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Postnatal growth and differentiation of muscle fibres in the mouse

II. A histochemical and morphometrical investigation of dystrophic muscle

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INTRODUCTION

Murine muscular dystrophy is an inherited, congenital disease. Abnormalities in muscle and nerve have been observed already perinatally (Banker, 1968; Biscoe, Caddy, Pallot & Pehrson, 1975; Meier, 1967; Meier, West & Hoag, 1965; Montgomery & Swenarchuk, 1978). The study of the course of the disease is not easy, because (early) pathologic processes coincide with normal postnatal growth and differentiation. Dystrophy in mouse muscle is characterised by necrosis, which results in a severe reduction in the number of muscle fibres. As a reaction to necrosis, regeneration occurs. This process, however, cannot compensate sufficiently for the loss, so that the course of the disease is progressive. Other compensatory mechanisms are hypertrophy and changes in contractile and/or metabolic properties of the remaining or regenerated muscle fibres. The evaluation of various effects of the pathological progression on muscle and muscle fibres is still more complicated, because it has to be assumed that the diseased animals will develop an aberrant use of their muscles. Also, they may become undernourished or hypoxic because of muscular weakness.

In the present paper, normal and dystrophic development are compared. Fibre number, fibre size (growth), and fibre type (differentiation) were the bases used to describe the course of the disease.

MATERIALS AND METHODS

Dystrophic (dy/dy) animals were obtained by crossing heterozygous (Dy/dy) mice of the Bar Harbor strain ReJ 129. Homozygous (Dy/Dy) mice served as controls. In dy/dy mice dystrophy becomes evident no earlier than about 2 weeks postnatally. To obtain muscles of younger dystrophic animals, the amputation method described by Platzer & Chase (1964) was used. The animals had free access to food and water. Three muscles of the hind leg (the soleus, plantaris and gastrocnemius) were processed for histochemical and light microscopic–morphometric evaluation. Muscle fibres were classed according to the activities of succinate dehydrogenase (SDH),

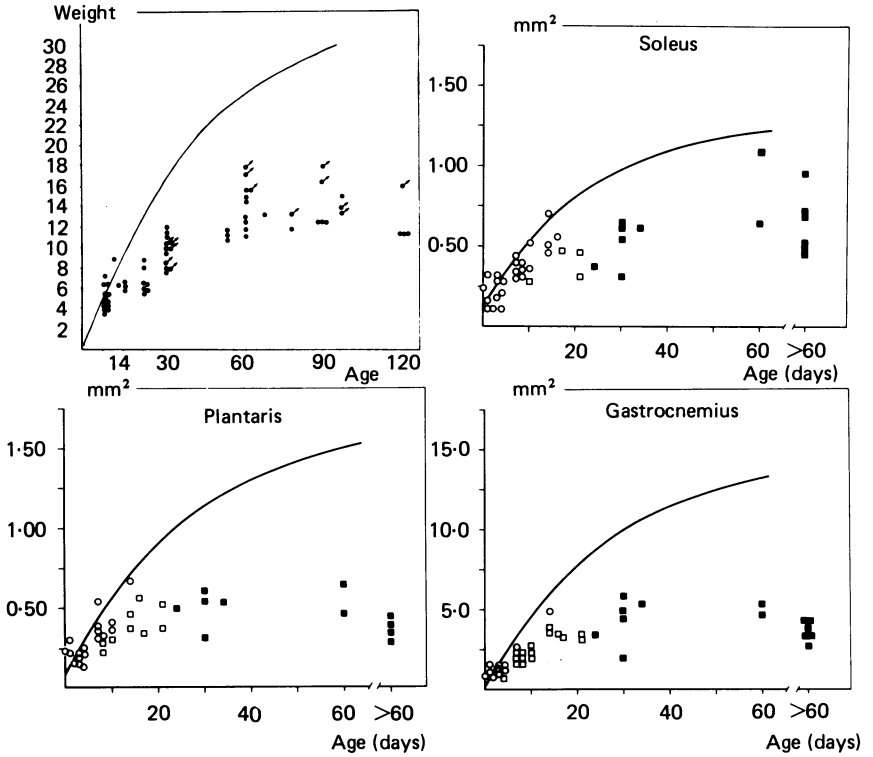


Fig. 1. Cross sectional area of soleus, plantaris and gastrocnemius muscles, expressed in mm². Drawn line, growth curve of normal muscles. Individual values of dystrophic muscles are given. ○, Not significantly different from normal; □, significantly different; ■, individual values of animals older than 3 weeks. (In the age groups 22–34 days, and 60 days and older, the differences from control values were significant, see Table 1). Body weights (in grams) of dystrophic animals compared with average litter weights of normal mice (drawn line, curve of least squares). Animals older than one month; ♂, male; ●, female.

α-glycerol-phosphate-dehydrogenase (GPOX) and myosin ATPase (after pre-incubation at pH 4·35). Detailed information was presented in the publication on normal mice (Wirtz, Loermans, Peer & Reintjes, 1983). In the muscle complex, five regions were discerned (i.e. soleus muscle, plantaris muscle regions I and II and gastrocnemius muscle regions I and II). Because in older dystrophic muscles the 'white' aspect was mostly lost, region II of the plantaris and gastrocnemius muscles were often difficult to circumscribe. In those situations, the most peripheral region of the muscle was considered to represent region II. In a few cases, the remnants of both regions I and II were pooled to obtain a sufficient number of muscle fibres to be classed. Cells that could not be clearly identified as muscle fibres (e.g. myoblasts) were not included in the counts. Relative numbers of fibres with central nuclei were assayed on sections of animals of different ages, stained with haematoxylin and eosin. The fibres were typed histochemically. The data were pooled and are represented in Table 3.

Starting from a linear relation between the cross sectional surface area of the muscle and the age of normal animals up to 3 weeks, a regression line was drawn. Subsequently, with a t test (Snedecor & Cochran, 1971) each dystrophic animal was tested for significant ($P < 0{\cdot}05$) deviation from this line. Animals older than 3 weeks

Table 1. *Cross sectional area of normal and dystrophic muscles, expressed in mm²* (*all P values between normal and dystrophic* $< 0{\cdot}01$)

	Age group 22–34 days		⩾60 days	
	Mean S.D.	*n*	Mean S.D.	*n*
Normal soleus muscle	0·98±0·10	3	1·29±0·16	9
Dystrophic soleus muscle	0·51±0·14	6	0·69±0·23	8
Normal plantaris muscle	1·28±0·34	3	1·61±0·20	9
Dystrophic plantaris muscle	0·50±0·10	6	0·40±0·12	8
Normal gastrocnemius muscle	10·87±0·98	3	14·36±1·01	9
Dystrophic gastrocnemius muscle	4·25±1·41	6	3·97±0·83	8
Normal muscle complex	13·13±1·41	3	17·26±1·22	9
Dystrophic muscle complex	5·27±1·63	6	5·06±1·14	8

were classed in two age groups (22–34 days and 2 months and older); Student's *t* test for two random samples was applied.

RESULTS

The results will be dealt with by focusing on the differences between normal and dystrophic development. For the normal development the reader is referred to Wirtz *et al.* (1983).

Muscle growth and fibre number

In our strain, we did not observe differences in body weight between normal and dystrophic mice during the first two weeks postnatally, but during the third week dystrophic mice did not gain much in weight, whereas normal animals passed through a growth spurt. After the third week, the former showed only a moderate growth rate until 2–3 months of age (Fig. 1). Muscle growth was evaluated by means of cross sections (Fig. 1). During the first days postnatally, values did not differ significantly from normal, although the majority of measuring points fell below the growth curve for normal muscle. At about one week of age, some data on the dystrophic material deviated significantly from the control curve, while dystrophic muscles more than 14 days old fell only incidentally within the range of normal muscle (Table 1). Following severe necrosis during the second week, the muscles atrophied between two weeks and one month of age. At one month, muscle growth was somewhat restored, presumably because of fibre hypertrophy and the occurrence of regeneration.

During the second month (soleus muscle: second and third month) there was a stationary phase in growth that was followed by a period during which the muscles atrophied slowly but steadily.

In dystrophic animals, the total number of fibres (Table 2, Fig. 2) is determined by loss through necrosis and gain via regeneration. Regeneration was closely related to necrosis not only chronologically, but also as to location (Figs. 6–9). Regenerating fibres were not observed during the first week, but within a few days following necrosis they were found to fill up the gaps between surviving fibres. In animals older than three weeks of age, regeneration was observed much less frequently.

In the soleus muscle, there were no significant differences in fibre number between

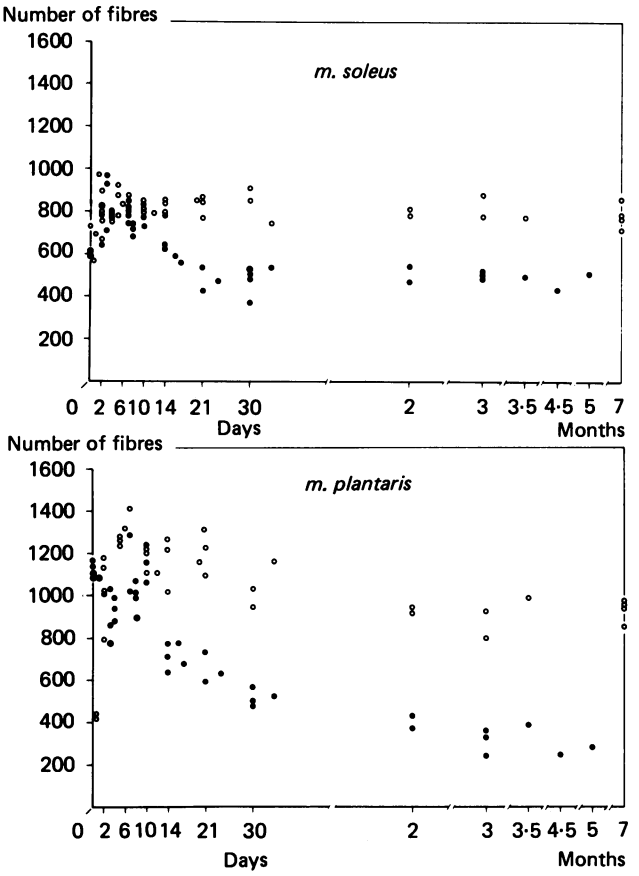


Fig. 2. Number of fibres in the soleus and plantaris muscles of dystrophic animals (●) and normal animals (○) during development. Each dot represents one muscle.

normal and dystrophic muscles during the first week. From 10 to 14 days postnatally there was a steady drop until day 21. Subsequently, there was a stabilisation at about 450 fibres, compared with about 800 in the normal animals. In the plantaris muscle, the dystrophic values tended to be lower already shortly after birth. About day 10 there was a sudden drop in fibre number, and during this period necrosis was severe. From day 14 on there was a slower, but steady decrease. In dystrophic animals of 4–5 months, about 300 fibres, compared with about 900 in controls, were counted. In the gastrocnemius muscle especially the central regions were severely attacked during the second week. As a consequence of this, regeneration was extensive (Fig. 8) so that in many instances only a few of the pre-existing fibres remained. However, individual differences in the progression of the disease were large.

Growth and differentiation of muscle fibres

In Figure 3 the frequencies of seven fibre classes are plotted against the age groups. No new fibre types, i.e. histochemical combinations, were found in dystrophic muscle compared with normal muscle as a whole. Certain muscle fibre types were found in dystrophic muscles at an age when they were not observed in normal muscles, or they were present in unusual quantities. During the first two weeks,

Table 2. *Number of fibres in normal and dystrophic soleus and plantaris muscles and corresponding two sided P values between normal and dystrophic animals*

(Comparison of normal and dystrophic muscles with the rank-sum test of Wilcoxon for two samples, applied for each age group.)

Age group	0–2	3–7	8–14	15–21	22–34	⩾60
Soleus muscle, dystrophic						
Median	638	802	724	544	478	500
Min., max.	601, 816	702, 972	627, 836	429, 584	367, 533	434, 540
n	5	10	9	4	6	8
P value	0·46	0·52	<0·01	0·02	0·02	<0·01
Soleus muscle, control						
Median	713	849	810	850	850	784
Min., max.	568, 948	756, 916	749, 856	771, 863	749, 908	719, 878
n	7	6	7	4	3	9
Plantaris muscle, dystrophic						
Median	1116	938	1002	704	513	341
Min., max.	986, 1153	782, 1294	640, 1236	590, 774	402, 630	237, 425
n	6	9	9	4	6	8
P value	0·42	<0·01	0·06	0·02	0·02	<0·01
Plantaris muscle, control						
Median	906	1263	1139	1197	1028	940
Min., max.	388, 1341	1236, 1414	1016, 1272	1094, 1319	946, 1163	793, 996
n	6	5	7	4	3	9

differentiation proceeded almost normally, but, at about day 14, when 'adult fast' fibres normally occur, deviations became clearly visible. Transitional fibres increased most markedly in the gastrocnemius muscle region I (5·33 and 17% at days 10, 14 and 21, respectively). Rare fibres (L–N, O–R) also increased. Their nature was not clear, but it has been assumed that they represent transitional fibres, such as the D–G fibres. Intermediate fibres (H–K) were also numerous, compared with the controls, especially in the plantaris and gastrocnemius muscles of older animals. The fibre population in the old dystrophic muscles (135, 150 days) was composed as follows (the normal percentages for animals of 90 and 210 days in parentheses):

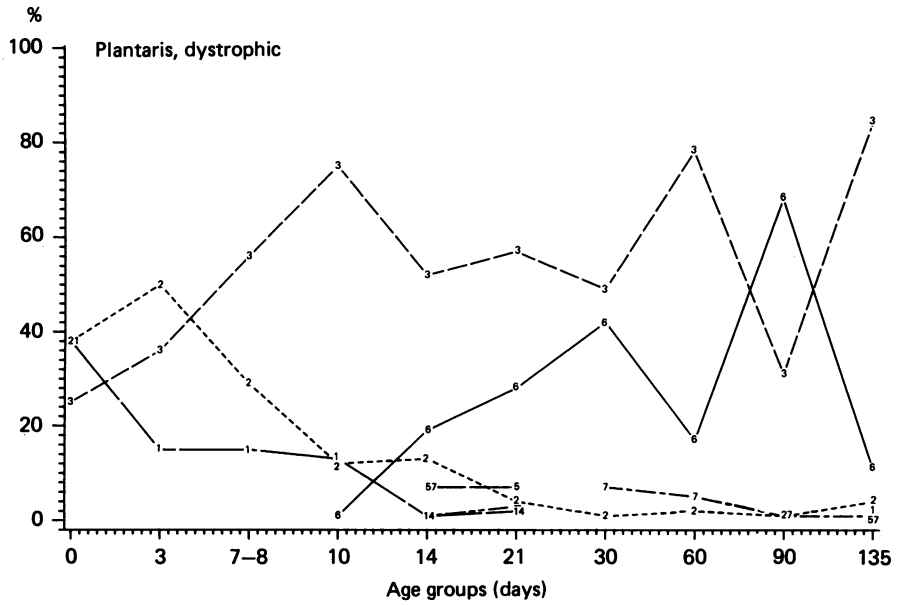
soleus muscle: A–C: ±55% (±45%); D–G: ±4% (−); H–K: ±20% (±3%); T–V: ±20% (±48%);

plantaris muscle: A–C: ±2% (±2%); D–G: ±4% (−); H–K: ±93% (±18%); S–V: nihil (±40%); W–Z: nihil (±40%);

gastrocnemius muscle region I: A–C: nihil (−); D–G: nihil (−); H–K: ±70% (±25%); S–V: ±27% (±15%); W–Z: nihil (±60%);

gastrocnemius muscle region II: A–C: nihil (−); D–G: nihil (−); H–K: ±94% (±5%); S–V: ±4% (−); W–Z: ±2% (±95%).

The average growth per individual fibre type (Fig. 4) was not markedly abnormal during the first two months, but, in the age groups of 14 and 21 days, there were abnormally large numbers of small 'transitional' and 'rare' fibres. Hypertrophy of the A–B fibres was observed in the soleus muscle, and hypertrophy of the S–U fibres



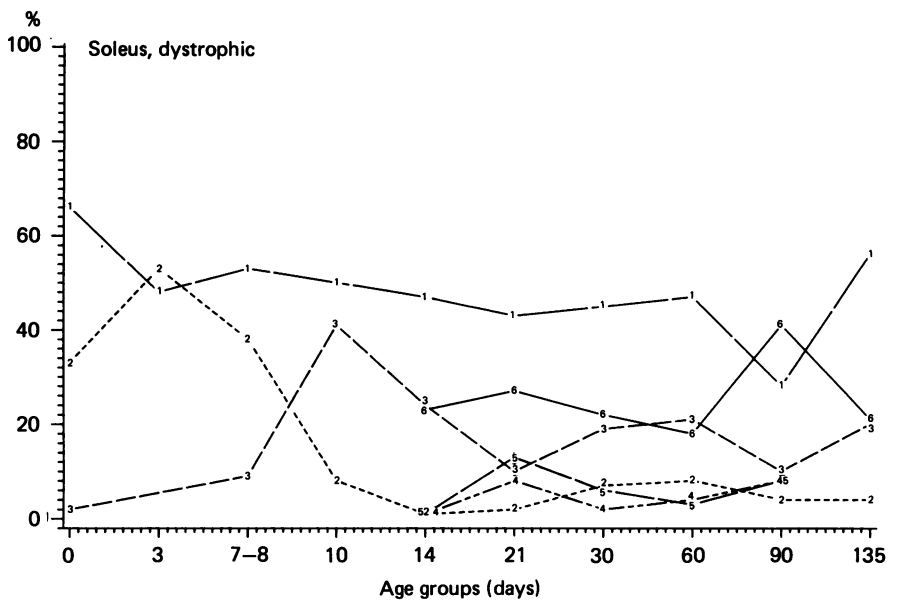
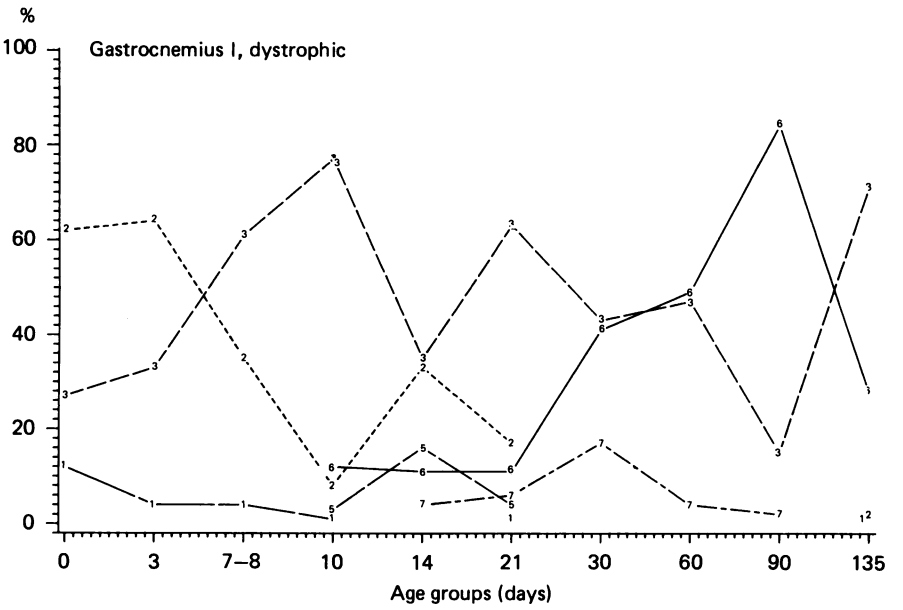


Fig. 3(*a*)

Fig. 3(*a–b*). Frequencies of seven fibre classes are plotted against the age groups for the soleus, plantaris muscles and gastrocnemius muscle regions I and II. (1) fibre class A–C; (2) fibre class D–G; (3) fibre class H–K; (4) fibre class L–N; (5) fibre class O–R; (6) fibre class S–V; (7) fibre class W–Z. In the graphs, the shifts between the fibre types during postnatal development are visualised. Characteristic for the dystrophic situation, compared with the differentiation of normal muscle, are: (1) the high level of 3; (2) the second peaks of 2 and 3, and where present 7,



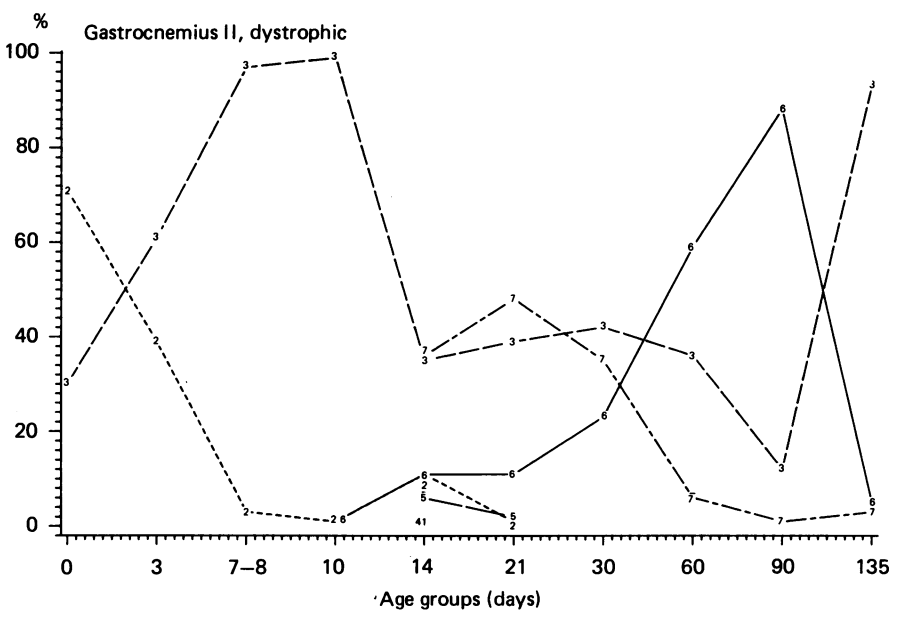


Fig. 3(*b*)

in animals older than 10 days of age; (3) the increase of 6 between 60 and 90 days; (4) the chiasma of 3 and 6 in animals of 90 days and older. These changes are presumed to represent respectively: 1. disturbed maturation; 2. regeneration; 3. functional compensation for loss of class 7 fibres; 4. atrophy. These features are revealed most clearly in the plantaris muscle and gastrocnemius muscle region I. It should be noted that the figures have to be read against a decreasing total number of fibres in the muscles. Number of animals, 22.

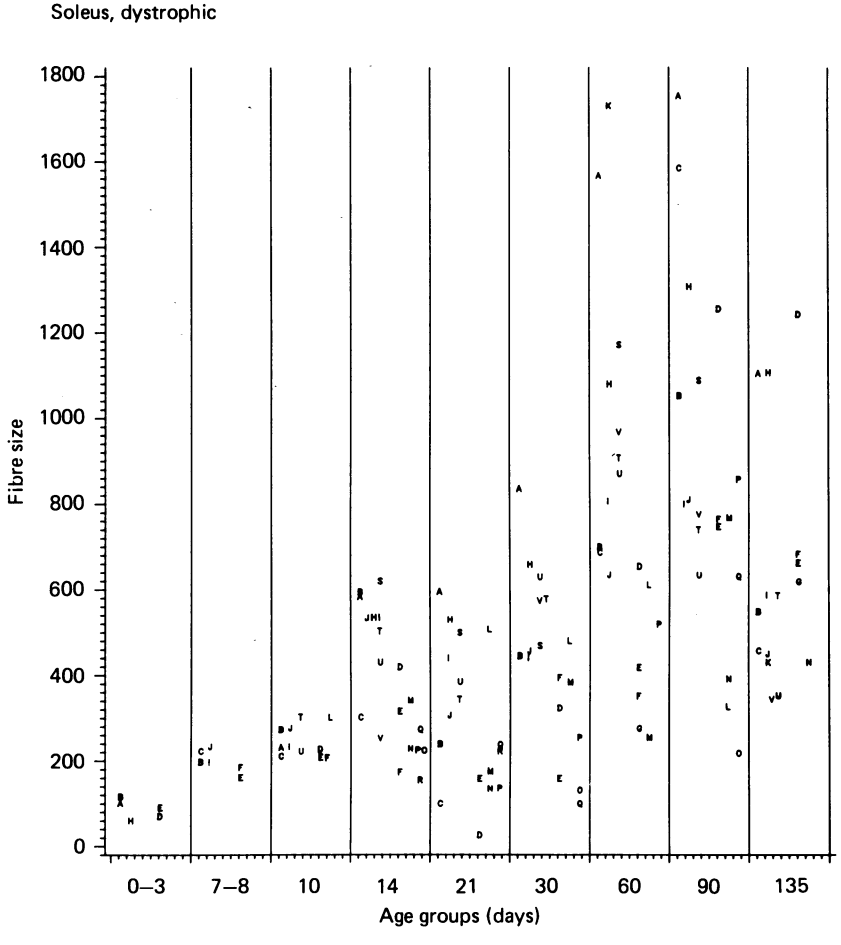

Soleus, dystrophic
Fibre size
1800
1600
1400
1200
1000
800
600
400
200
0
0–3
7–8
10
14
21
30
60
90
135
Age groups (days)

Fig. 4(*a*)

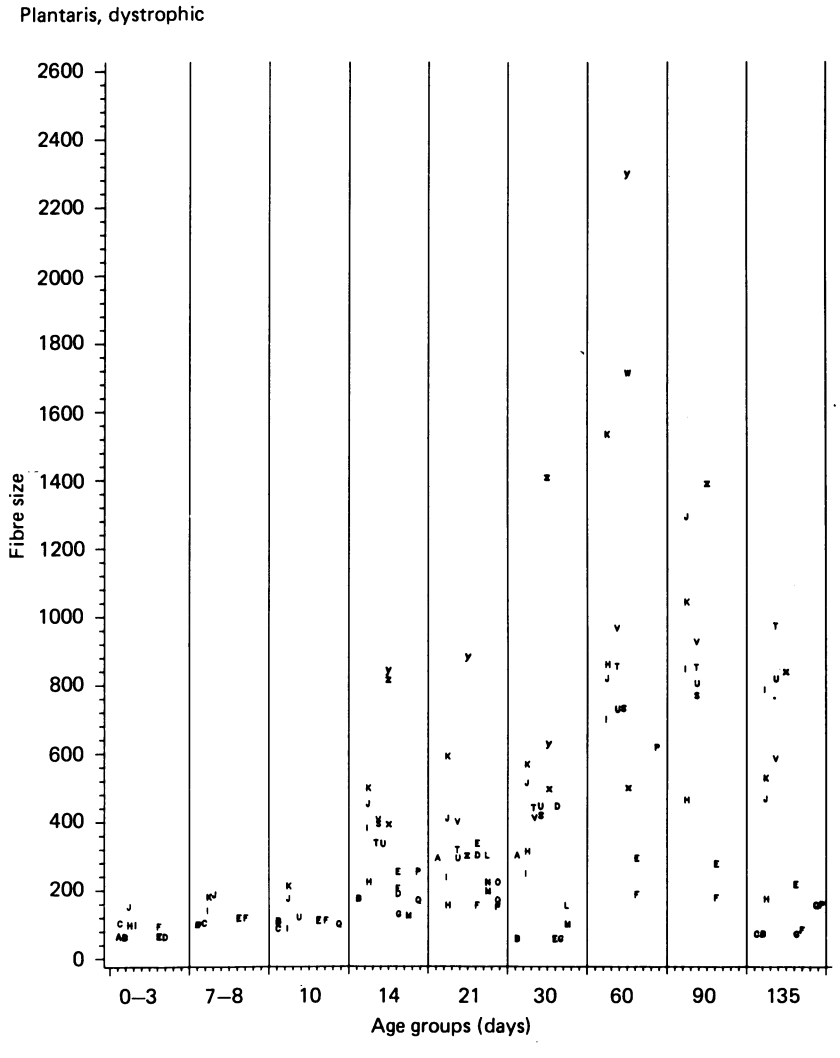

Plantaris, dystrophic
Fibre size
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0–3
7–8
10
14
21
30
60
90
135
Age groups (days)

Fig. 4(*b*)

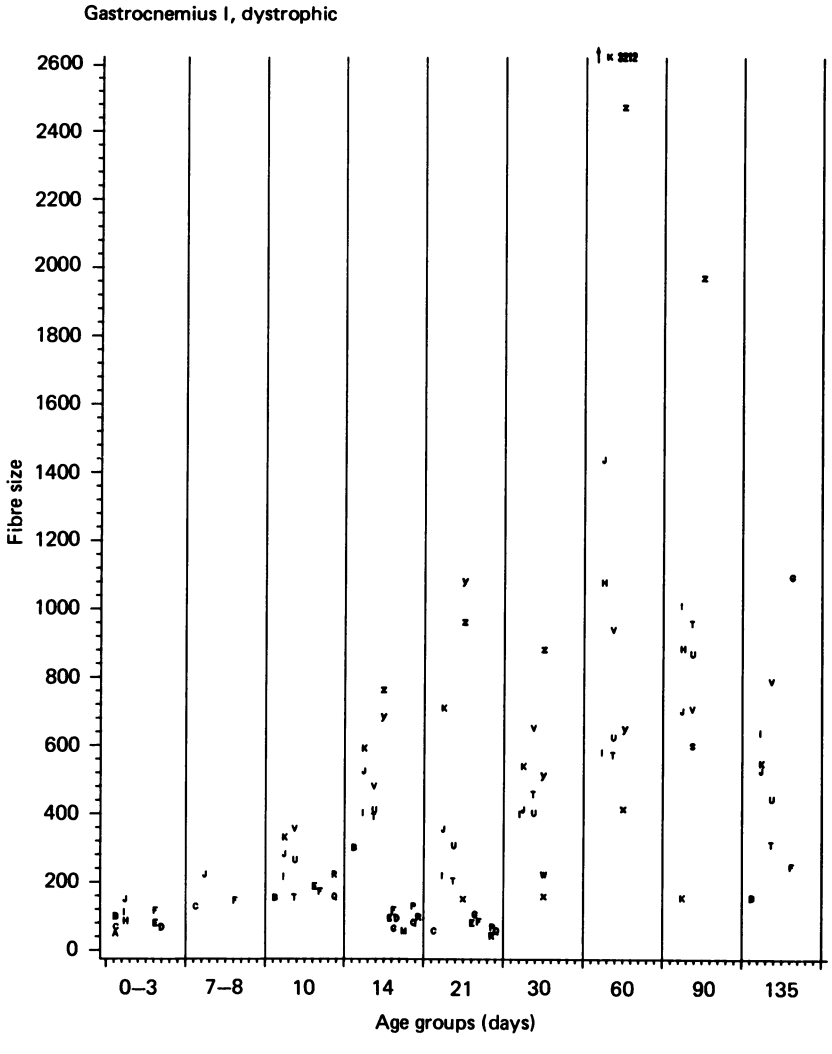


Fig. 4(*c*)

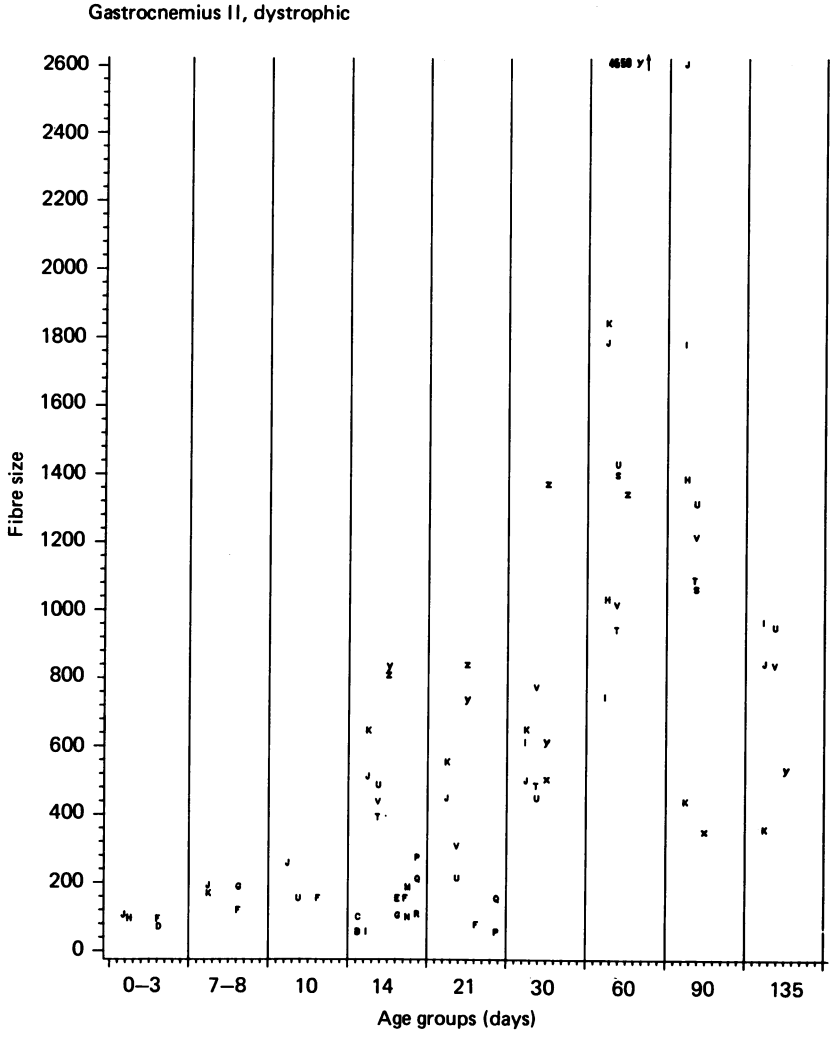


Fig 4(*d*)

Fig. 4(*a–d*). Relation between fibre type and fibre size (in μm^2) in different regions of the muscle complex. Note that growth of fibres was restricted up to about 2 months of age. Thereafter there was general atrophy

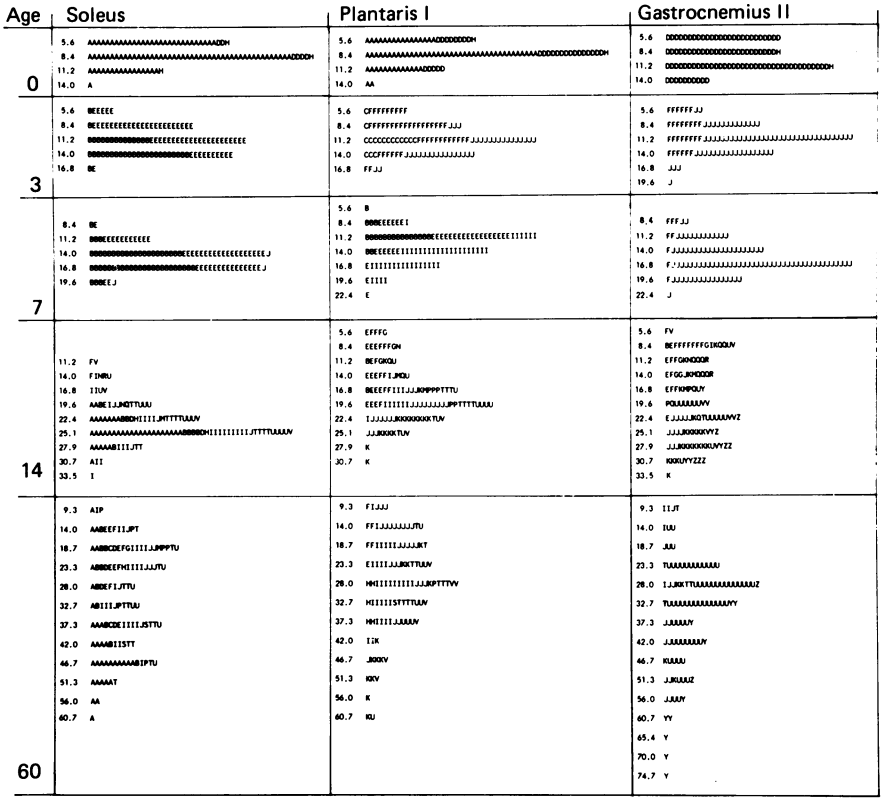


Fig. 5. Examples of fibre differentiation during postnatal development of dystrophic muscles. One animal each was chosen to represent an age group. It should be noted that there may be considerable differences in the histograms of individual, diseased animals. Fibre size is given in arbitrary units.

Table 3. *Fibre types with central nuclei (age groups have been put together)*

Muscle	Total fibres with central nuclei counted	Fibres falling in each fibre type group (%)						
		A–C	D–G	H–K	L–N	O–R	S–V	W–Z
Soleus muscle	113	21·3	9·7	15·9	9·7	8·9	34·5	—
Plantaris muscle	150	—	18	39·3	—	4	37·3	1·3
Gastrocnemius muscle	143	—	22·4	24·5	—	5·6	22·4	25·2

in the plantaris muscle, at the ages of 60 and 90 days. In the gastrocnemius muscle, some fibres of the W–Z type were hypertrophied, but the average fibre size was not increased.

After two months, a general fibre atrophy developed. Fibre growth in the normal gastrocnemius muscle was usually completed after about two months, while the fibres in the soleus and plantaris muscles continued to grow during the entire period of observation. Figure 5 shows that, especially after the second week, the normal fibre distribution was largely lost, and the average size of the total fibre population was markedly reduced (e.g. a shift to the left in the plantaris muscle at 60 days). Fibres with central nuclei (Table 3) were scarce in normal animals older than two

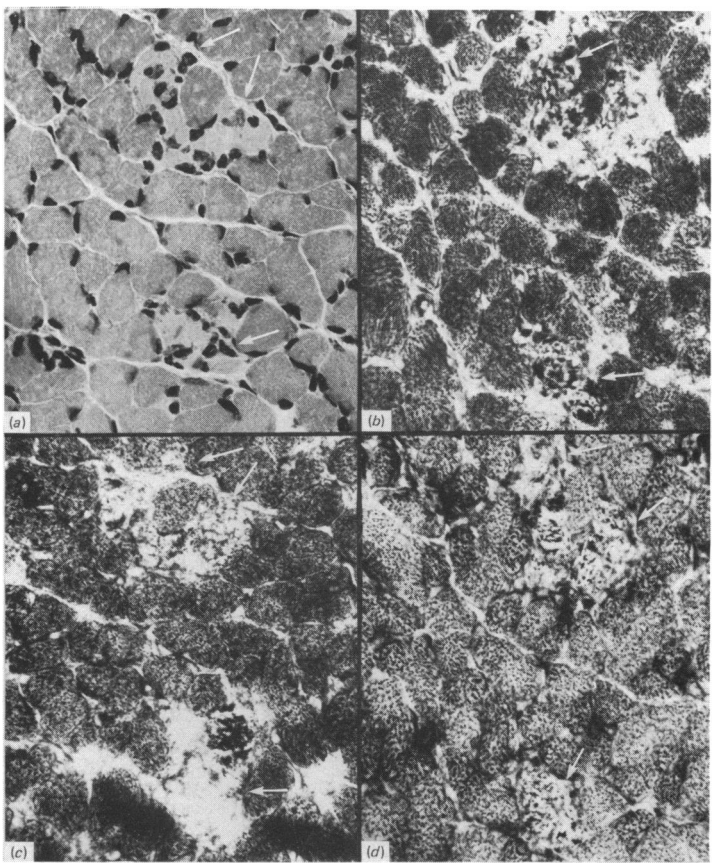


Fig. 6 (*a–d*). Dystrophic gastrocnemius muscle, age 10 days. Early necrosis (arrows) with infiltration of macrophages. Affected fibres have a deviant staining pattern. Serial sections, magn. ± 260×. (*a*) HE; (*b*) GPOX; (*c*) SDH; (*d*) diaphorase.

weeks, but in dystrophic muscles they were a common feature. Histochemical classification of these fibres did not bring out a preference for any fibre type. If the assumption of Schmalbruch (1976, 1980) is correct, that central nuclei are more likely an indication for regeneration than a result of redispositioning in pre-existing fibres, then this would imply that regenerated fibres in dystrophic mouse muscle are capable of differentiation (histochemically) into 'adult' fibres.

DISCUSSION

The primary factor causing muscular dystrophy in the mouse is unknown, although there are indications that a defect in the plasmalemma of dystrophic genotypes might be responsible (Strickland, Hudson & Thakar, 1979). In mice, early alterations in the myelination of axons at the lumbosacral spinal roots (Biscoe *et al.* 1975; Bradley & Jenkison, 1973; Jaros & Bradley, 1978), and perinatal focal muscle fibre damage (Banker, 1968; Meier, 1967; Meier *et al.* 1965; Platzer, 1979; Platzer & Chase, 1964) have been described. Also, a reduction in the number of myelinated axons in the nerves supplying the hind leg muscles has been reported (Montgomery & Swenarchuk, 1978). Whereas, in normal muscle, postnatal develop-

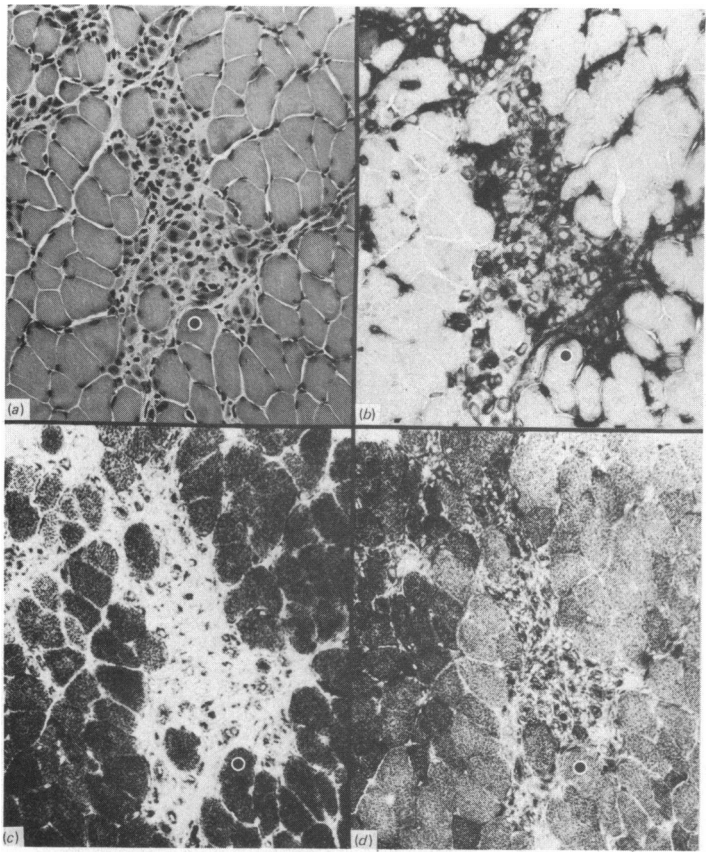


Fig. 7 (*a–d*). Dystrophic gastrocnemius muscle age 14 days. Groups of regenerating muscle cells between pre-existing fibres. Serial sections, magn. ± 130×. (*a*) HE; (*b*) ATPase; (*c*) GPOX; (*d*) SDH.

ment is characterised by growth and differentiation of muscle fibres, in dystrophic muscles, wasting of fibres and formation of new fibres via regeneration processes also influence the profile of the fibre population. Moreover, secondary processes may occur, such as changes in the histochemical character or the size of fibres, due to the altered situation in the dystrophic muscles (e.g. altered neural influence, disuse). Interpretation of the observed phenomena is complicated, because the magnitude of their contributions is not known, nor is the period during which they exert their influence.

Our fibre counts are in agreement with those reported in literature (Montgomery & Swenarchuk, 1978; Rowe & Goldspink, 1969). Especially during the second week, but also during the third week, considerable fibre loss was recorded, whereas thereafter there were only limited focal lesions. The reason for the severe loss during the early postnatal period is not known, but it coincides with the appearance of 'adult' fibre types as well as with the first locomotory activity of the animals. Also, there is no literature available concerning the possible influence of the neural abnormalities on the development of dystrophic muscle fibres.

It has been stated (Cosmos, Butler, Mazliak & Allard, 1980) that 'white' fibres are especially susceptible to damage during early development and that 'slow' (soleus muscle) fibres are more resistant (Butler & Cosmos, 1977). On closer examination,

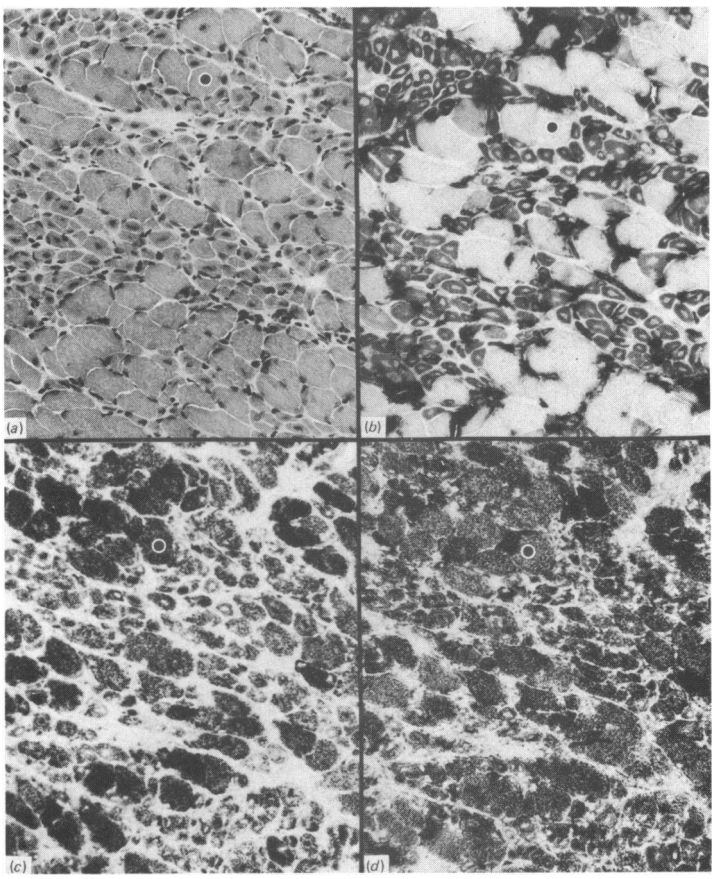


Fig. 8 (*a–d*). Dystrophic gastrocnemius muscle, age 14 days. Regenerated young muscle fibres between pre-existing fibres. They have central nuclei and a medium brown ATPase. Serial sections, magn. ± 130×. (*a*) HE; (*b*) ATPase; (*c*) GPOX; (*d*) SDH.

however, the situation appears to be more complex. In our material, including the soleus muscle, and especially during the early period (i.e. the second week, during the formation of 'adult' fibre types) there was a marked reduction in fibre number, which was *not* related to any fibre type in particular. In the plantaris and the gastrocnemius muscles, there was a deficiency of 'white' fibres. The period of severe necrosis coincided with the development of these fibres. It is difficult to decide whether the W–Z fibres were affected as soon as they developed, or were not formed because differentiation from intermediate fibres (H–K) was hampered. Our interpretation of the data is that differentiation into 'white' fibres was only partly successful. Disuse is not a likely causal factor, because animals of this age generally show normal walking behaviour. During a later stage of the disease, practically all type W–Z fibres disappeared. Regeneration was closely linked to necrosis. During the third week, a large population of new fibres (D + G) developed, especially in the mixed areas (plantaris muscle, and especially in the central parts of the gastrocnemius muscle). Summers & Parsons (1978) noted much regenerative activity during the second week and also reported that regeneration was often abortive. In our material, we could follow the differentiation of the regenerating fibres to the H–K type.

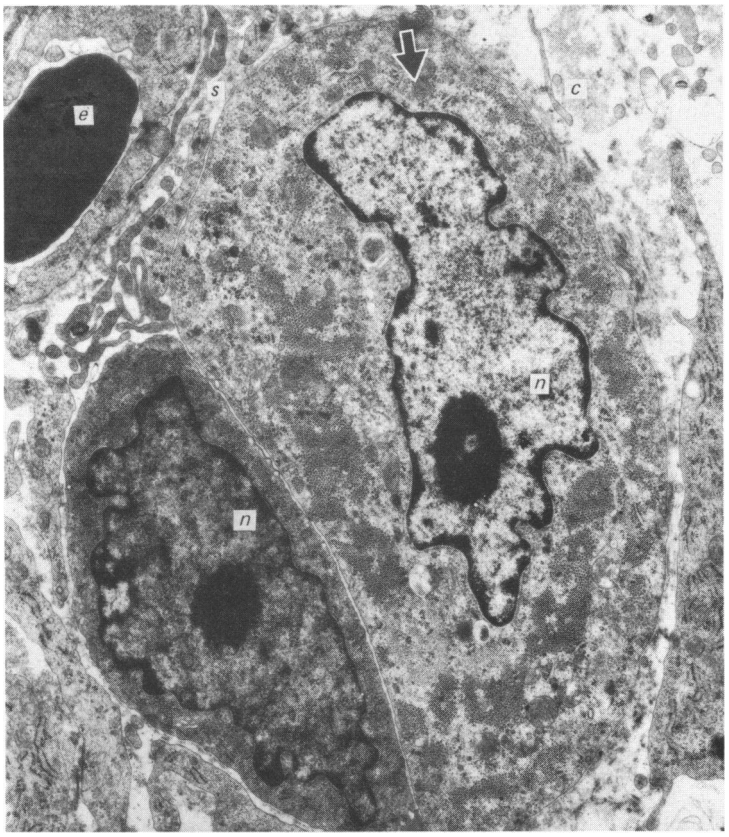


Fig. 9. Dystrophic gastrocnemius muscle, age 14 days. Ultrastructure of regenerating muscle cells in a necrotic area. Collagen (*c*) and (myo-) fibrillar waste material (*s*) in the intercellular space. *E*, erythrocyte; *n*, central nuclei of regenerating cells; arrow, myofibrils. Magn.± 8800×.

Up to that point, differentiation appeared normal, but little could be said about the fate of these fibres during a later stage, because reliable methods to discriminate between pre-existing and regenerated fibres are not available. If it is accepted that most muscle fibres with central nuclei are regenerated fibres (Schmalbruch, 1980), then our observation that there was no preference of central nuclei for any particular fibre type would indicate that newly formed fibres have the potential to mature histochemically. Growth of the (pre-existing) non-affected fibres was quite normal. However, in many instances the average size of all fibres in the population was reduced when compared with normal muscles. Within a fibre type there was a wide range in size between hypertrophied (when present) and small (mostly regenerated) fibres. The latter lowered the average size of several fibre types. Only in some fibre types, and only during certain periods, was hypertrophy observed. The relation between muscle force and body weight in dystrophic muscles during development is not known, but both muscle cross sectional area and body weight were reduced. After two months, a general fibre atrophy was observed that could be explained by a combination of disuse, malnutrition caused by muscular weakness, and (possibly) hypoxia, leading, in turn, to a deterioration of the physical condition of the animals. Simultaneous with fibre atrophy, the 'chess board' pattern of the metabolic enzymes

became less defined. Combined with the increase of 'intermediate' (H–K) fibres in the plantaris and gastrocnemius muscles, and the disappearance of almost all 'white' (W–Z) fibres, this resulted in a rather 'juvenile' and 'oxidative' aspect of the muscles. This is in agreement with the observations of Brust (1966) who described increased resistance to fatigue at an age of 2½–3 months. The muscles 'reacted more like the soleus'.

Dystrophic postnatal development can, therefore, be characterised by a rather normal development during the early postnatal period, followed by a severe progression of the disease during the second week, closely followed by regeneration, a relative stabilisation during the second half of the first month and the second month, and a general atrophy and de-differentiation of the histochemical profile in older animals.

SUMMARY

Postnatal development of three hind leg muscles, the soleus, plantaris, and gastrocnemius, of dystrophic mice (ReJ 129) was investigated with histochemical and morphometric methods. The results were compared with normal postnatal development. Especially during the second week postnatally, there was severe fibre necrosis with no apparent preference for any particular fibre type. This period of necrosis was shortly followed by a wave of regeneration during the third week that could not, however, compensate for the loss of fibres. In dystrophic animals of 4–5 months of age, the number of fibres was reduced by 40–70 %. Cross sectional areas of dystrophic muscles rarely, if ever, exceeded values for normal animals 14 days of age, while body weights were also drastically reduced. Growth and differentiation of the non-affected fibres proceeded almost normally during the first month. During the second month, the 'slow' fibres in the soleus muscle, and the 'fast-oxidative-glycolytic' fibres in the plantaris muscle were hypertrophied, while, incidentally, some 'fast-glycolytic' fibres showed hypertrophy; but in this case the average size of the fibre type was not increased. After two months, a general fibre atrophy was observed. The fate of the regenerated fibres was difficult to trace, especially in muscles older than one month. It is assumed that a number of them were capable of developing into 'adult' fibre types histochemically. During the course of the disease the percentage of 'intermediate' fibres increased markedly, whereas nearly all 'fast-glycolytic' fibres disappeared. Because of these shifts in fibre profiles, the plantaris and the gastrocnemius muscles obtained a rather 'juvenile' and 'oxidative' aspect. Changes in the histochemical character of the soleus muscle were less spectacular. In dystrophic muscles, no *new* fibre types were found, compared with normal muscles. Rather, fibre types were present at the wrong moment, or occurred in quantities unusual for the age concerned.

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