Histochemical differentiation of skeletal muscle in foetal and newborn mice

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WITH ONE PLATE

INTRODUCTION

In earlier investigations of muscle development, morphological criteria, such as diameter and staining with routine methods, have been used for classifying different fibre types.

In human foetal muscle three fibre sizes are seen from the 15th week (Cuajunco, 1942). The largest fibres seem to be the centre of each primary muscle bundle. They were denoted as B fibres by Wohlfart (1937), who considered that they were also functionally different from the smaller ones forming around them. Tello (1922) and Cuajunco (1942) supported the widely held opinion that the smaller fibres are formed from the larger ones by longitudinal splitting. However, Couteaux (1941) claimed that the small fibres belong to a new generation differentiating from interstitial cells.

Histochemical studies on foetal muscle are rare. However, during the last years a good deal of work has been carried out on the histochemistry of adult skeletal muscle. Nachmias & Padykula (1958) presented a survey of several histochemical reactions in different muscle fibres. They found that in some muscles up to three distinct fibre types could be distinguished. Dubowitz & Pearse (1960) studied the correlation between oxidative enzymes and phosphorylase and concluded that in higher vertebrates there are two main types, one rich in oxidative enzymes but poor in phosphorylase (type I) and the other vice versa (type II). Engel (1962) added ATP-ase, which was found to give a strong reaction in type II fibres. Staining for oxidative enzymes has revealed the presence of three different fibre types in various vertebrates (Ogata & Mori, 1964). The three types are also distinguished by their different content of phosphorylase, glycogen and lipids and by their different uptake of labelled palmitic acid (Wirsén,

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Their myoglobin content can be examined histochemically (Drews & Engel, 1961) and the commonly used classification into 'red', 'intermediary' and 'white' fibres has recently proved valid for both extra- and intrafusal fibres (Wirsen, 1964a). The difference in oxidative enzyme content of muscle spindle fibres was described earlier by Ogata & Mori (1962).

In human foetal muscle, from the 14th to the 44th week, Fenichel (1963) attempted to classify Wohlfart's B fibres histochemically. He found that from the 17th week dark and light fibres could be seen after staining with phosphotungstic acid-haematoxylin. Comparing this staining with the distribution of ATP-ase activity in some of the foetuses, he concluded that the B fibres represented a size variation of type I. Dubowitz (1963) studied the enzymatic maturation in man, rat, guinea-pig, hamster and rabbit. The muscle fibres were differentiated at birth except in the rat, in which the differentiation started 2 or 3 days after birth and appeared to be completed at an age of 14 days. Dubowitz concluded that the maturation at birth depends on the length of the gestation as well as on the motor activity displayed by the newborn.

In a preliminary experiment we found that, in mice, the different fibre types could be easily identified at birth. Thus, in this species it would be possible to study the development of a heterogeneous fibre population from the very beginning.

**MATERIAL AND METHODS**

Mice of the A/Jax and CBA strains were used. They were mated overnight and the day when the vaginal plug was observed was denoted as the zero day of gestation. The mothers were killed by dislocation of the atlas on the 14th to 21st gestation day and the foetuses were removed and frozen in liquid nitrogen or fixed in formol sucrose solution (Holt & Hicks, 1961). The cranial half of newborn, 1-day and 7-day mice was prepared in the same way. Serial sections of the thoracic region were made at 15 μ in a cryostat (W. Dittes, Heidelberg, Germany).

The fresh-frozen material was stained according to the following methods: for succinic dehydrogenase with nitro-BT dissolved with N,N-dimethylformamide as suggested by Pearson (1958), for lipids with Sudan Black B modified after Meier (1958), for glycogen with periodic acid-Schiff (PAS) in 70 per cent. ethanol (Mowry et al., 1952); control sections were incubated in 1 per cent. diastase (Merck, Darmstadt, Germany) in saline as well as in water only at 20° for 1 hr. before PAS staining (Graumann & Clauss, 1959), for phosphorylase (Takeuchi & Kuriaki, 1955, modified by Eränkö & Palkama, 1961) controlled by omitting glucose-1-phosphate in the incubation medium, and for non-specific esterase (Pearse, 1960).

Sections from the formol-sucrose-fixed material were stained for myoglobin (Drews & Engel, 1961), lipids, glycogen and non-specific esterase.
PLATE

C. WIRSEN AND K. S. LARSSON

(Facing page 761)
RESULTS

Staining for succinic dehydrogenase and lipids did not reveal any distinct difference between fibre types before birth and will therefore not be considered further. Esterase activity was present in motor end-plate regions and also in nerve fibres from the 14th gestation day. Peroxidase activity interpreted as due to myoglobin was not demonstrable until after birth. PAS-positive, diastase-digestible material was uniformly distributed in the myotubes on the 15th to 16th gestation days. Differences in glycogen content between fibres were observed from the 17th to 19th gestation day, at least in the A/Jax strain. The intensity of the reaction varied in different experiments. A positive phosphorylase reaction appeared in the myotubes on the 16th gestation day. This method has proved the outstanding one for demonstrating metabolic differences between fibres. The results obtained with this method will therefore be described in detail from the 16th gestation day, i.e., when different types of developing muscle fibres can be distinguished histochemically.

16th gestation day

Many of the muscles contained only strongly phosphorylase-positive myotubes. These will be denoted as primary fibres. In certain muscle groups, however, small fibres, of which several were in the myotube stage, appeared in close contact with the large primary fibres. These fibres, which showed a weaker reaction, will be denoted as secondary fibres (Plate, Fig. A).

17th and 18th gestation day

The number of secondary fibres had increased but the diameter was still less in most muscles than that of the primary ones. However, there were large variations between different animals as well as between the muscles in individual

EXPLANATION OF PLATE

Fig. A. Mouse foetus, 16th gestation day. Large primary myotubes surrounded by smaller secondary. One ‘giant’ fibre of the primary type (right) resembles the B fibre of Wohlfart. Phosphorylase. 300×.

Fig. B. Mouse foetus, 17th gestation day. Primary (dark grey) and secondary (light grey) fibres. ‘Triads’ with a small phosphorylase-negative myotube are seen. Phosphorylase. 300×.

Fig. C. Same animal as in Fig. B. In one of the muscle spindles (centre), a phosphorylase-negative intrafusal fibre is seen. Phosphorylase. 300×.

Fig. D. Mouse foetus, 19th gestation day. Tertiary fibres (light) are seen close to primary and secondary fibres. Phosphorylase. 300×.

Fig. E. Mouse foetus, 20th gestation day. A typical muscle spindle (centre) with three types of intrafusal fibre. Phosphorylase. 300×.

Fig. F. One-day-old mouse. A well-developed muscle spindle with four—two primary (dark), one secondary (light grey) and one tertiary (very weakly stained)–intrafusal fibres. The extrafusal fibres are polygonal as in adult musculature. Phosphorylase. 300×.
animals at this age. In some muscles, secondary fibres were scarce and their
diameter was only a quarter of that of the primary ones. In general,
however, this stage was characterized by a rapid development of the secondary
fibres, which in certain muscle groups had become as large as or even larger
than the primary fibres. Occasionally, small phosphorylase-negative myotubes
were seen close to or interposed between primary and secondary fibres so as to
form a sort of triads. No developed phosphorylase-negative fibres were ob-
served in the muscles on the 17th and 18th day of gestation (Plate, Fig. B).

Muscle spindles were seen developing in many of the muscles. As a rule,
they contained two to four fibres. The more primitive ones consisted of one
primary and one secondary fibre surrounded by a very thin capsule. In others,
small myotubes—usually of the secondary type—appeared between these two
fibres, but in one case, a phosphorylase-negative myotube was observed as well
(Plate, Fig. C).

19th gestation day

Secondary fibres were now well developed in practically all muscles. In some,
most of the fibres of both types were in the myotube stage, whereas in others only
occasional myotubes were seen. A third fibre type, the tertiary fibre, which was
practically phosphorylase-negative, was now seen developing between the
primary and secondary fibres (Plate, Fig. D). At this stage, the muscle fibres
were getting more closely packed together and their shape had changed from
rounded to polygonal as in adult musculature.

Several muscle spindles were found. They were now more distinctly separated
from the surrounding musculature and contained up to five fibres.

20th and 21st gestation day

In some muscles, the diameter of the secondary fibres was equal to or even
exceeded that of the primary fibres. The intercellular space was markedly
reduced. Myotubes were still seen among primary and, occasionally, secondary
fibres. Some of the smaller tertiary fibres were also in the myotube stage.

The spindles contained three to five fibres. In some, all three types could
be distinguished (Plate, Fig. E).

Newborn and 1 day

All three fibre types were seen in several muscles. There was no longer any
strict correlation between fibre diameter and histochemical type. Only occasional
myotubes were found. The spindles contained four to five fibres (Plate, Fig. F).

6–7 days

At this stage, the musculature had practically attained the adult appearance.
In certain muscles the tertiary fibres were the largest ones. A few myotubes
were still seen among primary fibres. Muscle spindles with up to six fibres and
with all three types represented were seen.
DISCUSSION

The present results show that the trunk musculature begins to take an adult appearance already at birth in both mouse strains studied. Differences in the predominance of glycolytic mechanisms, as indicated by the phosphorylase content in three distinct fibre populations, present themselves with the development of secondary fibres from the 16th and of tertiary fibres from the 19th gestation day. During the foetal development of fibre populations, there is thus a gradual transition from glycolytic to presumably oxidative metabolism, reflected by the strong phosphorylase reaction of the primary fibres as compared to the moderate staining of the secondary and the very weak reaction of the tertiary fibres. However, in certain respects, such as different contents of lipids, esterase, oxidative enzymes and myoglobin, the histochemical differentiation characteristic of adult musculature is not clearly demonstrable even at 1 week of age.

A direct correlation between gestation length and maturation of the musculature as proposed by Dubowitz (1963) does not obtain, since the differentiation appears to be much more advanced at birth in the mouse than in the rat in spite of the same gestation period, about 21 days. Nor is the more advanced maturity of the musculature in a newborn mouse reflected in a more vigorous motor activity than that displayed by the less mature rat.

The development of new sets of myotubes around larger and more developed ones, which in the two mouse strains takes place between the 16th day and birth in the trunk muscles, closely resembles the so-called 'Myotubenvermehrungsstadium' (myotube multiplication stage) (Tello, 1922), described in 15-week human foetuses by Cuajunco (1942). This increase in the number of developing fibres has been attributed, as mentioned before, either to longitudinal splitting of primary myotubes (Tello, 1922; Cuajunco, 1942), or to differentiation of new fibres from interstitial myoblastic elements (Couteaux, 1941).

The splitting hypothesis is not supported by the present results for the following reasons:

(1) With the newly formed secondary fibres there also appears a different reaction intensity for phosphorylase, whereas fibres formed by splitting off from the large primary myotubes would be expected to show the strong phosphorylase reaction of their 'mother' fibres.

(2) In straight cross-sections there are always distinct boundaries between closely attached fibres. Due to attenuation of the overlying fibre in oblique sections, one may get a false impression of 'budding' of small fibres from the larger ones and of a cytoplasmic continuity between them. However, in longitudinal sections the different fibre types are always distinctly separated.

Couteaux's statement that new fibres develop from interstitial myoblastic cells thus seems to be confirmed and extended. The new sets of fibres bring about not only an increase in the number of muscle fibres, but also new metabolically
different types. These new types, the secondary and the tertiary, appear to develop in the same way as the primary, i.e., via the myotube stage into adult polygonal fibres with laterally placed nuclei. As judged from our observations, this development becomes more accelerated for each new type. Thus, primary fibres can be seen retaining their myotube appearance up to late stages (cf. Boyd, 1960).

Whereas the small myotubes seen on the 16th day are likely to develop into secondary fibres, the fate of the phosphorylase-negative ones appearing in a few muscle groups on the 17th day is still a question left open for discussion. The position between or close to primary and secondary fibres may well suggest their being future tertiary fibres. However, it may also take a certain time before early secondary myotubes get their characteristic phosphorylase content. Moreover, in the present material there is a latency from the 17th until the 19th day before developing tertiary fibres are first observed. As on the 20th and 21st days all developmental stages of the tertiary fibres can be seen, there is reason to believe that the three fibre types represent three different populations, each with its own inherent metabolic qualities.

Three types of fibre with different reactivity for phosphorylase staining are found also in adult musculature of various species. The phosphorylase content of each type is inversely correlated to that of oxidative enzymes, lipids and also myoglobin. Therefore, the three foetal fibre populations, primary, secondary and tertiary, could be tentatively classified as corresponding to the ‘white’, ‘intermediary’ and ‘red’ fibres of adult musculature.

The B fibre of Wohlfart has been defined as (1) the large central myotube in each primary muscle bundle of a developing embryo or foetus, (2) large, often rounded fibres that can be easily distinguished from the surrounding smaller fibres, especially in older foetuses and infants and also in certain conditions later in life (Wohlfart, 1937). With regard to their staining reactions, the large rounded ‘B fibres’ in the diaphragm of the dog are often intermediary (Wirsen, 1964a). However, in the flight muscles of the pigeon, the very large, rounded fibres are white. Fenichel (1963) observed that the largest fibres in 22-week foetuses gave a weak ATP-ase reaction, but did not present any histochemical data of the large central myotubes in the 17th week. In our material, the largest fibres at early stages all show a strong phosphorylase reaction. The present observations also leave some doubt as to whether large fibres with a certain staining quality at 22 weeks can be said to represent a later developmental phase of the largest ones at 17 weeks. In the mouse, the largest fibres observed at birth belong in some muscles to the secondary or even to the tertiary type. Such a reversal of the original correlation between diameter and histochemical type as has taken place within a few days in the mouse, may well be completed within 5 weeks in the human foetus.

The muscle spindles are stated to develop during the myotube stage through the morphogenetic influence from outgrowing sensory nerve endings (Zelenà,
Most authors agree that the number of intrafusal fibres increases during the development of the spindle. However, from earlier investigations it cannot be deduced which myotube types are represented. Since muscle spindles appear in the limb muscles of the rat on the 19th gestation day (Zelenà, 1957), i.e., before the fibres are histochemically differentiated (Dubowitz, 1963), there is reason to believe that the bundle of muscle cells that gets enclosed within the developing spindle capsule consists of myotubes and myoblasts with the same inherent metabolic qualities as the surrounding extrafusal fibres. It has been shown (Ogata & Mori, 1962; Wirsén, 1964a) that in adult animals the intrafusal fibres represent the same histochemical types as are found in the adjacent musculature. This heterogeneity is first seen in mouse foetuses on the 17th gestation day, i.e., the stage dominated by the rapid development of the secondary fibres. Likewise, three different types of intrafusal fibre are clearly distinguished as tertiary fibres develop in the surrounding musculature. Admittedly, the present material is too restricted to make justifiable an analysis of successive developmental phases. But there seems to be nothing in the findings against the concept that extra- and intrafusal fibres differentiate in principally the same way.

**SUMMARY**

1. The histochemical differentiation of developing muscle fibres in two mouse strains has been studied from the 14th gestation day to 1 week after birth.

2. Of the various staining methods used, phosphorylase staining proved the best one for distinguishing between the three types of fibre present in vertebrate muscles. Differences in glycogen content were also noticed, whereas the developing fibres could not be classified according to their content of oxidative enzymes, esterases and lipids, as is the case in adult muscles. Myoglobin was not demonstrable until after birth.

3. A positive esterase reaction at motor end-plate regions and in nerve fibres was noticed throughout the series.

4. A positive phosphorylase reaction appeared on the 16th gestation day. It was strong in the large myotubes, which are denoted as the primary type. Smaller (secondary) fibres with weaker staining intensity developed close to them. From the 19th day, tertiary fibres with practically no positive reaction for phosphorylase were seen developing close to the primary and secondary fibres.

5. The same heterogeneous development was noticed in muscle spindles.

6. It is proposed that the three different fibre types of adult vertebrate musculature develop as three distinct populations. Earlier theories of longitudinal splitting of developing myotubes do not seem plausible in view of the present findings.
Différenciation histochemique du muscle squelettique chez les foetus et les nouveaux de souris

1. On a étudié la différenciation histochemique des fibres musculaires en cours de développement chez deux lignées de souris, du 14e jour de la gestation à la première semaine après la naissance.

2. Parmi les diverses méthodes de coloration utilisées, la coloration de la phosphorylase s'est révélée la meilleure pour distinguer les trois types de fibres présentes dans les muscles de Vertébrés. Des différences dans la teneur en glycogène ont aussi été notées, tandis que les fibres en cours de développement n'ont pu être classées selon leur teneur en enzymes oxydatives, estérases et lipases, comme c'est le cas pour les muscles d'adulte. La myoglobine n'a pu être mise en évidence jusqu'après la naissance.

3. Une réaction positive pour l'estérase dans les régions terminales motrices (plaque) et dans les fibres nerveuses a été notée dans toute la série.

4. Une réaction positive pour la phosphorylase est apparue le 16e jour de la gestation. Elle était forte dans les grands myotubes, qui constituent le type primaire. Des fibres plus petites (secondaires) avec une intensité de coloration plus faible se sont développées à côté d'eux. A partir du 19e jour, des fibres tertiaires n'ayant pratiquement pas de réaction positive pour la phosphorylase ont été vues se développant à côté des fibres primaires et secondaires.

5. On a noté le même développement hétérogène dans les fuseaux musculaires.

6. On suggère l'idée que les trois types différents de fibres de la musculature des Vertébrés adultes se développent comme trois populations distinctes. Des théories plus anciennes sur la fissuration longitudinale des myotubes en cours de développement ne semblent pas plausibles à la lumière des résultats présents.

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REFERENCES


WIRSEN, C. (1964b). To be published.


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