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# Pre- and post-rigor fixation of muscle

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## INTRODUCTION

Surprisingly little information is available in the scientific literature concerning the effects of fixation and subsequent processing of biological tissue for light and electron microscopy. Information about the effects of different fixatives on cell size is particularly scarce. Of course it is not always necessary to obtain the absolute size of cells or organelles because measurements from experimental tissues are often compared with those from control tissues and any distortion or shrinkage due to fixative may be presumed to effect the experimental and control tissues to the same extent. There are, however, many cases when absolute measurements of cell size are required. These include the determination of the relationship between cell size and the functional output of the tissues, e.g. the relationship of the force developed by skeletal muscle to the cross-sectional area of the fibres. Another example is in animal breeding experiments when an attempt is made to relate changes in tissue size to parameters such as changes in cell number and changes in cell size, and to determine how much of the variation is due to the individual parameters.

For most tissues it is difficult to measure cell size accurately without serially sectioning and reconstructing the tissue. However, this particular problem is not encountered in striated muscles in which the fibres run from tendon to tendon in a parallel fashion. In this latter case fibre size may be determined from a single transverse section. Previous published work on the effects of fixation on muscle fibres (Goldspink, 1961; Hegarty, 1970) indicates that many of the common histological methods cause considerable distortion and shrinkage.

Hegarty (1970) claimed that the peripheral fibres of muscles fixed post-rigor are smaller than those fixed pre-rigor. He argued that because of the slow penetration of most fixatives the innermost fibres of muscles, even those fixed immediately after death, shrink more than the peripheral fibres, as the former are fixed in a post-rigor condition by the time the fixative has penetrated to the centre of the tissue. Because of this he advocated that muscle should be fixed post-rigor rather than in the fresh state.

In view of the lack of available information it was felt that the effects of fixation on skeletal muscle should be further examined, particular attention being paid to possible differences between pre- and post-rigor tissue. In studies on human muscles and muscles from agricultural animals it is often necessary to work on post-mortem material, and so information concerning the effects of post-rigor fixation of tissue would be particularly valuable.

## MATERIALS AND METHODS

### *Muscles*

Mature male mice of the 129 Re strain weighing between 30 and 35 g were killed by dislocation of the cervical vertebrae and pinned to a cork board with their forelimbs in the extended position. The biceps brachii muscle from one side of the animal was exposed and freed from its surrounding connected tissue. The muscle was then either fixed *in situ* by pipetting fixative over it for 10–15 minutes, or frozen by pouring cold Freon 12 (−160 °C) over it.

To test the effects of different fixatives on the pre- and post-rigor muscle fibres the carcass of the animal was left at room temperature for 5 hours. The contralateral muscle was then fixed or frozen in exactly the same way as the pre-rigor muscle had been.

In order to determine the effects of a longer post-mortem period on the size of the peripheral fibres another group of muscles was allowed to go into rigor by placing the carcass in a refrigerator at +4 °C for 24 hours, after freezing and removing the biceps brachii from one limb. At the end of the 24 hour period the muscle from the other limb was frozen in the same way and removed from the carcass. Both pre- and post-rigor muscles were then sectioned on a cryostat.

Once it had been established that the peripheral fibres decrease in size during rigor as stated by Hegarty (1970) and Hegarty & Hooper (1971), it was necessary to test the hypothesis that the smaller fibres, which are invariably found in the deeper regions of fixed muscle, are an artefact arising from the slow penetration of the fixative into the tissue. For this purpose muscles were removed and immersed immediately after death in Flemming's solution without acetic acid (F.W.A.) and the contralateral muscles were removed from the carcass 24 hours after death (4 °C) and treated in the same way. This fixative has a very slow rate of penetration. In this experiment the change in size of the inner fibres as well as the outer fibres was examined.

### *Histology*

The fixatives used were Carnoy (glacial acetic acid 10 %, ethanol 60 % and chloroform 30 %), F.W.A. (4 ml of 4 % osmic acid, 15 ml of 1 % chromic acid), and glutaraldehyde (2·5 % glutaraldehyde in 0·062 M phosphate buffer at pH 7·3 and containing 0·5 % glucose). In the case of the glutaraldehyde fixative the effect of the tonicity of the fixative on fibre size was investigated. For this experiment glutaraldehyde fixative contained the following concentrations of glucose: 0 %, 0·125 %, 0·250 %, 0·325 %, 0·500 %.

After the muscles had been fixed *in situ* the limbs were removed, pinned to a small piece of cork strip, and immersed in the fixative for a further hour. Following this period the muscles were dissected off the limb and re-immersed in fixative for one more hour in the case of the Carnoy and glutaraldehyde fixatives and a further 23 hours in the case of the F.W.A. After washing, the muscles were dehydrated in ethanol and embedded in paraffin wax. They were then sectioned transversely at a thickness of 7·5 $\mu$m, stained in Mallory's triple stain, and mounted in Canada balsam.

### *Frozen sections*

The biceps brachii muscles were frozen *in situ*, with the limb in the extended position, by pipetting Freon 12 at −160 °C over the upper part of the limb. They were chipped out with a pre-cooled solid scalpel and clamped, whilst still frozen, to a cold microtome chuck. They were then sectioned transversely at a thickness of 10 $\mu$m using a Pearse-Slee cryostat. Sections from the centre of the muscle belly were picked up on clean coverslips and mounted in glycerine jelly. Measurements of fibre size from the unfixed and unstained frozen sections were carried out as soon as possible after sectioning, using phase-contrast optics.

### *Electron microscopy*

Some of the muscle that had been fixed in the various glutaraldehyde solutions was prepared for electron microscopy by teasing the muscle apart and embedding single fibres or small bundles of fibres in Araldite. The Araldite blocks were sectioned at about 50 nm on a Reichert ultramicrotome and, after staining in lead citrate and uranyl acetate solutions, the sections were examined and photographed using a JEM 7a electron microscope.

### *Measurements*

For the measurement of mean fibre size a transverse section was selected from each muscle and the diameters of 100 fibres in it were taken by averaging the largest and smallest diameter measurements of each fibre. Measurements were made either directly from the sections using an ocular micrometer, or from photographs of regions of sections using micrometer calipers. In all cases the fibre samples were obtained by traversing the muscle several times, except for the 24 hour post-mortem experiment, in which only the peripheral fibres of the muscles were included in the sample. The measurements using the light microscope were all carried out at a magnification of × 300.

The Z and A filament lattices of the myofibrils were measured from electron micrographs using micrometer calipers. Measurements were made only on myofibrils that were sectioned in the true transverse plane. Three muscles were used for each fixative, and approximately five measurements were made on three fibres of each muscle. Measurements of lattice spacing were made at a final magnification of about × 150000. The exact magnification was obtained in each case by photographing a carbon replica of a 2160 lines/mm diffraction grating.

## RESULTS

The results of the experiments involving the fixation of muscle pre- and post-rigor (5 hours after death at room temperature) are shown in Table 1, which shows that the mean fibre diameter varied according to the fixative used. As compared with the frozen sections all the fixatives resulted in a decreased mean fibre diameter, the least shrinkage being caused by the Flemming's without acetic acid. However, at 5 hours *post mortem* there was apparently no significant difference between the muscles fixed pre- and post-rigor ($P$ is considerably greater than 0·05 using the one sample $t$ test

Table 1. *Effect of different fixatives on the size of muscle fibres fixed pre- and post-rigor (5 hours after death)*

(Fibre diameters given in μm.)

| Carnoy | | F.W.A. | | Glutaraldehyde | | Frozen sections | |
|---|---|---|---|---|---|---|---|
| Pre | Post | Pre | Post | Pre | Post | Pre | Post |
| 24·8±0·4 | 27·6±0·5 | 40·3±1·1 | 39·1±1·4 | 27·7±0·4 | 26·6±0·6 | 42·6±0·8 | 36·6±0·8 |
| 28·6±0·7 | 28·1±0·7 | 39·0±0·9 | 34·5±0·9 | 29·7±0·6 | 24·5±0·5 | 33·7±0·9 | 38·1±1·0 |
| 28·6±0·5 | 41·3±0·9 | 36·0±0·8 | 30·3±0·8 | 19·9±0·4 | 25·6±0·5 | 46·7±1·0 | 37·6±0·6 |
| 25·6±0·5 | 24·2±0·6 | 28·8±0·6 | 28·7±0·6 | 39·4±0·7 | 28·4±0·6 | 41·9±0·8 | 35·4±0·5 |
| 27·0±0·6 | 25·6±0·5 | 35·1±0·8 | 27·4±0·6 | 28·0±0·5 | 35·6±0·7 | 45·7±1·0 | 50·0±0·9 |
| Means 26·9 | 29·4 | 35·8 | 32·0 | 28·9 | 28·2 | 42·1 | 39·5 |

Table 2. *Sizes of outer and inner fibres in sections of frozen and* F.W.A.*-fixed muscles taken immediately after death and 24 hours post-mortem (carcass maintained at 4 °C) in μm*

| F.W.A. sections | | | | Frozen sections | | | |
|---|---|---|---|---|---|---|---|
| Outer fibres | | Inner fibres | | Outer fibres | | Inner fibres | |
| Pre | Post | Pre | Post | Pre | Post | Pre | Post |
| 43·3±0·8 | 35·8±0·8 | 33·7±0·7 | 31·2±0·6 | 61·7±1·2 | 49·2±1·4 | 28·1±0·7 | 31·8±0·7 |
| 42·6±0·7 | 37·5±0·8 | 38·2±0·7 | 32·9±0·6 | 60·1±1·5 | 41·7±0·7 | 49·6±1·2 | 35·5±0·6 |
| 51·1±0·5 | 41·9±0·8 | 39·4±0·8 | 32·2±0·6 | 55·1±1·9 | 58·1±0·9 | 45·8±0·8 | 40·8±0·7 |
| 40·0±0·6 | 34·9±0·6 | 33·6±0·5 | 31·8±0·5 | 53·5±0·5 | 49·8±0·9 | 40·7±0·5 | 39·3±0·5 |
| 43·9±0·6 | 34·5±0·6 | 35·6±0·6 | 30·3±0·6 | 48·5±0·9 | 47·5±0·7 | 38·4±0·8 | 33·0±0·6 |
| 50·4±0·6 | 43·0±0·8 | 38·9±0·7 | 32·0±0·6 | 58·1±1·0 | 38·5±0·6 | 41·1±0·6 | 33·6±0·7 |
| Means 45·2 | 37·9 | 36·7 | 31·7 | 56·1 | 47·5 | 40·6 | 35·7 |
| Difference −15·7% | | −13·6% | | −15·3% | | −12·1% | |

on the differences between pairs. This was so whether the results were compared in fixative groups or as a whole).

In the second experiment (Table 2), which was carried out as described by Hegarty (1970), except that frozen sections were used as well as F.W.A. fixed sections, the peripheral fibres of the post-rigor muscles were found to be significantly smaller ($P < 0.01$), in agreement with Hegarty's finding. However, this was also true of the innermost fibres and those in fixed and frozen muscle. It is difficult to see, therefore, how Hegarty's suggestion about the effect of fixation on fibre size can be true, as the change in fibre size is apparent in the frozen as well as in the fixed sections.

The effects of altering the osmolarity of the glutaraldehyde fixative on fibre size and ultrastructure are shown in Table 3. The osmolarity of the fixative apparently had no effect on fibre size in freshly fixed muscles. Also the filament lattice spacings were not altered, except perhaps in the case of the Z disc lattice, which showed reduced spacing when the fixative contained no glucose.

Table 3. *Effect of alterations in the osmolarity of glutaraldehyde on the structure of muscle*

| Fixative | Z disc lattice (nm) | Mean | M line lattice (nm) | Mean | Fibre size ($\mu$m) | Mean |
|---|---|---|---|---|---|---|
| Glutaraldehyde, no glucose | 13·0±0·2<br>12·5±0·3<br>13·6±0·3 | 13·0 | 39·0±1·2<br>32·0±0·6<br>36·4±0·6 | 35·8 | 27·6±0·5<br>31·6±0·6<br>35·0±0·6 | 31·4 |
| Glutaraldehyde, ±0·125 % glucose | — | — | — | — | 36·1±0·6<br>28·2±0·5<br>26·6±0·7 | 30·3 |
| Glutaraldehyde, ±0·25 % glucose | 16·9±0·2<br>18·2±0·3<br>17·5±0·5 | 17·5 | 32·4±0·5<br>34·3±0·7<br>39·2±0·6 | 35·3 | 23·3±0·6<br>27·7±0·5<br>31·5±0·7 | 27·5 |
| Glutaraldehyde, ±0·325 % glucose | — | — | — | — | 32·0±0·8<br>24·1±0·5<br>26·2±0·7 | 27·4 |
| Glutaraldehyde, ±0·5 % glucose | 16·4±0·3<br>16·2±0·3<br>18·6±1·0 | 17·1 | 32·7±1·2<br>34·4±0·5<br>34·3±0·4 | 33·8 | 29·4±0·8<br>27·4±0·5<br>32·1±0·6 | 29·6 |

## DISCUSSION

The results indicate that the type of fixative used is of great importance when determining cell size, and that the chemical nature of the fixative seems to be more important than its osmolarity. Of the fixatives used the best one for preserving muscle fibre size and shape appears to be F.W.A., confirming earlier work on fixation of muscle by Goldspink (1961). This fixative, however, still gave a slightly smaller mean fibre diameter than the frozen sections, although it must be borne in mind that the diameter of the frozen fibres was probably slightly enlarged, because water expands by about 4 % when it is converted into ice.

During a 5 hour post-mortem period the development of rigor had apparently no effect on fibre size of either fixed muscles or frozen sections. As the extent of rigor is considerable in mouse muscles at room temperature 5 hours after death it is probable that the fibre shrinkage is not associated with the rigor process itself but with later post-mortem changes. However, if the muscles were allowed to go into rigor over a longer period of time but at a lower temperature there was a significant decrease in fibre size. Hegarty attributed the smaller fibre diameter of the innermost fibres to the fact that fibres were being fixed in a post-rigor state. He also stated that with fixatives of slow penetration, such as F.W.A., some of the more central fibres of the muscle will be fixed in the post-rigor state even if the muscle is immersed in the fixative immediately after death. Furthermore, he suggested that this was the reason why some muscles – for example, the mouse biceps brachii – show a bimodal distribution of fibre size. However, some muscles exhibit a bimodal distribution of fibre size whether the measurements are made on fixed or frozen sections (Howells & Goldspink, unpublished findings). Also Hegarty used an unusual strain of mice, bred

for largeness and exhibiting a unimodal distribution of fibre sizes (Luff & Goldspink, unpublished findings).

In the experiments reported here the outer fibres and the inner fibres decreased by about the same percentage in the frozen sections as in the fixed tissue. Therefore Hegarty's hypothesis is apparently not valid.

The reason why the fibres decrease in size after death is probably the result of the decreased water-binding capacity of the cellular proteins (Bendall & Wismer-Pedersen, 1962) causing the water to pass from the intracellular compartment into the extracellular spaces.

In conclusion, it seems that as far as fibre size is concerned, the chemical nature of the fixative is more important than its osmolarity, and contrary to what Hegarty (1970) advocates, the muscle should always be fixed in the fresh state whenever possible. However, if the muscles are fixed after death, the degree of shrinkage of the outer and inner fibres seems to be consistent.

## SUMMARY

The effects of several fixatives on fresh and post-rigor muscles were investigated by comparing the size of the fibres with those of frozen sections. As far as shrinkage was concerned, the chemical nature of the fixatives was more important than the osmolarity. Myofilament lattice spacings were measured in fibres that had been fixed in glutaraldehyde of different osmolarities. Only the Z disc lattice changed appreciably.

The effects of different post-mortem conditions on fibre size were investigated in fixed and frozen sections. A shrinkage of about 15% occurred in a 24 hour post-mortem period, although no fibre shrinkage was evident at 5 hours post-mortem. This indicates that the shrinkage is not associated with the rigor process itself but with the loss of the water-binding capacity of the proteins. During the 24 hour period the inner fibres were found to shrink to about the same extent as the peripheral fibres in frozen as well as fixed muscles. This finding contradicts a recent suggestion that the innermost fibres in fixed tissue are disproportionately small because of slow penetration by the fixative. The results also contradict the suggestion that it is more desirable to fix muscle post-rigor for purposes of fibre measurements.

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