Histochemistry 65, 193–205 (1980)

Histochemistry

The Reliability of Histochemical Fibre Typing of Human Necropsy Muscles

Per-Olof Eriksson[1], Anders Eriksson[2], Margareta Ringqvist[1], and Lars-Eric Thornell[3]

[1] Department of Stomatognathic Physiology
[2] Institutes of Pathology and Forensic Medicine
[3] Institute of Anatomy, University of Umeå, S-901 87 Umeå, Sweden

Summary. The reliability of muscle fibre typing of post mortem specimens was investigated with special reference to the influence of time and temperature. In specimens stored at +4° C, muscle fibre typing could be reliably performed up to at least ten and fifteen days post mortem for the masseter and biceps brachii muscles respectively. The corresponding figures for storage at room temperature were three and six days. The difference in the preservation of enzyme activity between masticatory and limb muscles might be related to the demonstrated difference in the fibre type composition and thus the enzyme content and energy sources.

Introduction

Analysis of muscle biopsies treated by histochemical methods has become a routine method in evaluation of muscle structure and function in health and disease (Dubowitz and Brooke, 1973). Human limb muscles are usually accessible for biopsy, while studies of the human masticatory muscles have been hampered because of their complex structure and topography. A preferential alternative for investigating the histochemical appearance of the functionally different parts of the human masticatory muscles is to use specimens obtained post mortem.

To provide a basis for a comprehensive study on the fibre type composition of the human masticatory muscles, we have systematically investigated the reliability of muscle fibre typing of post mortem specimens with special reference to the influence of time and temperature.

Materials and Methods

Muscle Samples. Specimens from the superficial part of the left masseter and the left biceps brachii muscles were obtained within 24 h of sudden death from three previously physically healthy subjects,

0301-5564/80/0065/0193/$02.60

two males (aged 30 and 40 years) and one female (aged 19 years). Strips from each of the two muscles were divided into portions equal in size (approximately $5 \times 5 \times 5$ mm). Ten portions from each muscle were placed in a humified chamber and stored in the refrigerator (+4° C, 100% relative humidity) for one to ten days post mortem. Another ten portions from each muscle were stored at room temperature (+21° C, 100% relative humidity) for the same periods. All pieces were located with the fibres lying parallel to and in contact with the bottom of the chamber. Each day (1 to 10), muscle samples from both temperature groups were mounted for transverse sectioning and frozen in isopentane chilled with liquid nitrogen. In one case (female 19 years) four pieces from each muscle were stored at +4° C and 100% relative humidity also for extended periods (15, 20, 25 and 30 days post mortem).

Enzyme Histochemistry. The sections were stained for the demonstration of myofibrillar ATPase at pH 9.4 (Padykula and Herman, 1955) and after acid preincubation (Brooke and Kaiser, 1970) and for the demonstration of NADH-tetrazolium reductase (Nyström, 1968). In addition, a modified Gomori trichrome staining was used (Engel and Cunningham, 1963).

Examination. The sections were analyzed in a Leitz Dialux-20 light microscope. Sections stained with Gomori trichrome were analyzed for the assessment of cell borders and nuclear staining. NADH-tetrazolium reductase and ATPase were used for differentiation of fibre types (Dubowitz and Brooke, 1973; Ringqvist, 1974).

Results

With increased storage time, the depth of staining became weaker and uneven and the fibre size decreased. This was especially true for storage at room tempera-

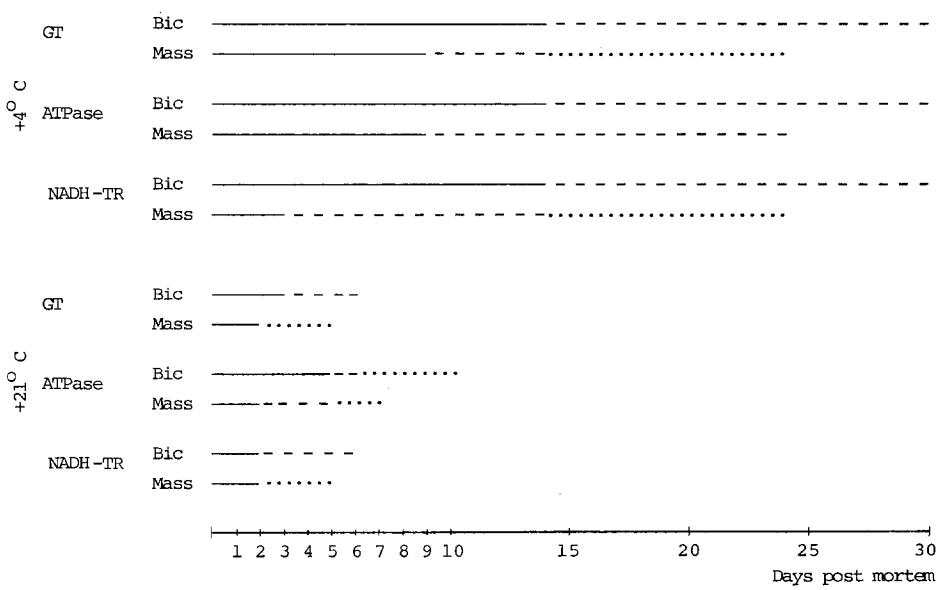

Fig. 1. The influence of time and temperature upon the ability to distinguish cell borders and nuclei (Gomori trichrome, GT) and different fibre types (ATPase and NADH-TR) in post mortem specimens of human limb (biceps brachii, *Bic*) and masticatory (masseter, *Mass*) muscles. The results of the analyses of one female (aged 19 years) and two male (aged 30 and 40 years) subjects are summarized. Cell borders, nuclei and different muscle fibre types are in all areas clearly distinguishable (—), in general or in large areas (more than half of the section) distinguishable (---), or distinguishable only in small areas (less than half of the section) (···)

ture. The morphological changes observed, were found to be variable within and between the different muscles. The results of the analyses of the three cases are summarized below and in Fig. 1.

Storage at +4° C

ATPase (pH 9.4; pH 4.6; pH 4.3)

Biceps brachii. 1–10 days post mortem: Different muscle fibre types (type I, IIA, IIB and, relatively few in number, type IIC) were clearly observed in a mosaic pattern (Fig. 2A–C). In two subjects (female 19 years and male 30 years) fibres intermediately stained for ATPase at pH 9.4, and strongly stained after acid preincubation at pH 4.3 and pH 4.6 were observed (Fig. 2A–C). These fibres showed a stronger activity for NADH-tetrazolium reductase than the type I fibres (Fig. 2D) and were more intensely stained by the modified Gomori trichrome than the other fibre types. The frequency of these intermediately stained fibres was estimated to be approximately 3% of the total fibre population.

15–30 days: In places, or in small areas (less than half the area of the section), fibres with a weak reaction product were seen. Different muscle fibre types could still in general, or in large areas (more than half the area of the section) be distinguished (Fig. 3A–B).

Masseter. 1–9 days post mortem: Muscle fibres with a weak (type I), intermediate and strong (type II) reaction for ATPase at pH 9.4 could clearly be distinguished. The variability in size and distribution was great. The type II fibres, as a rule very small compared with the type I fibres, could be subdivided into one group in which the staining reaction was inhibited at pH 4.3, type IIB, and a second group in which the reaction was partially inhibited at pH 4.3, type IIC. Only three fibres in the whole material (in one and the same subject) were identified as type IIA (staining reaction inhibited at pH 4.6). In addition, in all three subjects there were occasional, relatively large, usually round fibres (range 50–80 μm) with a strong reaction for all stains.

10–20 days: In places, or in small areas, fibres with a weak staining reaction and sometimes with areas devoid of reaction product were seen. Different muscle fibre types were still in general or in large areas distinguishable (Fig. 4A).

25–30 days: In general, the reaction products were very weak and areas lacking enzyme activity could be seen in the fibres. Different muscle fibre types could only be distinguished locally.

NADH-Tetrazolium Reductase

Biceps brachii. 1–10 days post mortem: Different muscle fibres with different degrees (three or more) of activity could clearly be distinguished (Fig. 2D).

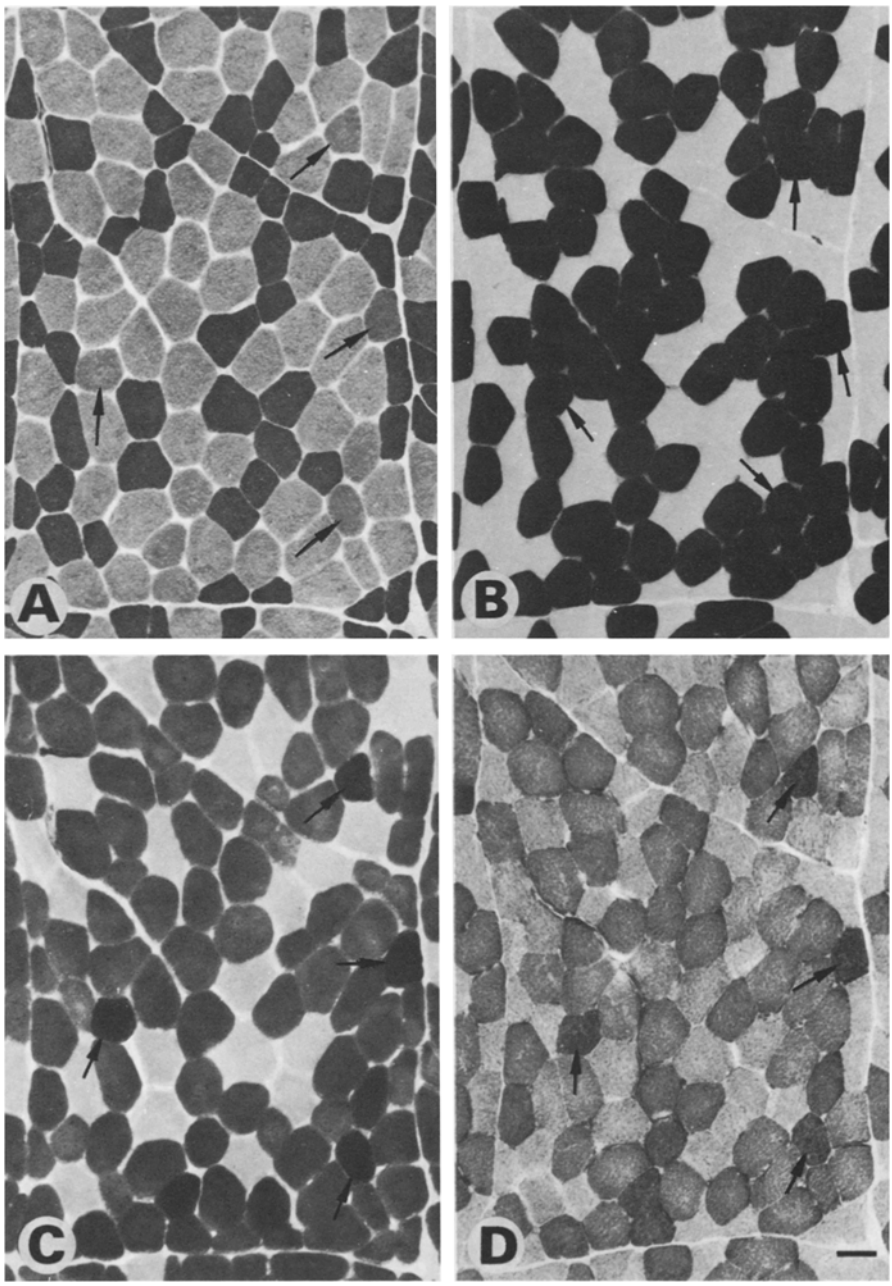

Fig. 2A–D. Biceps brachii muscle one day post mortem. Sections stained for the demonstration of ATPase pH 9.4 (**A**), ATPase pH 4.3 (**B**), ATPase pH 4.6 (**C**) and NADH-TR (**D**). Type I, type IIA, type IIB and ATPase intermediately stained fibres can be clearly distinguished in a mosaic pattern. Type IIB are smaller than the other fibres. Fibres intermediately stained for ATPase at pH 9.4 (arrows) are strongly stained after acid preincubation at pH 4.3 and 4.6 and show a stronger NADH-TR activity than the type I fibres. Bar, 50 μm. ×100

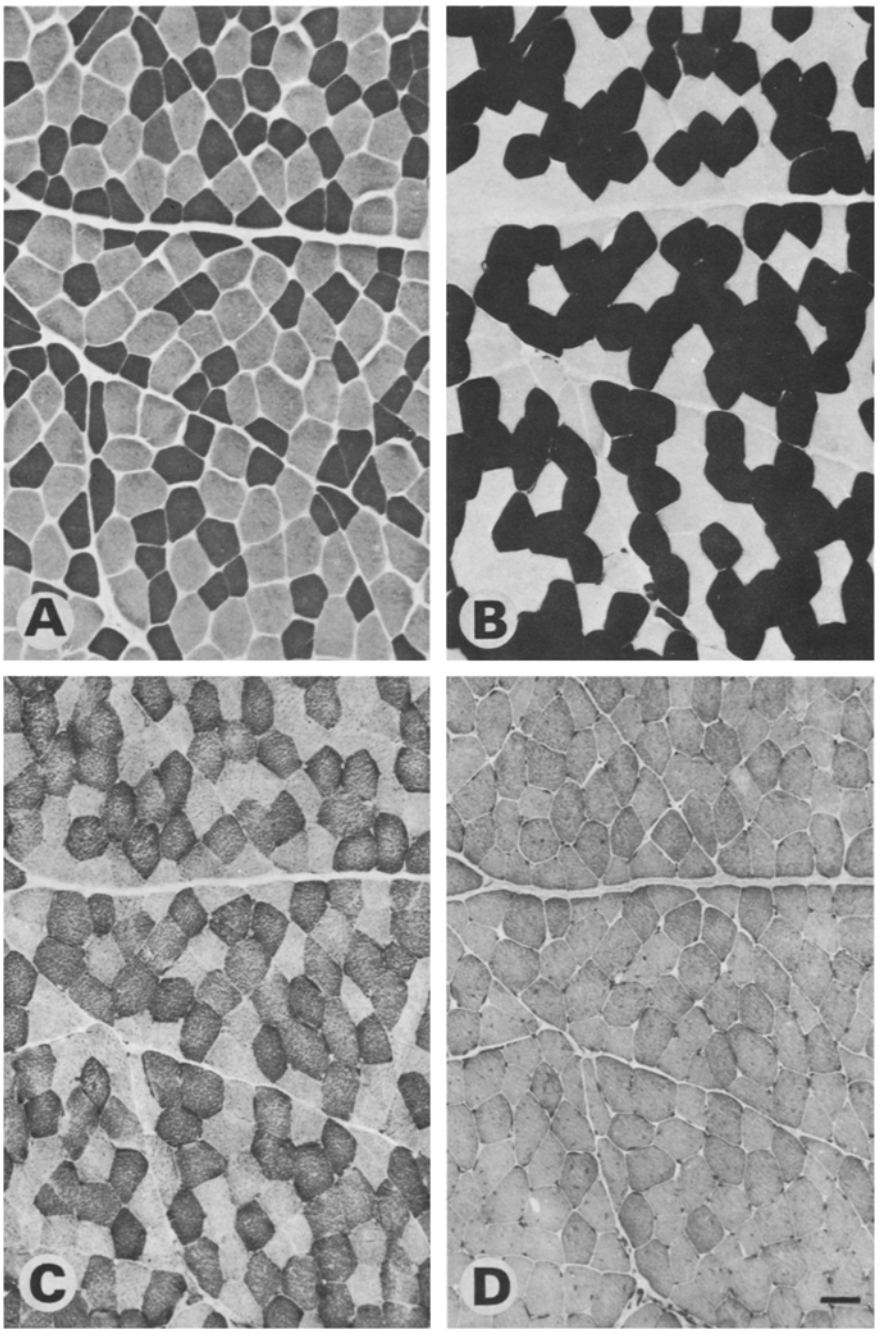

Fig. 3A–D. Biceps brachii muscle thirty days post mortem after storage at +4° C. Sections stained for the demonstration of ATPase pH 9.4 (**A**), ATPase pH 4.3 (**B**), NADH-TR (**C**) and with Gomori trichrome (**D**). Cell borders, muscle nuclei and different muscle fibre types are in general clearly distinguishable. Fibres with three or more degrees of activity can be seen in NADH-TR. The decrease of the mean diameter was estimated to be about 18% compared with the fibres measured one day post mortem. Bar, 50 μm. ×100

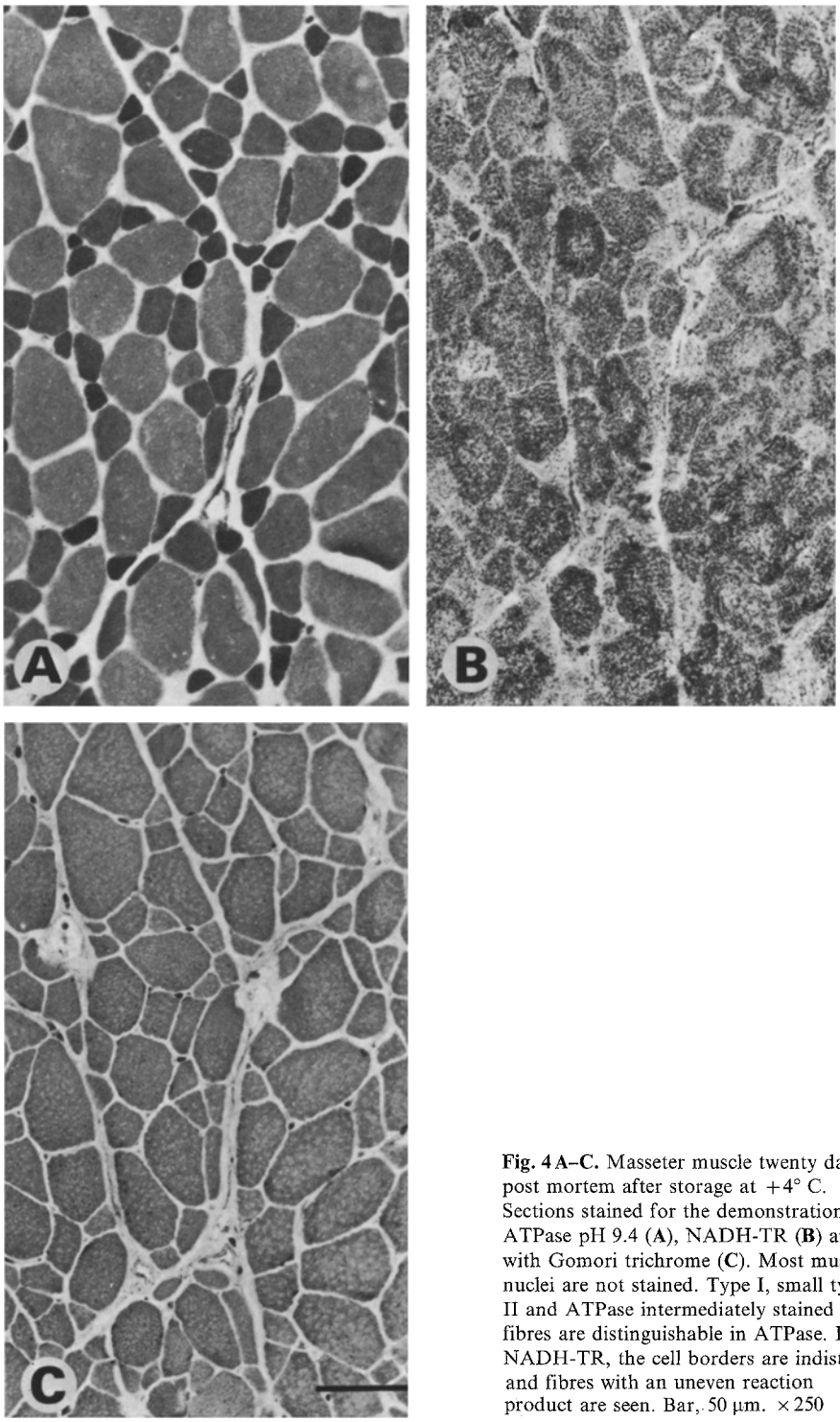

Fig. 4 A–C. Masseter muscle twenty days post mortem after storage at +4° C. Sections stained for the demonstration of ATPase pH 9.4 (**A**), NADH-TR (**B**) and with Gomori trichrome (**C**). Most muscle nuclei are not stained. Type I, small type II and ATPase intermediately stained fibres are distinguishable in ATPase. In NADH-TR, the cell borders are indistinct and fibres with an uneven reaction product are seen. Bar, 50 μm. ×250

15–30 days: In places, or in small areas, fibres with a weak and/or uneven reaction product (centrally lucent) were seen. Different muscle fibre types were still in general or in large areas distinguishable (Fig. 3C).

Masseter. 1–4 days post mortem: Different muscle fibre types with different degrees (three or more) of activity could be distinguished.

5–10 days: In places, or in small areas, fibres with a weak and/or uneven (centrally lucent) reaction product were seen. Different muscle fibre types were still in general or in large areas distinguishable.

15–20 days: Fibres with a weak and/or uneven reaction product were frequently seen (Fig. 4B). Different muscle fibre types could be distinguished only in small areas.

25–30 days: In general, the staining reaction was very weak and/or uneven with only peripheral activity. Different muscle fibre types could not be distinguished.

Gomori Trichrome

Biceps brachii. 1–10 days post mortem: The cell borders and the muscle nuclei were clearly distinguishable.

15–30 days: In places or in small areas the cell borders were indistinct and the nuclei did not stain. In general, or in large areas, the cell borders and the nuclei were distinguishable (Fig. 3D).

Masseter. 1–9 days post mortem: The cell borders and the muscle nuclei were clearly distinguishable.

10 days: In places, the cell borders were indistinct and the nuclei did not stain.

15–20 days: In large areas the cell borders were indistinct and there was no nuclear staining (Fig. 4C).

25–30 days: In general, the cell borders were indistinct and the nuclei did not stain.

Storage at +21° C

ATPase (pH 9.4; pH 4.6; pH 4.3)

Biceps brachii. 1–5 days post mortem: Different muscle fibre types could clearly be distinguished in a mosaic pattern. In one case (male 30 years), muscle fibres in parts of the sections showed an inverse reaction for ATPase at pH 9.4 after storage for four days or more.

6 days: In places, or in small areas, the reaction product was weak and areas lacking enzyme activity could be seen in the fibres. Different muscle fibre types could still in general, or in large areas, be distinguished (Fig. 5A–B).

7–10 days: In large areas, the muscle fibres showed a weak staining reaction and contained areas devoid of reaction product.

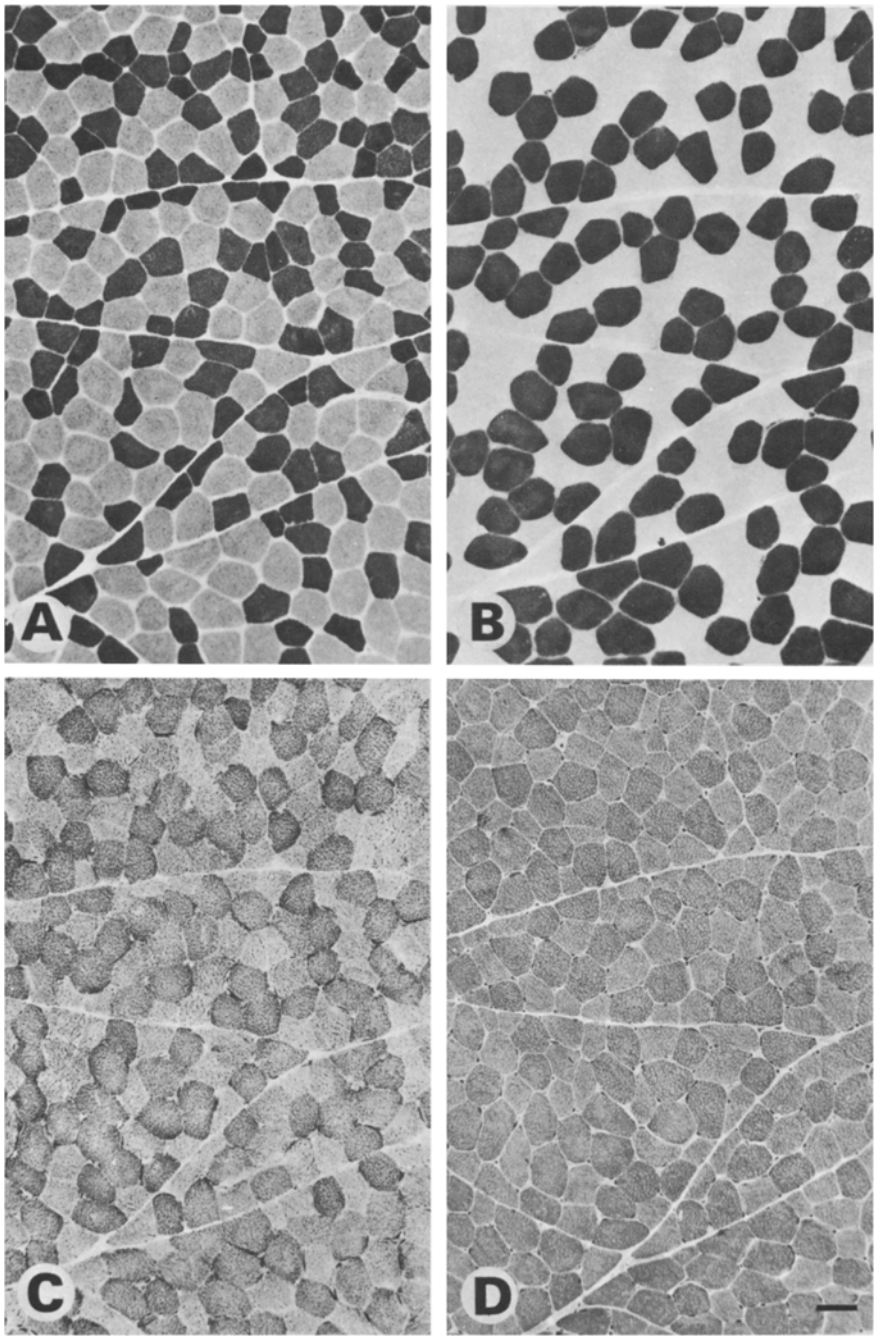

Fig. 5A–D. Biceps brachii muscle six days post mortem after storage at +21° C. Sections stained for the demonstration of ATPase pH 9.4 (**A**), ATPase pH 4.3 (**B**), NADH-TR (**C**) and with Gomori trichrome (**D**). In places the muscle nuclei are not stained. Cell borders and different fibre types are in general clearly distinguishable. In NADH-TR, three or more degrees of activity can be seen. The decrease of the mean diameter was estimated to be about 25% compared with the fibres measured one day post mortem. Bar, 50 μm. ×100

Masseter. 1–2 days post mortem: Type I, small type II and intermediately stained muscle fibres could clearly be distinguished.

3–5 days: In places, or in small areas, the reaction product was weak and areas lacking enzyme activity could be seen in the fibres. Different muscle fibre types were still in general, or in large areas, distinguishable (Fig. 6A–B). In one case (male 30 years), fibres in parts of the sections showed an inverse reaction for ATPase at pH 9.4 after storage for four days or more.

6–7 days: In large areas, the muscle fibres showed a weak reaction product and areas lacking enzyme activity.

8–10 days: In general, the reaction product was very weak or absent. Different muscle fibre types were only locally distinguishable.

NADH-Tetrazolium Reductase

Biceps brachii. 1–2 days post mortem: Different muscle fibre types with different degrees (three or more) of activity could clearly be distinguished.

3–6 days: Fibres with a weak and/or uneven reaction product were seen in places or in small areas. Different muscle fibre types with different degrees (three or more) of activity could still in general, or in large areas, be distinguished (Fig. 5C).

7–10 days: In general, the reaction product was weak to very weak and/or uneven with only peripheral activity. Different muscle fibre types could only be distinguished locally.

Masseter. 1–2 days post mortem: Different muscle fibre types with different degrees (three or more) of activity could be distinguished.

3–5 days: Fibres with a weak and/or uneven reaction product were frequently seen. Different muscle fibre types with different degrees (three or more) of activity could be distinguished only in small areas (Fig. 6C).

6–10 days: In general, the reaction product was weak to very weak and/or uneven with only peripheral activity. Different muscle fibre types could not be distinguished.

Gomori Trichrome

Biceps brachii. 1–3 days post mortem: The cell borders and the muscle nuclei could clearly be distinguished.

4–6 days: In places, or in small areas, the cell borders were indistinct and the nuclei did not stain. In general or in large areas, the cell borders and the nuclei were distinguishable (Fig. 5D).

7–10 days: The cell borders were generally indistinct and the nuclei did not stain.

Masseter. 1–2 days post mortem: The cell borders and the muscle nuclei could clearly be distinguished.

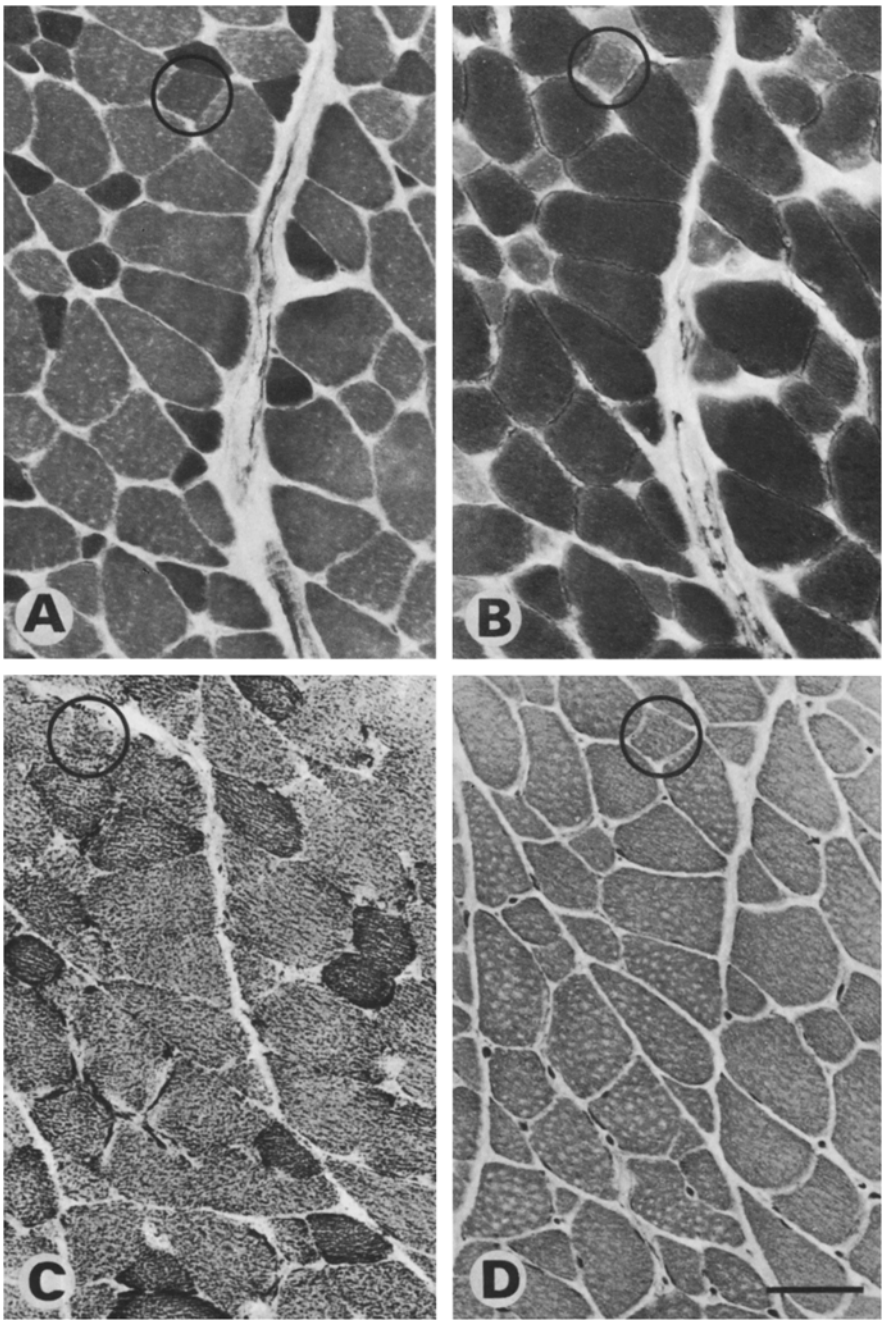

Fig. 6A–D. Masseter muscle five days post mortem after storage at +21° C. Sections stained for the demonstration of ATPase pH 9.4 (**A**), ATPase pH 4.3 (**B**), NADH-TR (**C**) and with Gomori trichrome (**D**). Most muscle nuclei are not stained. Type I and small type II fibres are clearly observed. An ATPase intermediately stained fibre is encircled. In NADH-TR, three or more degrees of activity can be seen in this part of the section. Bar, 50 μm. ×250

3–5 days: In large areas, the cell borders were indistinct and most nuclei did not stain (Fig. 6D).

6–10 days: In general, the cell borders were indistinct, the nuclei did not stain and vacuoles could be seen in the fibres.

Discussion

Starting at the moment of death, a series of post mortem events develops in more or less orderly fashion including loss of enzyme activities. The reliability of histochemical fibre typing of post mortem muscles depends, of course, on the extent of such cellular alterations. Although histochemical fibre typing of human necropsy muscles has been performed previously (Jennekens et al., 1971 a, b; Johnson et al., 1973; Polgar et al., 1973; Serratrice et al., 1976; Susheela and Walton, 1969; Wachstein and Meisel, 1955), no systematic analysis of the influence of time and temperature on the reliability of the method has been reported.

The present study indicates that muscle fibre typing can be reliably performed up to at least ten and fifteen days post mortem for the masseter and biceps brachii muscles respectively, when the specimens are stored at +4° C. The corresponding figures for storage at room temperature were three and six days. When necropsy specimens are used for diagnosis of neuromuscular disease or research, the samples are most often obtained well within these time limits.

The morphological changes, which occur in muscle tissues during rigor process and autolysis, are known to be highly variable within and between different muscles and have been related to the activity of ATPase and the levels and decrease of high energy phosphates and glycogen (Bendall, 1973; Goldspink et al., 1973; Heffron and Hegarty, 1974; Heffron and McLoughlin, 1971; McNaughtan 1978a, b; Tarrant et al., 1972a, b).

From our study it is also obvious that post mortem degradation varies within and between different muscles. The myofibrils were more resistant than the mitochondria as expressed by preserved ATPase activity and destroyed NADH-tetrazolium reductase activity. Myofibrillar ATPase activity was also more resistant than membranes and nuclei, as fibre typing could be performed in sections stained for myofibrillar ATPase, while in serial sections stained for Gomori trichrome, membranes and nuclei did not stain. These findings can be correlated with ultrastructural investigations where changes in post mortem skeletal muscles have been demonstrated in the mitochondria prior to any observable changes in myofibrillar proteins (Hegarty et al., 1978). Also, in the central part of transplanted cat muscles, fibre typing has been successfully performed in sections stained for myofibrillar ATPase, despite necrosis of the muscle fibres with demolition of all membranes, as shown by electron microscopy (Schiaffino et al., 1975).

The staining and fibre size pattern we have found in our biceps brachii and masseter samples within 24 h post mortem are similar to those in biopsy specimens (Dubowitz and Brooke, 1973; Ringqvist, 1974; Serratrice et al., 1976). The demonstrated differences between the masseter and biceps brachii concern-

ing post mortem preservation of enzyme activity might be related to the different fibre composition and thus to differences in enzyme content and energy sources.

Our observation of a decrease in fibre diameter with increased time of storage (cf. Figs. 2A, 3A and 5A) is in accordance with previous studies, where a decrease in fibre diameter of about 15% was shown within 24 h post mortem (Goldspink et al., 1973; Heffron and Hegarty, 1974). Fibre diameter measurements in autopsy material thus are not directly comparable with measurements in fresh frozen biopsies, which has not always been taken into consideration by other authors (Jennekens et al., 1971a; Polgar et al., 1973; Serratrice et al., 1976).

Intermediately stained fibres in standard ATPase staining of human limb muscles have been infrequently observed or discussed. We sometimes observe ATPase intermediately stained fibres in our routine biopsies, which we use for diagnostic and research purposes (unpubl. observations). Karpati et al. (1975) have, with the myofibrillar ATPase reaction at pH 9.4, observed intermediately stained fibres in morphologically normal human muscle biopsies and proposed that they be called type IB fibres. Askansas and Engel (1975) did not observe any subtypes of type I fibres in the ATPase stained sections but noticed two distinct type I subtypes by oxidative enzyme reactions. Recent classification of fibre types in human limb muscles by photometric evaluation of staining intensity (Dahl, 1979) shows a subgroup of fibres with staining properties as for the intermediately stained fibres which we have seen in the biceps brachii. The variation of histochemical fibre typing between various laboratories might reflect slight methodological differences.

The large fibres we found locally in our masseter samples, and which showed a strong reactivity for all stains as compared with the other fibres, might be a contraction artefact (McNaughtan, 1978a; Schmalbruch, 1973) induced by excision and fixation. Compared with the biceps brachii, the masseter muscle has a more complicated structure and thus more manipulation was needed for dissection and mounting.

Conclusions

While fibre typing in different muscles is reliable several days post mortem on the basis of myofibrillar ATPase activity, the use of NADH-tetrazolium reductase activity is less reliable. The speed with which decomposition develops differs within and between various muscles and probably reflects differences in fibre type composition and thus in enzyme and energy content.

Acknowledgements. The skilful technical assistance of Miss Inga Johansson is gratefully acknowledged. – Financial support was provided by the Swedish Medical Research Council (12X-3934 and 24X-4808), the Muscular Dystrophy Association of America and the Faculty of Odontology, University of Umeå, Sweden.

References

Askansas V, Engel WK (1975) Distinct subtypes of type I fibres of human skeletal muscle. Neurology 25:879–887

Bendall JR (1973) Post mortem changes in muscle. In: Bourne GH (ed.) The Structure and function of muscle. Academic Press, New York London, pp 244–309

Brooke MH, Kaiser KK (1970) Muscle fiber types: How many and what kind? Arch Neurol 23:369–379

Dahl HA (1979) Basal muskelmorfologi – Muskelfibertyper og andre faktorer av betydning for musklenes funksjonelle egenskaper. Scand J Rheumatol Suppl 30:13-22

Dubowitz V, Brooke MH (eds.) (1973) Muscle biopsy – A modern approach. WB Saunders, London, pp 34–73

Engel WK, Cunningham GC (1963) Rapid examination of muscle tissue. An improved trichrome method for fresh-frozen biopsy sections. Neurology 13:919–923

Goldspink G, Gelder S, Clapison L, Overfield P (1973) Pre- and post-rigor fixation of muscle. J Anat 114:1–6

Heffron JJA, Hegarty PVJ (1974) Evidence for a relationship between ATP hydrolysis and changes in extracellular space and fibre diameter during rigor development in skeletal muscle. Comp Biochem Physiol 49:43–56

Heffron JJA, McLoughlin JV (1971) The relationship between the rate of adenosine triphosphate hydrolysis and glycolysis post mortem in skeletal muscle. Proc 2nd Int Symp Conditon Meat Quality Pigs, Zeist, pp 109–117

Hegarty PVJ, Dahlin KJ, Benson ES (1978) Ultrastructural differences in mitochondria of skeletal muscle in the prerigor and rigor states. Experientia 34:1070–1071

Jennekens FGJ, Tomlinson BE, Walton JN (1971a) The sizes of the two main histochemical fibre types in five limb muscles in man. An autopsy study. J Neurol Sci 13:281–292

Jennekens FGJ, Tomlinson BE, Walton JN (1971b) Data on the distribution of fibre types in five human limb muscles. An autopsy study. J Neurol Sci 14:245–257

Johnson MA, Polgar J, Weightman D, Appleton D (1973) Data on the distribution of fibre types in thirty-six human muscles. An autopsy study. J Neurol Sci 18:111–129

Karpati G, Eisen AA, Carpenter S (1975) Subtypes of the histochemical type I muscle fibers. J Histochem Cytochem 23:89–91

MacNaughtan AF (1978a) A histological study of post mortem changes in the skeletal muscle of the fowl (*Gallus domesticus*). I. The muscle fibres. J Anat 125:461–476

MacNaughtan AF (1978b) A histological study of post mortem changes in the skeletal muscle of the fowl (*Gallus domesticus*). II. The cytoarchitecture. J Anat 126:7–20

Nyström B (1968) Histochemistry of developing cat muscles. Acta Neurol Scand 44:405–439

Padykula HA, Herman E (1955) The specificity of the histochemical method for adenosine triphosphatase. J Histochem Cytochem 3:170–183

Polgar J, Johnson MA, Weightman D, Appleton D (1973) Data on fibre size in thirty-six human muscles. An autopsy study. J Neurol Sci 19:307–318

Ringqvist M (1974) Fiber types in human masticatory muscles. Relation to function. Scand J Dent Res 82:333–355

Schiaffino S, Sjöström M, Thornell L-E, Nyström B, Hackelius L (1975) The process of survival of denervated and freely autotransplantated skeletal muscle. Experientia 31:1328–1330

Schmalbruch H (1973) Contracture knots in normal and diseased muscle fibres. Brain 96:637–640

Serratrice G, Pellissier JF, Vignon C, Baret J (1976) The histochemical profile of the human masseter. An autopsy and biopsy study. J Neurol Sci 30:189–200

Susheela AK, Walton JN (1969) Note on the distribution of histochemical fibre types in some normal human muscles. A study on autopsy material. J Neurol Sci 8:201–207

Tarrant PJV, McLoughlin JV, Harrington MG (1972) Anaerobic glycolysis in biopsy and post-mortem porcine longissimus dorsi muscle. Proc Royal Irish Acad 72B: 55–73

Tarrant PJV, Hegarty PJV, McLoughlin JV (1972) A study of the high energy phosphates and anaerobic glycolysis in the red and white fibres of porcine semitendinosus muscle. Proc Royal Irish Acad 72c: 229–251

Wachstein M, Meisel E (1955) The distribution of histochemically demonstrable succinic dehydrogenase and of mitochondria in tongue and skeletal muscles. J Biophys Biochem Cytol 1:483–487

Received November 19, 1979