Enzyme histochemistry of skeletal muscle

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Part I   Developing animal muscle

This is the first of three papers on the application of enzyme histochemical techniques to the study of developing and diseased muscle. This paper will deal with developing muscle in various laboratory animals, the second with developing human muscle, and the third with hereditary neurogenic atrophies in infancy and childhood.

In 1678 Stefano Lorenzini had already observed that animal muscle could be subdivided into red and white muscle on the basis of differences in colour (Ciaccio, 1898). The extensive studies of Ranvier (1873, 1874, 1880) as well as of other physiologists (Kölliker, 1856; Grützner, 1884; Knoll, 1891) during the last century helped to establish a firm correlation between the morphology of muscle and its function. It was shown that the red muscle fibres had a slow speed of contraction, but were capable of sustained activity, while the white fibres had a fast speed of contraction and were mainly adapted for short bursts of rapid activity.

Following the introduction of a reliable histochemical method for the demonstration of succinate dehydrogenase in tissue sections (Seligman and Rutenburg, 1951), a number of authors demonstrated a variation in the content of this enzyme in the individual muscle fibres in various animals (Padykula, 1952; Wachstein and Meisel, 1955; Buño and Germino, 1958; Nachmias and Padykula, 1958; Ogata, 1958; George and Scaria, 1958). In general, red muscle contained mainly fibres with a high content of succinate dehydrogenase and white muscle a high proportion of fibres poor in succinate dehydrogenase.

In a comparative study of human and various animal muscles (Dubowitz and Pearse, 1960a, 1960b, 1961), it was observed that in serial section the fibres rich in succinate dehydrogenase also had a high content of other oxidative enzymes such as cytochrome oxidase, nicotinamide-adenine dinucleotide (NADH) diaphorase, and NAD-linked lactate dehydrogenase, but were poor in phosphorylase. In contrast, the fibres with a weak reaction for succinate dehydrogenase also had a low content of the other oxidative enzymes but were rich in phos-

phorylase. It was suggested that these fibre types be referred to as type I and type II respectively. Type I fibres corresponded to 'red' muscle and probably depended on oxidative metabolism (Krebs cycle) for their energy, whereas the type II fibres, corresponding to 'white' muscle, probably obtained their energy mainly from the anaerobic glycolytic pathway. In most muscles, fibres were also observed with an intermediate activity between the strong and the weak fibres, for a particular enzyme reaction.

Pearse (1961) demonstrated a high content of mitochondrial, non-coenzyme linked, α-glycerophosphate dehydrogenase in type II fibres, and recently Blanchaer, van Wijhe, and Mozersky (1963) and van Wijhe, Blanchaer, and Jacyk (1963) have shown that, with the addition of phenazine metho-sulphate to the histochemical reagents, a high content of NAD-linked α-glycerophosphate dehydrogenase as well as lactate dehydrogenase is found in type II fibres. These observations provide further evidence that type II fibres selectively utilize the substrates of the glycolytic cycle in their metabolism. Engel (1962) has also observed a high content of myosin adenosine triphosphatase (ATPase) in type II fibres.

Attempts have recently been made, on the basis of the relative content of various enzymes, to subdivide further the gastrocnemius and soleus muscles of the rat into three fibre types (Stein and Padykula, 1962) or even eight fibre types (Romanul, 1964). While recognizing that fibres do occur with an intermediate activity for the different enzyme reactions, and that additional permutations are possible with the use of a larger number of enzyme methods, I think that the subdivision into two types, with a limited number of enzyme methods, is useful in the comparative study of normal and diseased human and animal muscle.

MATERIAL AND METHODS

An initial study was made of two newborn rats, less than 24 hours old, killed by decapitation. Sections of limb and trunk muscle were prepared for histochemical assessment

along the lines previously described (Dubowitz and Pearse, 1964).

The following enzyme reactions were performed:— NADH-diaphorase, succinate dehydrogenase; NAD-linked α-glycerophosphate, isocitrate, malate and lactate dehydrogenases; phosphorylase and adenosine triphosphatase (ATPase).

Subsequently, the limb and trunk muscles of two series of rats, aged from 1 to 14 days, were investigated by the same techniques. In each series, one rat at each day of age was studied, and muscles were obtained from both sides of the body.

A comparative study was made of newborn mice, hamsters, rabbits, and guinea-pigs, less than 24 hours old. Only the following enzymes, considered to be sufficiently representative, were assessed: NADH diaphorase, lactate dehydrogenase, phosphorylase, and ATPase.

Two litters of guinea-pig foetuses were studied along similar lines. In the first litter, of 35 to 40 days' gestation, three of the five foetuses (weighing 22·2, 22·0, and 21·8 g. respectively) were dissected, and in the second litter, of approximately 50 to 55 days' gestation, two out of three foetuses (weighing 112 and 98 g. respectively) were studied. In each foetus sections were made of limb and trunk muscles from both sides of the body.

RESULTS

RAT   In the newborn rat all muscle fibres gave a uniformly positive result with the various enzyme reactions, and there was no subdivision into different fibre types. Individual muscle fibres were rounded in section, and tended to be arranged in small clusters rather than compact bundles.

In the developing rats from 1 to 14 days of age, no clear-cut dividing line could be drawn between the phase of uniform activity and the phase of complete differentiation. The process appeared to be a gradual one and to occur initially in random parts of the muscle and subsequently to extend to the whole muscle (Fig. 1). Some muscles showed early evidence of differentiation with one enzyme (especially ATPase), and complete uniformity with the others.

Animals from smaller litters grew faster and also showed earlier differentiation than smaller rats of similar age from other litters.

By the seventh day most muscles were showing some variation in enzyme content between fibres and by the 14th day the majority had a pattern similar to adult muscle.

MOUSE   Muscle from the newborn mouse showed a uniform activity of fibres for the various enzyme reactions comparable to that of the rat.

RABBIT   The muscle from newborn rabbits showed a differentiation into fibre types as in the adult animal, although the contrast between strongly and weakly reacting fibres was less striking than in the adult animal.

HAMSTER   Newborn hamsters also showed differentiation of the muscle into fibre types, but this was less clearly defined than in the newborn rabbit.

GUINEA-PIG   The muscle in newborn guinea-pigs resembled adult muscle and was fully differentiated into type I and type II fibres.

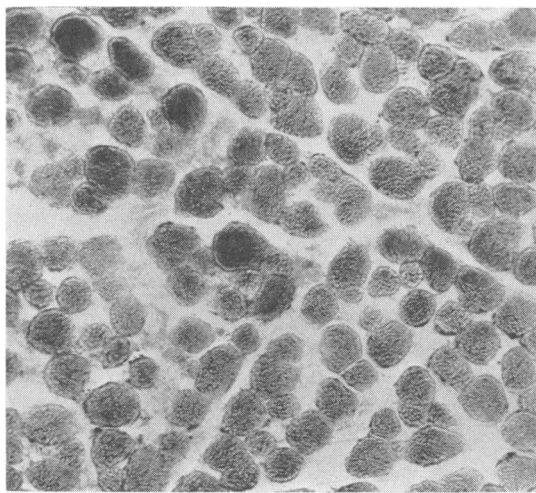In the foetuses of 35 to 40 days' gestation, most

FIG. 1.   *Rat, aged 4 days, upper forelimb. Overall uniform enzyme activity, with isolated stronger fibres.* × 280.
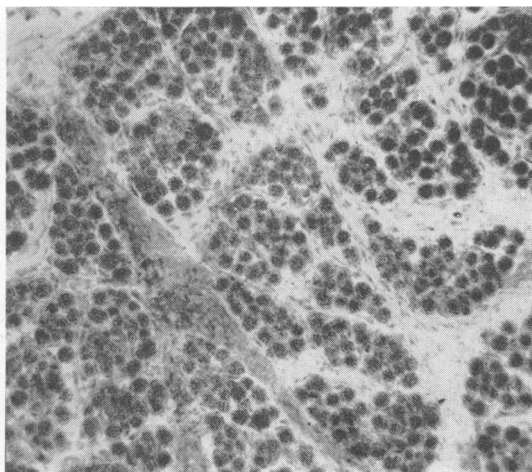

FIG. 2.   *Guinea-pig foetus of 35 to 40 days' gestation. Triceps. Overall uniformity of enzyme activity. Some bundles more strongly reacting. NADH diaphorase* × 100.
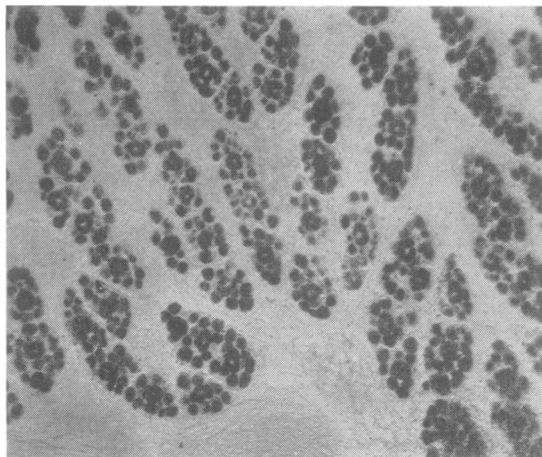
FIG. 3. *Guinea-pig foetus of 35 to 40 days' gestation. Calf muscle. Bundles subdivided into smaller groups. Strongly and weakly reacting fibres. Phosphorylase × 100.*
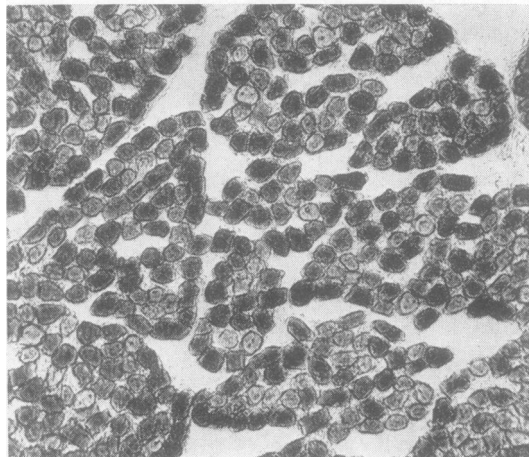

FIG. 4. *Guinea-pig foetus of 50 to 55 days' gestation. Triceps. Strongly and weakly reacting fibres; checker-board pattern. Phosphorylase × 100.*

muscles showed complete uniformity of fibres with the NADH-diaphorase reaction, apart from some variation in overall stain of some whole bundles as compared with others (Fig. 2). In occasional muscles there was some variation between individual muscle fibres within a bundle.

With phosphorylase and ATPase, in contrast, most muscles showed subdivision of fibres into strongly and weakly reacting ones. However, it did not give the clear-cut checkerboard pattern of mature muscle (Fig. 3).

The muscle bundles tended to be subdivided into small subgroups of 20 to 30 fibres and these frequently contained a large central fibre with a strong phosphorylase or ATPase activity (Fig. 3).

At 50 to 55 days' gestation, all the muscles showed clear-cut differentiation into type I and type II fibres with the various enzyme reactions and showed the checkerboard pattern of mature muscle (Fig. 4).

DISCUSSION

There is a striking difference in the maturation of skeletal muscle in different species of animals. Thus, in the guinea-pig at birth the muscle already shows full differentiation into fibre types. This process has already occurred by approximately three-quarters of the way through pregnancy. At the other extreme, the rat and mouse show no differentiation of their muscle into fibre types at birth and the process is only complete in the rat by about 2 weeks of age. The hamster and rabbit show differentiation at birth but this is less striking than in the guinea-pig.

There appears to be some correlation between the

differentiation of the muscle at birth and the general maturity of the animal. The guinea-pig at birth is a fairly active and mobile animal, with a mature, well-developed fur. The rat and mouse, on the other hand, have no fur at birth and are relatively sluggish and immobile. The rabbit and hamster have a better fur and are more active than the rat but not quite to the extent of the guinea-pig.

There is also some correlation between muscle differentiation and the length of gestation in the case of the guinea-pig (68 days), the rabbit (30 days), the rat (21 days), and the mouse (20 days). However, the hamster, which is more mature than the rat, has a gestation of only 16 to 19 days.

SUMMARY

In skeletal muscle from adult animals at least two fibre types can be recognized by various histochemical enzyme stains.

In developing muscle there is a difference between various species. In the guinea-pig the muscle is fully differentiated at birth and the process starts between half to three-quarters of the way through pregnancy. In the rat and mouse the muscle is undifferentiated at birth. In the rat differentiation is complete by about 2 weeks of age.

In newborn rabbits and hamsters the muscle is already differentiated but not as extensively as in the guinea-pig.

There is some correlation between the presence of differentiation in the muscle at birth and the general maturity and mobility of the animal, and also the length of gestation.

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Part II   Developing human muscle

In human muscle, as in animal, at least two fibre types can be recognized with histochemical enzyme techniques (Dubowitz and Pearse, 1960, 1961). Type I fibres are rich in certain oxidative enzymes such as succinate dehydrogenase, cytochrome oxidase, and NADH (nicotinamide-adenine dinucleotide) diaphorase, but poor in phosphorylase, whereas type II fibres have a high content of phosphorylase as well as other enzymes connected with the glycolytic cycle, such as mitochondrial α-glycerophosphate dehydrogenase (Pearse, 1961) and lactate dehydrogenase (Blanchaer, van Wijhe, and Mozersky, 1963; van Wijhe, Blanchaer, and Jacyk, 1963), but are poor in Krebs cycle oxidative enzymes. Type I fibres, corresponding to 'red' muscle of animals, probably obtain their energy mainly from oxidative metabolism, while type II fibres, corresponding to 'white' muscle, depend mainly on the anaerobic metabolism of the glycolytic pathways.

In the present study, which is along similar lines to the preceding paper on animal muscle (Part I), an attempt has been made to follow the changes in the enzyme histochemistry of muscle during the course of normal development.

MATERIAL AND METHODS

INFANCY AND CHILDHOOD   Specimens of muscle were obtained from 13 children, with no neurological disease, ranging in age from 2 weeks to 8 years. Six were biopsies and included deltoid, internal oblique, rectus femoris, erector spinae, and gluteus maximus. The remainder were obtained at necropsy. In each of the latter cases the gastrocnemius and quadriceps were studied and in one child a large selection of different muscles was obtained.

NEWBORN FULL-TERM INFANTS   Twenty-one muscle specimens, mainly gastrocnemius and quadriceps, were obtained from 12 newborn infants, of over 36 weeks' gesta-

tion and 2,500 g. birth weight, who had died within 48 hours of birth.

NEWBORN PREMATURE INFANTS   Forty muscle specimens, mainly gastrocnemius and quadriceps, were obtained at necropsy from 21 newborn premature infants, ranging in birth weight from 830 to 2,278 g. With one exception all had died within 48 hours of birth.

FOETUSES   An extensive selection of limb and trunk muscles was dissected from 17 human foetuses. The majority of these resulted from therapeutic abortions but a few followed spontaneous abortions. Details of these foetuses, together with the estimated gestational age, based on the data of Arey (1954), are included in Table I.

TABLE I

ESTIMATED GESTATION OF HUMAN FOETUSES

Index	Estimated Gestation[1]	Weight (g.)	Crown-rump Length (mm.)	Total Length (mm.)	Post-mortem Delay (hr.)[2]
1 LOD(2)	25	666 (24)[3]	200 (24)	295 (24)	24
2 MAL	23	615 (24)	210 (24)	305 (24)	8
3 DOW	25	550 (23)	209 (24)	327 (26)	15
4 EYR	23	410 (21)	195 (23)	280 (23)	7
5 DON	24	314 (20)	180 (22)	250 (20)	6
6 STE	17	260 (19)	160 (20)	250 (20)	11
7 DEN	19	170 (18)	140 (19)	210 (19)	—
8 LOD(1)	18	144 (17)	130 (18)	190 (18)	—
9 ROD	20	124 (17)	135 (18)	190 (18)	3
10 LLE	16	—	—	—	—
11 ROB	19	65 (14)	105 (16)	142 (16)	6
12 COL	—	61 (14)	95 (15)	130 (15)	2
13 HAR	15	58 (14)	105 (16)	140 (16)	4
14 WIL	12	37 (13)	87 (14)	120 (14)	4
15 ROT	12	18 (12)	75 (13)	98 (13)	6
16 PEA	11	18 (12)	65 (12)	90 (12)	—
17 SME	12	18 (12)	—	—	5

[1]Obstetrical. All ages in weeks.
[2]Time lapse between death of foetus and dissection of muscle.
[3]Estimated gestation.

All the above muscle specimens were rapidly frozen in liquid nitrogen, sectioned in a cryostat (cold microtome), and NADH diaphorase, lactate dehydrogenase, phosphorylase, and adenosine triphosphatase (ATPase) assessed. Details of the techniques and references to the methods have been given previously (Dubowitz and Pearse, 1964).

RESULTS

In the infants and children, irrespective of age, the muscle showed a checkerboard pattern, with subdivision into at least two fibre types, as in adult muscle (Figs. 1 and 2). The biopsy material gave better results than the necropsy material. In the latter the phosphorylase reaction was frequently negative or very weak and the ATPase reaction frequently produced a diffuse surface precipitate. Although the reactions for NADH diaphorase and lactate de-
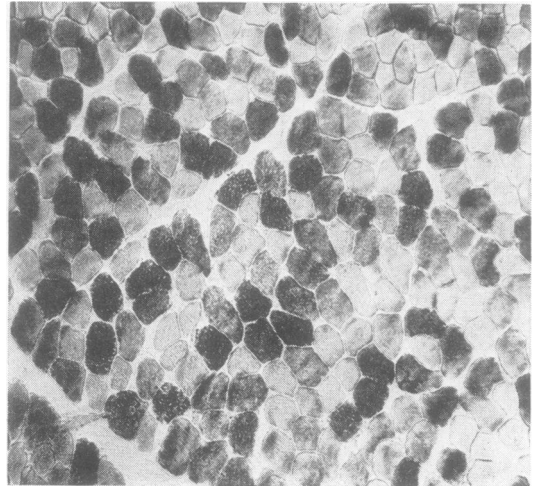

FIG. 1.   *Normal child, aged 5. Internal oblique, showing checkerboard pattern of strongly and weakly reacting fibres. Phosphorylase × 100.*
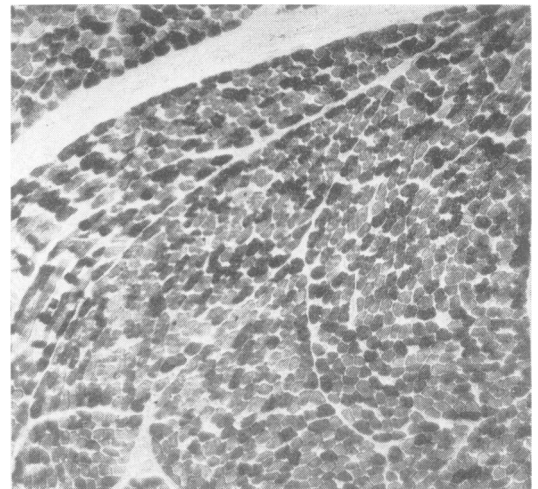

FIG. 2.   *Normal child, aged 4 weeks, glúteus maximus. Checkerboard pattern as in Fig. 1, but note smaller fibre diameter. Phosphorylase × 100.*

hydrogenase were consistently positive in necropsy material, the stain was often diffused, obscuring the outline of the muscle fibres.

The fibre diameter in the children over 6 months usually ranged from 20μ to 50μ (Fig. 1), while that of the infants under that age was usually between 10 and 25μ (Fig. 2).

In the newborn full-term infants the pattern of fibre differentiation was similar to that of the older infants. The NADH diaphorase gave the most

consistently positive results. A correlation of the intensity of reaction and the presence of differentiation for the various enzymes is given in Table II.

TABLE II

CORRELATION OF REACTION INTENSITY AND FIBRE DIFFER-
ENTATION FOR VARIOUS ENZYMES IN NEWBORN FULL-TERM
INFANTS

Enzyme Reaction	Fibre Differentiation	Grade of Reaction Intensity		
		2	1	0
NADH-diaphorase	2	14[1]	—	—
	1	2	2	—
	0	—	2	—
Phosphorylase	2	3	1	—
	1	—	8	—
	0	—	—	9
ATPase	2	9	—	—
	1	9	—	—
	0	1	2	—

[1]Numbers refer to number of muscles.

The newborn premature infants showed a similar pattern of differentiation into fibre types, but the results were less consistent than in the full-term infants. Table III correlates the strength of reaction and presence of differentiation in these muscles.

TABLE III

CORRELATION OF REACTION INTENSITY AND FIBRE DIFFEREN-
TIATION FOR VARIOUS ENZYMES IN NEWBORN PREMATURE
MUSCLE

Enzyme Reaction	Fibre Differentiation	Grade of Reaction Intensity		
		2	1	0
NADH-diaphorase	2	6[1]	—	—
	1	10	10	—
	0	4	10	—
Phosphorylase	2	10	—	—
	1	3	15	—
	0	3	6	3
ATPase	2	6	—	—
	1	8	10	—
	0	5	9	—

[1]Numbers refer to number of muscles.

With one exception, all the newborn infants had an approximately equal proportion of type I and type II fibres in the muscle, as in adult muscle. The exceptional one was a newborn premature infant, of approximately 26 weeks' gestation, weighing 840 g., in whose muscles there was a striking preponderance of type II fibres as compared with type I fibres (approximately 20 to 1).

The foetuses could be divided into two distinct groups on the basis of their enzyme histochemistry. The five larger foetuses (Table I, nos. 1-5), ranging in gestation from approximately 20 to 26 weeks, all had a very similar pattern. This was best shown with the NADH diaphorase reaction. The muscle con-
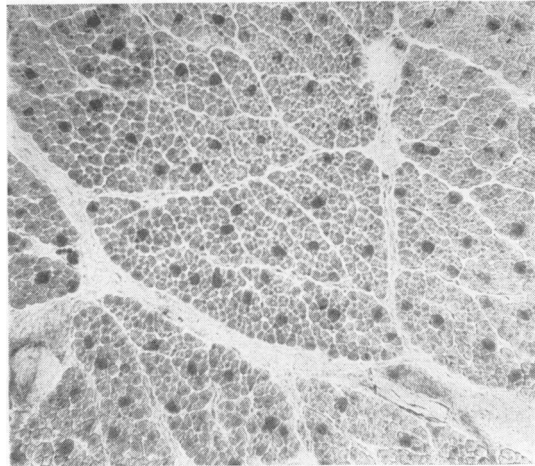

FIG. 3. *Human foetus (4, EYR), 21 to 23 weeks' gestation. Triceps. Note small proportion of large dark fibres. NADH diaphorase × 100.*

sisted of two groups of fibres, one giving a very intense reaction and the other a moderate reaction. However, the very strongly reacting fibres only formed a small proportion of the total (between 3 and 10%), and were usually at the upper range in fibre diameter (Fig. 3). This pattern was consistently present in all the skeletal muscles and also in the diaphragm and was similar to that noted in the small premature infant referred to above.

With the phosphorylase and ATPase reactions the results were not as consistent. However, a number of muscles with a good reaction showed an exact reciprocal to the NADH diaphorase reaction, with a predominance of strongly reacting fibres and a small proportion of large diameter, weakly reacting ones (Fig. 4).

The small proportion of fibres, strong in NADH diaphorase and weak in phosphorylase and ATPase, therefore corresponds to the type I fibres of mature muscle, while the remaining fibres with a higher content of phosphorylase and ATPase correspond to type II.

In the smaller foetuses, ranging from 12 to 20 weeks' gestation, the above pattern was not present. In some muscles there was a complete uniformity of enzyme activity in all the fibres (Fig. 5). In many of these there was some variation in overall stain between one whole bundle and another but the individual fibres within the bundle were uniform.

Some muscles showed the presence of weakly or strongly reacting fibres for a particular enzyme but this did not form a pattern similar to the older foetuses or a checkerboard pattern. The stronger or weaker fibres tended to occur together in clusters.
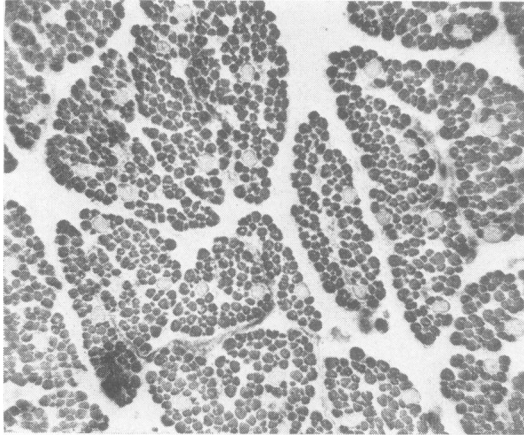
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FIG. 4. *Human foetus (4, EYR), 21 to 23 weeks' gestation. Pectoralis major. Note small proportion of large pale fibres. ATPase × 100.*
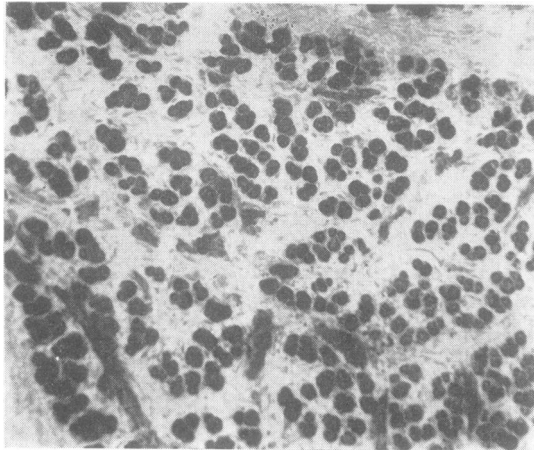

FIG. 5. *Human foetus (14, WIL), 12 to 14 weeks' gestation. Glutei, showing overall uniform activity; some bundles stronger than others. Fibres not in compact bundles. ATPase × 100.*

On serial section, in a number of instances, fibres with a stronger activity of one enzyme were also found to have a higher content of the other enzymes, so that they did not correspond to the type I or type II fibres of mature muscle.

In these younger foetuses the muscle fibres were not arranged in compact bundles but tended to occur in widely separated small groups.

DISCUSSION

On the basis of the enzyme histochemistry developing human muscle can be divided into three distinct phases.

In phase I, from early foetal life until approximately 20 weeks, there is no clear-cut subdivision of the muscle into different fibre types. The variation in enzyme content seen with some reactions in some of the muscles does not follow any set pattern but groups of fibres at random appear to have a slightly stronger or weaker reaction. These fibres cannot be correlated with type I and type II fibres of mature muscle.

In phase II, from approximately 20 to 26 weeks' gestation, there is a clear-cut subdivision of the muscle into two fibre types, which correspond in enzyme content to the type I and type II fibres of mature muscle. However, the type I fibres comprise only a very small proportion of the total.

In phase III, from about 30 weeks to full term, the muscle shows a pattern of differentiation similar to
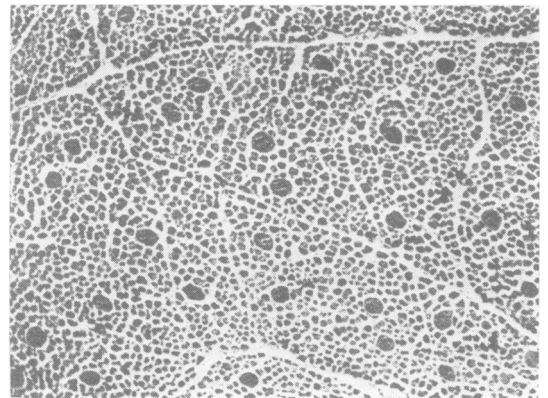

FIG. 6. *Copy of part of a photomicrograph (Fig. 17) of Wohlfart (1937). Child aged 4 months. Sartorius, showing the larger 'b' fibres. Stained, fixed material. × 80.*

that of mature adult muscle, with an approximately equal proportion of type I and type II fibres.

There is a striking correlation between our observations on the distribution of type I and type II fibres, and the observations of Wohlfart (1937) on the histology of foetal muscle. Wohlfart noted that in the sartorius of the newborn infant approximately 1% of fibres were of larger diameter. He called these 'b' fibres and the remainder 'a' fibres (Fig. 6). In a 20 cm. foetus, *i.e.*, about 18 weeks' gestation, the 'b' fibres comprised 4·5% of the total. In a 38 cm. foetus (about 30 weeks' gestation) the proportion of 'b' fibres had dropped to about 1% and remained at that level to term. In the sartorius 'b' fibres could be recognized in children up to 11 years of age, but in many other muscles, including the gastrocnemius, the rectus femoris, and the biceps, they had already disappeared by birth.

In foetuses of less than 18 weeks' gestation, Wohlfart noted some variation in fibre diameter but was unable to differentiate 'b' fibres from the rest, even in the sartorius.

Wohlfart concluded that the 'b' fibres were a separate type and that their eventual disappearance might be due to a relative increase in the size of 'a' fibres. In some muscles the 'b' fibres disappeared earlier than in others.

In phase II of our developmental study the fibres rich in NADH diaphorase (type I) correspond in distribution to Wohlfart's 'b' fibre (compare Figs. 3 and 6). In phase III, however, the muscle shows a subdivision into type I and type II fibres but these are present in approximately equal numbers and it is not possible to recognize the 1% of 'b' fibres observed by Wohlfart. If indeed these larger 'b' fibres are still present in this phase, they may comprise the larger fibres among the type I fibres.

Recently Fenichel (1963) reassessed the status of the Wohlfart 'b' fibres. He measured the fibre diameter in fixed material from the quadriceps, psoas, biceps, and triceps of foetuses and newborns, and also studied the ATPase reaction in fresh frozen sections from five foetuses.

His approach was essentially a statistical one, and on the basis of his measurements on foetuses from 20 weeks' gestation to full term, he plotted curves for fibre size against total number of fibres. These fell into one of three patterns, *viz.*, bimodal (23% of specimens), skewed curve (53%), and symmetrical curve (24%). With the ATPase reaction all specimens showed light and dark fibres. All large fibres corresponding to Wohlfart's 'b' fibres gave a weak reaction for ATPase. However, when plotting the distribution of fibre size for the light and dark fibres he obtained a similar peak diameter.

Fenichel concluded that the two fibre types recognizable in mature muscle with ATPase were present early in human gestation. He considered that the giant fibres of Wohlfart ('b' fibres) were merely an extreme size of one fibre type (histochemical type I), which extended over a wide range of fibre size. At its lower limits this fibre type was identical in size with Wohlfart's 'a' fibres. He suggested that 'the b fibre of Wohlfart is not a fibre type *sui generis* but is instead a reflection of a minor size difference between histochemical type I and type II fibres during foetal and neonatal life'.

My own observations on developing muscle agree with Fenichel's conclusions only in phase III of foetal development. During this phase (30 weeks to full term) the distribution of type I and type II fibres is similar to mature muscle, and Wohlfart's 'b' fibres, if present at all, probably comprise the upper extreme in size of type I fibres.

In phase II of foetal development (approximately 20 to 26 weeks) however, the Wohlfart 'b' fibres correspond in size and distribution to all the type I fibres in the various muscles studied. In this phase they therefore do not appear to comprise only the larger diameter fibres of type I fibres, but the whole group.

In phase I (12 to 20 weeks' gestation) no fibres corresponding to Wohlfart's 'b' fibres were recognizable, as noted also by Wohlfart himself.

No explanation is apparent for the mechanism of changeover from the small proportion of type I fibres during phase II of development to the approximately equal proportion of type I and type II fibres subsequently.

In the same way that Wohlfart considered the most plausible explanation for the disappearance of the 'b' fibres was a relative increase in size of the 'a' fibres, so the increase in number of type I fibres may be due to the conversion of some of the type II fibres to type I. This process may be under some neural influence of the central nervous system, as suggested by the physiological studies of Buller, Eccles, and Eccles (1960).

As will be shown in Part III in the next issue of this Journal, the recognition of different phases in the normal process of development is of great practical importance. The application of these enzyme histochemical methods to diseased muscle enables a hypothesis to be formulated as to the stage of development of the foetus at which the pathological process may have started.

SUMMARY

Enzyme histochemical studies have been done on muscle from infants and children (new born full-term and premature infants) and human foetuses ranging in gestation from 12 to 26 weeks.

The process of development of muscle can be divided into three phases. In phase I (from approximately 12 to 20 weeks' gestation) there is no subdivision of the muscle into different fibre types. In phase II (approximately 20 to 26 weeks' gestation) the muscle is differentiated into type I and type II fibres, as in adult muscle, but the type I fibres comprise only a small proportion (3 to 10%) of the total. These type I fibres correspond in size and distribution to Wohlfart's 'b' fibres. In phase III (from 30 weeks to full term) the muscle is divided into type I and type II fibres, with an approximately equal distribution as in the muscle of older children and adults.

The recognition of different phases in development of muscle by histochemical methods may prove of value in postulating at what stage of development a pathological process may have started.

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