lowermost. Left. white areas visible in frozen muscle. Right. end-plates visible after staining for cholinesterase.

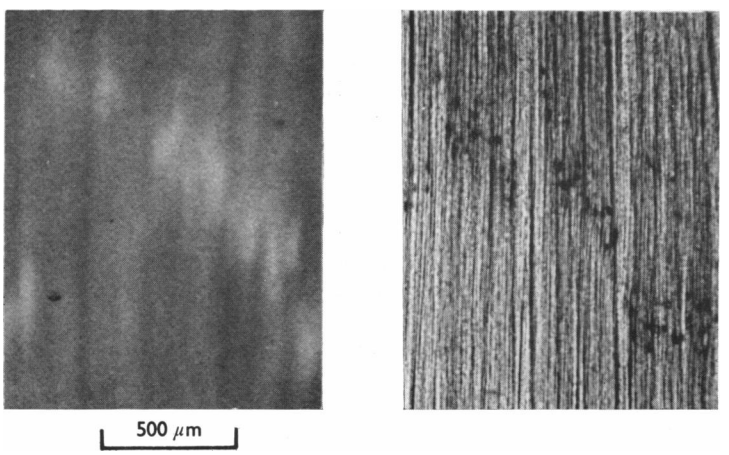

Fig. 7. Photomicrographs of rat latissimus dorsi muscle. Left. frozen muscle. Right. end-plates after staining for cholinesterase.

decreases, suggesting that at this temperature ice crystallization was commencing in the tissue. Furthermore, Williams & Merryman (1965) have shown that ice crystallization in rat muscle occurs predominantly between $-1°$ and -10 °C, so that there is a close correlation between the temperature range for ice crystallization, and that for the appearance of the white end-plate areas.

To test the hypothesis that the white areas were composed of ice crystals, muscles

were freeze-dried over phosphorus pentoxide at −30 °C for 1 month. The white areas disappeared in the dehydrated muscles, whose water content was reduced from 86·6 to 34·8%. This change in water content represents a twelve-fold diminution in tissue water when expressed as water per gram dry weight of muscle. The white areas

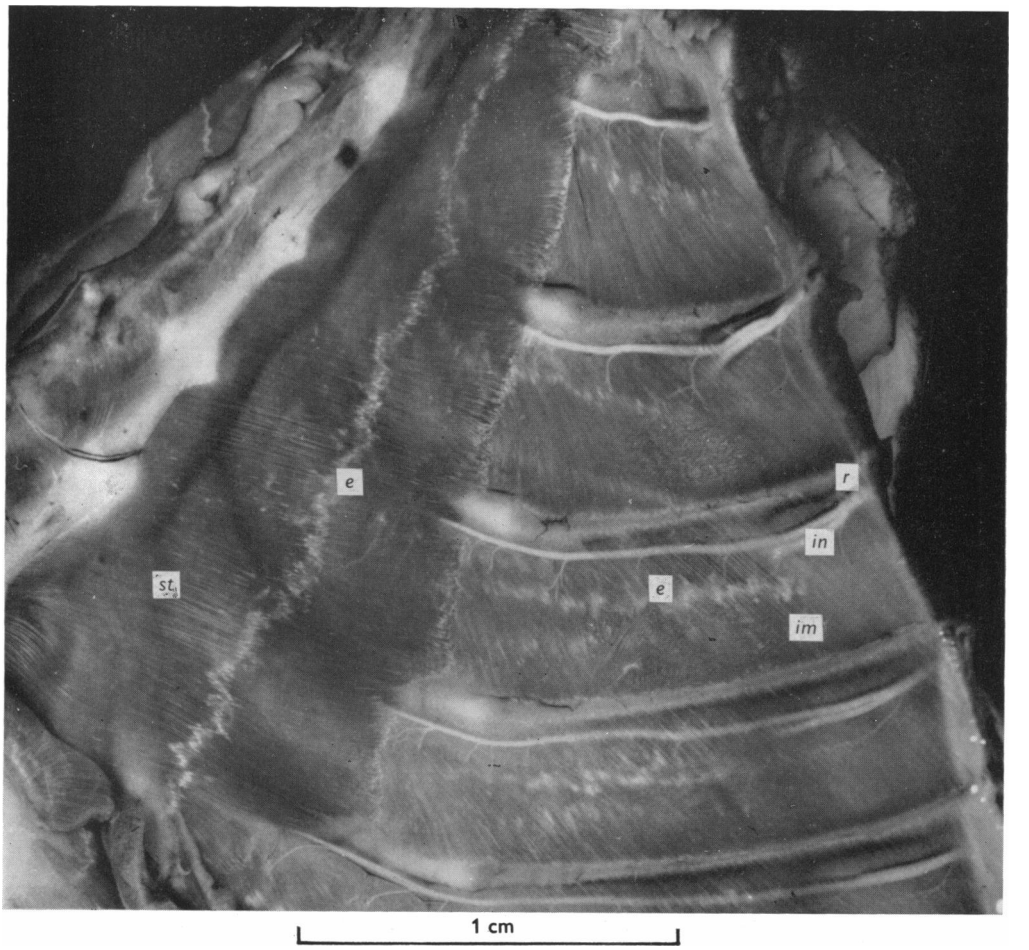

Fig. 8. Frozen rat thoracic cage, viewed from an internal aspect. On the left sternocostalis (*st*) may be seen with its white end-plate area (*e*). The internal intercostal muscles (*im*) may be seen between each rib (*r*), and nerve branches are passing from the intercostal nerve (*in*) to the end-plate region of the muscle (*e*).

remained unchanged in control samples kept at −30 °C over ice, and their water content was 83·8%. These experiments, therefore, confirmed the hypothesis that the white areas were composed of ice crystals.

Ice crystallization might well explain why the white ovals could not be clearly resolved under the light microscope, and if the end-plates merely served as centres of crystal nucleation, then the ovals might easily grow to be larger than the stained

end-plate area. But why should ice crystallization in the end-plate region produce a whiter appearance than crystallization elsewhere in the muscle? It seems likely that small ice crystals must be formed in greater numbers near the end-plates, for in this case there would be more reflecting surfaces. Smith & Smiles (1953) have shown that when tissue is cooled slowly large crystals form in the extracellular space, which expands at the expense of the intracellular water. Finally, the cell itself freezes, and small crystals are formed. It is not known how this process is affected by the motor end-plates. More small crystals may be formed at the end-plate, because there is more cytoplasm, even after cellular shrinkage. Alternatively, the end-plate or teloglial surface may promote the formation of numerous small crystals in the extracellular space.

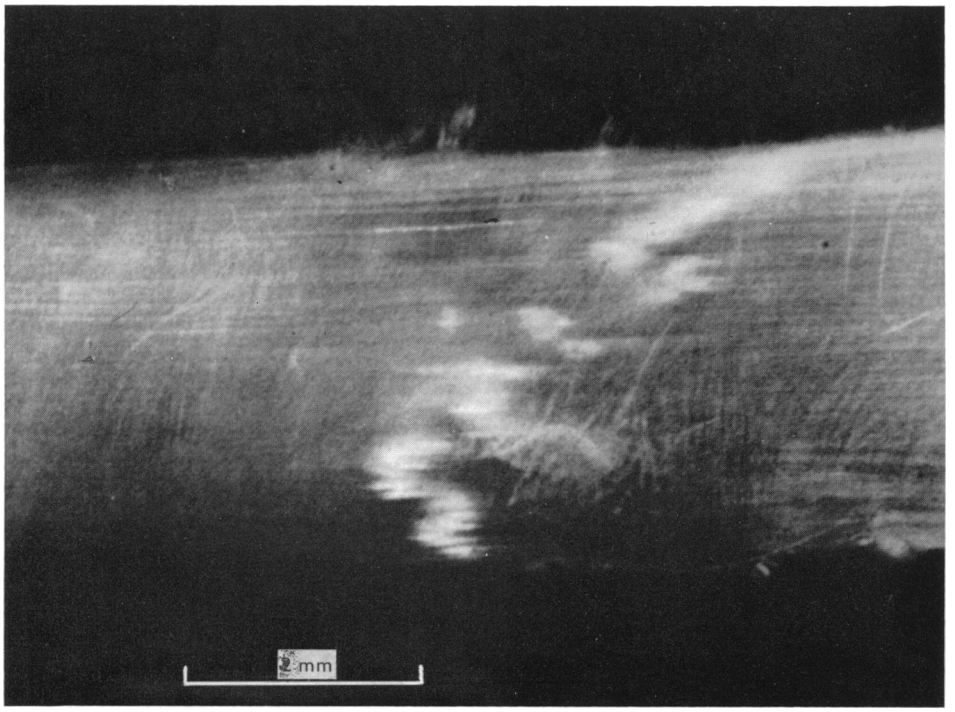

Fig. 9. Close-up view of central part of frozen rat epitrochlearis muscle. The white end-plate area crosses the muscle obliquely.

Wherever the small crystals may have been formed, however, there was still no direct evidence for their existence. If the whiteness were due to small crystals, though, one would predict that with rapid chilling the entire muscle would become white, because under these circumstances very small crystals are produced (Rey, 1960). Indeed, it was found that with rapid chilling in isopentane at -160 °C the whole surface of the muscle became white. The end-plate areas could not be distinguished, presumably because small crystals had been formed throughout the muscle, as well as at the end-plate. It was therefore concluded that the white areas seen with slow cooling were composed of numerous small ice crystals. This conclusion must remain

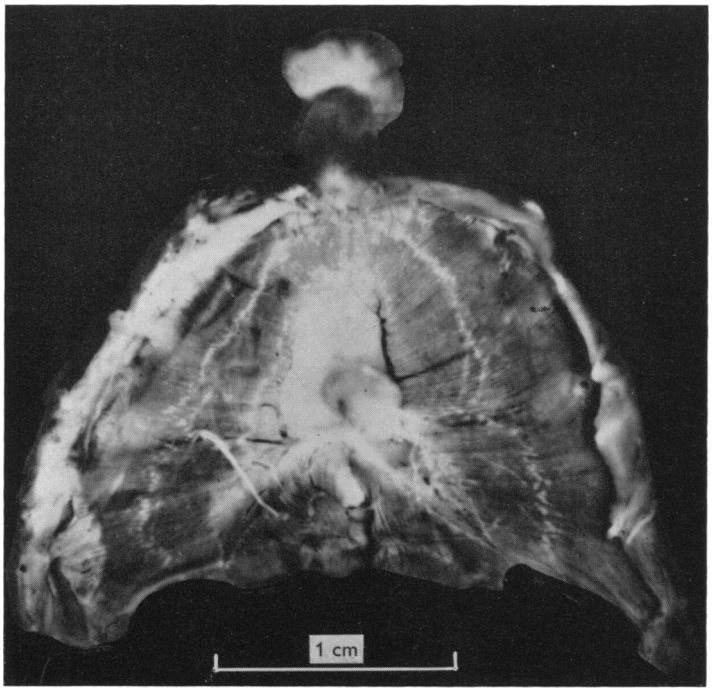

Fig. 10. Frozen rat diaphragm viewed from the pleural aspect. The ribs, xiphisternum and central tendon can be seen. The white end-plate areas are visible on each side, and so is the left phrenic nerve.

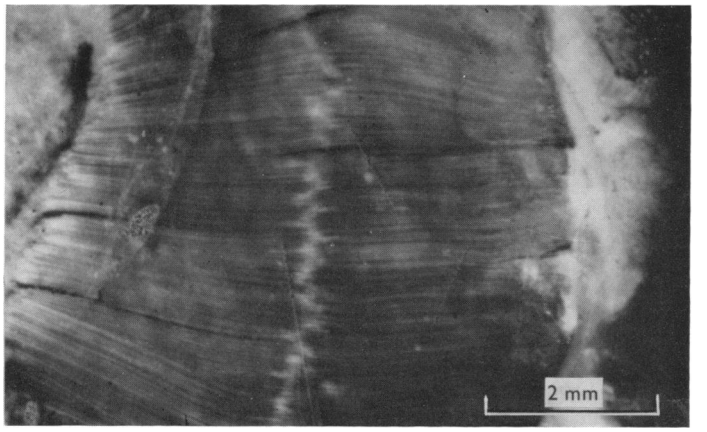

Fig. 11. Close-up view of frozen mouse diaphragm. Part of the right hemidiaphragm is seen from the pleural aspect. The rib is on the right, and the central tendon is on the left, and the white end-plate area forms a vertical line in the centre.

somewhat tentative, however, until it can be confirmed by more direct methods of study.

The white end-plate areas have also been demonstrated in rat muscles removed from animals injected, 1 h previously, with labelled decamethonium. Autoradiographic studies have shown that decamethonium enters muscle fibres maximally near the end-plates (Creese & Maclagan, 1967), and an attempt was made to measure the end-plate uptake of this drug, by excising the white areas from the frozen muscles. The muscles were cut into slices (Fig. 1), and a note was made of the slice containing the end-plates. The radioactivity was determined in slices of latissimus dorsi (Figs. 3, 6), epitrochlearis (Figs. 4, 9) and diaphragm (Figs. 5, 10). In each case the drug uptake was found to be maximal in the region of the motor end-plates.

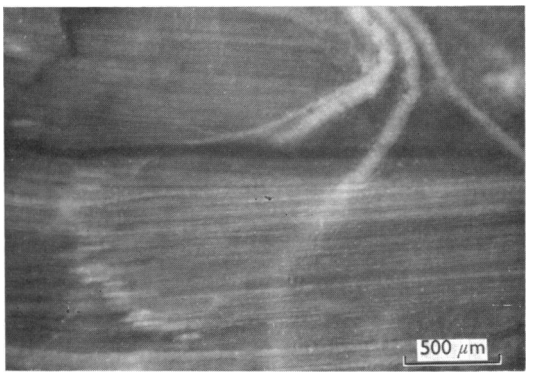

Fig. 12. Photomicrograph of frozen mouse diaphragm. The white end-plate area is seen on the left, composed of small ovals. Intramuscular branches of the phrenic nerve can be seen in the upper right of the plate.

This method of localizing end-plates is rapid and convenient. However, the method depends upon ice crystallization, which may directly or indirectly damage the tissue (Smith, 1961). In the present paper, where measurements are made of drug uptake, tissue damage is probably unimportant. For other studies however the end-plates must be localized without injury to the tissue. In such circumstances protective agents such as glycerol (Polge, Smith & Parkes, 1949) may be helpful, because ice crystals still form in their presence.

SUMMARY

1. In frozen rat muscles the end-plate region may be identified as a white area.

2. The white end-plate area appears as the tissue is freezing, but cannot be distinguished when muscle is rapidly chilled in isopentane at −160 °C.

3. When frozen muscles are dehydrated at −30 °C the white areas disappear.

4. This method of locating the end-plate region has been used to study the entry of labelled decamethonium into different parts of the muscle fibre.

5. One hour after the intravenous injection of radioactive drug into rats, several muscles were removed and frozen. When the end-plate area was cut from each frozen muscle it was found to have the highest uptake of drug.

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