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Biochemistry of Dystrophic Muscle

2. SOME ENZYME CHANGES IN DYSTROPHIC MOUSE MUSCLE*

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Following upon the discovery (Michelson, Russell & Harman, 1955) of a hereditary myopathy in a strain of mice, several workers have measured the activity of a number of enzymes in muscle from the affected animals. Weinstock, Epstein & Milhorat (1958) found a marked increase in acid-cathepsin activity and in cytochrome oxidase but no significant difference in aldolase or succinate oxidase; these results were obtained when comparison with normal muscle was made on the basis of either non-collagen nitrogen or total nitrogen. On the other hand, White (1959) reported a decrease of aldolase and of lactate dehydrogenase, when comparison was made on a total protein basis; isocitrate-dehydrogenase activity, on the other hand, was increased. α-Glucan phosphorylase and phosphoglucomutase are decreased when based on fresh weight and also, in the latter case, when based on non-collagen nitrogen (Leonard, 1957; Hazzard & Leonard, 1959). McCaman (1960) found a striking increase (about fourfold) in glucose 6-phosphate dehydrogenase, and smaller increases in two other NADP-linked enzymes, isocitrate dehydrogenase and glutathione reductase (all based on fat-free dry weight); in contrast, lactate dehydrogenase and α-glycerophosphate de-

* Part 1: Pennington (1961*a*).

hydrogenase (both NAD-linked) were decreased. In the previous paper of this series (Pennington, 1961*a*) it was reported that mitochondria isolated from dystrophic muscle showed a slightly higher adenosine-triphosphatase activity than normal (relative to mitochondrial nitrogen) but no change in succinate-dehydrogenase activity. Read (1962) has recently reported that the creatine-kinase activity (based on non-collagen nitrogen) of dystrophic mouse muscle is normal.

The work reported below comprises measurements of three enzymes not previously studied in dystrophic muscle, namely AMP deaminase, acid phosphatase and adenylate kinase. In addition, both acid- and alkaline-cathepsin activity have been measured, in view of the fact that Koszalka & Miller (1960) found a peak of cathepsin activity in rat skeletal muscle at pH 8·5–9·0. Finally, the sharp increase in glucose 6-phosphate-dehydrogenase activity (McCaman, 1960) has been confirmed.

A preliminary report on AMP-deaminase activity in dystrophic muscle has been published (Pennington, 1961*b*).

EXPERIMENTAL

Mice. These were of either sex, strain 129 (Roscoe B. Jackson Memorial Laboratory, Bar Harbor, U.S.A.). Some of the animals used were purchased directly from Bar Harbor, but the majority were bred from stock obtained from there; none, however, was more than three generations removed from the Bar Harbor stock. The presence of the disease is always evident when the animals are 2–3 weeks old. Affected mice show occasional dragging of a hind limb and convulsive vertical movement of the head. The 'normal' animals included some litter mates of the dystrophic mice; some of these 'normal' animals were probably heterozygotes, since the myopathy is inherited by an autosomal recessive gene. The mice were kept at 21° and were fed on 'breeding sow cubes' (J. N. Brown and Son, Minden Street, Newcastle upon Tyne) supplemented daily with fresh vegetables.

Preparation of muscle suspensions. Mice were killed by dislocating the neck and bled as completely as possible from the throat. Most of the muscle of the hind legs was removed and freed as completely as possible from fat and nerve. It was chopped very finely with scissors and homogenized with about 5 vol. of water in a Potter–Elvehjem homogenizer. The pestle was rotated at high speed for a total of about 3 min.; the tube was cooled in ice at intervals during this period. With one exception, enzyme assays were carried out on the whole suspension, suitably diluted with water. For assay of glucose 6-phosphate dehydrogenase the suspensions were first centrifuged (5 min. on a Beckman Spinco model 152 Microfuge); otherwise they were too turbid to assay. All assays were commenced within 0·5–2 hr. of preparing the suspension, which was kept in ice.

Determination of 'non-collagen nitrogen'. Owing to the increased quantity of connective tissue (which, however, never exceeds about 10% of the total protein) in dystrophic muscle, the reference base used for comparing muscle-enzyme activities was 'non-collagen N' (Lilienthal, Zierler, Folk, Buka & Riley, 1950). This was determined by digesting samples of the suspension overnight at 30° with 9 vol. of 0·1 N-NaOH; the digests were then centrifuged and the N of the clear supernatant was determined by a micro-Kjeldahl procedure (Johnson, 1941).

Determination of enzyme activities

AMP deaminase. The activity of this enzyme in muscle was measured in a medium (total volume, including tissue suspension, 1 ml.) containing (final concn.): citric acid–sodium citrate buffer, pH 6·7 (0·1 M with respect to total citrate); AMP (Na salt, 5 mM). The tubes were shaken gently at 37° for 10 min. Perchloric acid (1 ml.; 10%, w/v) was then added, the tubes were centrifuged and the NH_3 content of the supernatant was measured with Nessler's reagent. The measured activity was directly proportional to the amount of tissue present, provided that not more than about one-half of the AMP was deaminated.

Acid phosphatase. The method of Gutman & Gutman (1940) was used; this involves hydrolysis of phenyl phosphate at pH 4·9 and measurement, with the Folin–Ciocalteu reagent, of the phenol liberated. Incubation was for 20 min. at 37° with gentle shaking.

Adenylate kinase. This was determined spectrophotometrically by coupling with hexokinase and glucose 6-phosphate dehydrogenase, a method used by other workers (e.g. Oliver, 1955). The medium contained (final quantities): tris–HCl, pH 7·5 (0·1 M), $MgCl_2$ (5 mM), ADP (Na salt, 0·5 mM), NADP (0·5 mM), hexokinase (70 μg. of type IV, Sigma Chemical Co., St Louis, Mo., U.S.A., containing approx. 3×10^5 units/g.; 1 unit defined according to Kunitz & McDonald, 1946), glucose (0·2%), glucose 6-phosphate dehydrogenase (1 μg. purchased from C. F. Boehringer und Soehne, Mannheim, Germany, and containing approx. 4000 units/mg.; 1 unit defined according to Beisenherz *et al.* 1953). The total volume, including tissue suspension, was 0·4 ml.; the reaction was carried out at 21–22° in 1 cm. microcells in a Unicam SP. 500 spectrophotometer. The medium was allowed to stand for about 3 min. before adding the tissue, to allow utilization of the small amount of ATP in the ADP. Several readings were taken at 340 mμ between 2 and 10 min. after addition of the tissue; the increase was linear over this time-period and the activity was computed from the slope of the line. The commercial hexokinase and glucose 6-phosphate dehydrogenase used were shown to contain no myokinase activity under these conditions. The tissue suspensions, at the concentrations used, did not measurably oxidize $NADPH_2$.

Cathepsin. The tissue suspension was shaken gently in capped tubes for 1 hr. at 37° in a medium containing (final concn. after addition of tissue): acetic acid–sodium acetate buffer, pH 4·0 (0·1 M with respect to total acetate), or tris–HCl, pH 8·6, at room temperature (0·1 M); crystallized bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne) (0·77%). The total volume was 0·13 ml. Trichloroacetic acid (0·13 ml.; 10%) was added and the tubes were allowed to stand in ice for several minutes and centrifuged. The supernatant was assayed with the Folin–Ciocalteu reagent (Anson, 1938); a Beckman Spinco microcolorimeter, model 151, was used. The increase in colour obtained at 650 mμ, when compared with similar tubes

Table 1. *Enzyme activities in normal and dystrophic muscle*

Activities were measured as described in the text. The result in each case is the mean ±S.D., and number of mice in parentheses.

Age of mice (weeks)	AMP deaminase (μmoles/min./mg. of non-collagen N)		Acid phosphatase (μm-moles/min./mg. of non-collagen N)		Adenylate kinase (μmoles/min./mg. of non-collagen N)	
	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic
3–4	23·4±3·1 (4)	14·0±2·1 (4)	—	—	—	—
12–20	20·1±3·9 (6)	5·2±1·3 (5)	24·0±3·9 (7)	34·8±7·3 (8)	6·1±1·3 (5)	3·1±0·4 (4)

that were not incubated, was expressed in terms of tyrosine, which was used to construct the standard curve. For determining the pH–activity relationships, other buffers were used as described.

Glucose 6-phosphate dehydrogenase. The medium (total volume 0·4 ml.) contained: tris–HCl, pH 7·6 (0·1 M), $MgCl_2$ (10 mM), NADP (0·25 mM), glucose 6-phosphate (Na salt, 2 mM). Activity was computed from the constant rate of increase of E at 340 mμ, between 2 and 10 min. after addition of the tissue preparation, at 21–22°. No correction was made for the activity of 6-phosphogluconate dehydrogenase; under the conditions used its activity was found to be about 10% of that of glucose 6-phosphate dehydrogenase.

RESULTS

AMP deaminase. The activities obtained with dystrophic and normal muscle are shown in Table 1. There is evidently a marked fall in activity in the dystrophic muscle. The mice used were of two main age groups. In the older mice (12–20 weeks) the mean activity in the dystrophic muscle was only about one-quarter of that of the normals. The younger dystrophic mice (3–4 weeks) showed, however, a less-pronounced difference from normal.

In other experiments the deaminase activity of mixed homogenates from normal and dystrophic muscle was measured. In every case the combined activity equalled the sum of the separate activities; thus it is improbable that the low activity of dystrophic muscle was due to the presence of an inhibitor in the tissue.

The AMP-deaminase activity of muscle homogenates was diminished by approximately 15% if the homogenates were rapidly frozen and thawed three times before testing. The use of 0·5 M-potassium chloride as homogenizing medium gave activities about one-third less than those obtained when water was used.

No ammonia production was observed when AMP was replaced by adenosine in the incubation medium. Hence none of the observed activity is due to the combined action of 5′-nucleotidase and adenosine deaminase.

Acid phosphatase. The activity of muscle from normal and dystrophic mice (12–20 weeks old) is shown in Table 1. The dystrophic muscle is significantly ($P < 0·01$) more active.

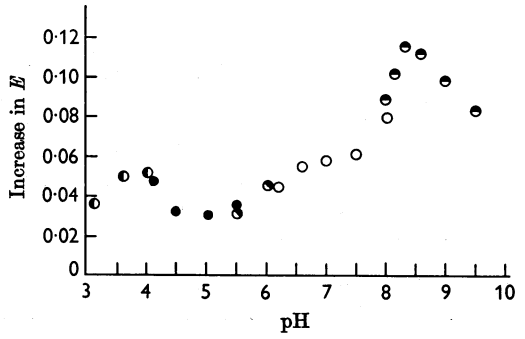

Fig. 1. Variation of cathepsin activity with pH. Activity was measured as described in the text. Buffers used: ◐, citric acid–sodium citrate (0·15 M with respect to total citrate); ●, acetic acid–sodium acetate (0·15 M with respect to total acetate); ◔, succinic acid–sodium succinate (0·15 M with respect to total succinate); ○, Na_2HPO_4–NaH_2PO_4 (0·15 M with respect to total phosphate); ◓, tris–HCl (0·15 M).

Adenylate kinase. Table 1 shows the results obtained on muscle from four dystrophic and five normal mice (12–20 weeks old). The mean activity of the dystrophic muscle is about one-half of the normal ($P < 0·01$).

Cathepsin activity. Fig. 1 shows the cathepsin activity of normal mouse muscle at various pH values. The experiments were with three mice (aged 4 months), and gave closely similar results. It is evident that, as found for rat muscle by Koszalka & Miller (1960), the greatest activity occurs at pH 8–9, with a smaller peak at about pH 4. The relatively high activity in the alkaline range was confirmed by the use of extinction measurements at 280 mμ, in place of the Folin–Ciocalteu reagent.

No activity was observed (pH 8·6) if the water homogenate was heated to 70° for 15 min. before testing.

The presence in the medium of ferrous sulphate (mM) or the detergent Triton X-100 (0·1%) did not alter the activity at either pH 4·0 or pH 8·6.

Muscle-cathepsin activity of a series of dystrophic and normal mice was measured at pH 4·0 and 8·6 and the results are presented in Table 2. It is

Table 2. *Acid- and alkaline-cathepsin activity in normal and dystrophic muscle*

Activity was measured at 37° as described in the text. Each pair of figures (pH 4·0 and 8·6) was obtained from a single animal.

Age of mice (weeks)	Acid-soluble 'tyrosine' formed (μg./hr./mg. of non-collagen N)			
	Normal		Dystrophic	
	pH 4·0	pH 8·6	pH 4·0	pH 8·6
3–4	19·8	13·3	19·6	28·5
	11·8	12·6	24·0	19·6
			21·9	16·7
12–20	6·6	16·0	39·2	55·4
	6·5	15·5	17·4	33·2
	8·2	21·4	24·9	31·4
	9·9	16·8	21·3	36·8

Table 3. *Activity of glucose 6-phosphate dehydrogenase in normal and dystrophic muscle*

Activity was measured at 21–22° as described in the text.

Age of mice (weeks)	NADP reduced (μm-moles/min./mg. of non-collagen N)	
	Normal	Dystrophic
12–20	9·3, 10·7, 7·5	29·5, 54·8, 54·0
3–4	10·4, 9·4	29·9, 29·8

evident that, in addition to the increase in activity in dystrophic muscle at pH 4·0, previously reported by others, a rise occurs also in the alkaline-cathepsin activity. In Table 2 it is shown that, for both normal and dystrophic mice, the ratio of cathepsin activity at pH 8·6 to the activity at pH 4·0 is greater in the older mice. Other experiments (unpublished) have confirmed this age relationship but have failed to indicate such a relationship in another strain (DAB) of mice.

Glucose 6-phosphate dehydrogenase. Table 3 shows the values obtained for this enzyme. The increase in dystrophic muscle is confirmed and shown to be very marked even at an early age.

Enzyme activities in blood

It is known that there is a relatively high activity of both acid phosphatase and glucose 6-phosphate dehydrogenase in erythrocytes. It was therefore important to estimate whether a substantial proportion of the measured activities in muscle could be accounted for by occluded blood. If so, any differences in the amount of blood in dystrophic and normal muscle could lead to false conclusions. Measurements on laked whole blood from a number of normal and dystrophic mice showed that the activity of acid phosphatase/ml. of blood was approximately seven times the activity/g. wet wt. of normal muscle. For glucose 6-phosphate dehydrogenase the corresponding ratio was 10, and for cathepsin (pH 4·0) the ratio was 3. Whole blood showed no cathepsin activity at pH 8·6. An approximately maximum value for the amount of blood in several samples of normal and dystrophic muscle was obtained by measuring E at 540 mμ after clarifying the homogenate by high-speed centrifuging; this was compared with the increase in E obtained when a known quantity of blood was added to the homogenate. In no case was the proportion of blood in the muscle, determined in this manner, greater than 3%. Other workers (e.g. Gitlin & Janeway, 1954) have reported values of a similar order for the amount of blood in the muscle of bled animals. Thus the differences in enzyme activities in dystrophic muscle cannot be due to differences in the amount of blood present in the muscle samples.

DISCUSSION

Of the five enzyme activities measured in the present investigation, three (acid phosphatase, cathepsin and glucose 6-phosphate dehydrogenase) show increased activity in dystrophic muscle, whereas the other two (AMP deaminase and adenylate kinase) are markedly decreased. Comparison on the basis of total protein, instead of non-collagen nitrogen, would undoubtedly have given very similar results since the amount of collagen, even in dystrophic mouse muscle, is small.

It is evident from these and previous results that many enzymes show altered activity in dystrophic muscle; possibly this would apply to the majority of muscle enzymes. There is no reason to suppose that any of the changes reported are closely linked to the genetic defect that causes the disease. It has not been demonstrated that any are specific for genetic muscular dystrophy and, in fact, some of the changes, e.g. cathepsin (Weinstock, Goldrich & Milhorat, 1955) and glucose 6-phosphate dehydrogenase (McCaman, 1961), have been shown to be similar in myopathy due to other causes. Further work should indicate the extent to which muscle degeneration follows a common biochemical pattern, whatever the ultimate cause. Studies at various stages of muscle disease should help to throw light on the relationships between the changes that occur.

Increases in other hydrolytic enzymes in dystrophic muscle have been reported (Tappel, Zalkin, Caldwell, Desai & Shibko, 1962). It is conceivable that such increases could result from damage to muscle lysosomes, with release of the enzymes, and that such damage may be an important factor in the cause of muscular dystrophy. However,

there is no good evidence that muscle fibres contain particles which serve as reservoirs of hydrolytic enzymes, corresponding to the lysosomes of liver. Moreover, homogenizing the tissue in water or treatment with detergent would, in any case, be expected to release the enzymes, if such bodies behaved like liver lysosomes.

Most workers agree that phagocytosis is a feature of the breakdown of dystrophic muscle. Consequently some of the enzyme increases observed may be due to enzymes in the macrophages. This may be true of glucose 6-phosphate dehydrogenase and isocitrate dehydrogenase, since Rubenstein & Smith (1962) reported histochemical evidence that macrophages in necrotic lesions of various tissues contained high activities of these enzymes. On the other hand, Weinstock, Marshall & Jenkins (1962) found that the electrophoretic behaviour on starch gel of cathepsins from dystrophic muscle (nutritional muscular dystrophy) differed from that of cathepsins from peritoneal macrophages. They observed also that the cathepsin activity differed from connective-tissue cathepsin in this respect, indicating that the increased cathepsin activity in dystrophic muscle is probably not a result of proliferation of connective tissue.

SUMMARY

1. The mean AMP-deaminase activity of muscle homogenates from mice with hereditary muscular dystrophy was about 60% of normal in mice 3–4 weeks old and about 25% of normal in mice 12–20 weeks old.

2. Acid-phosphatase activity in the dystrophic muscle was, on the average, about 50% higher than normal (12–20 weeks old).

3. Dystrophic muscle from mice 12–20 weeks old had a decreased adenylate-kinase activity (about one-half of normal).

4. An increase in cathepsin activity (pH 4) in dystrophic muscle was confirmed. Mouse muscle showed a second peak of cathepsin activity at pH 8–9; the activity at this pH was also increased in dystrophic muscle. The increases were greater in older mice.

5. Muscle-cathepsin activity at pH 8·6 relative to that at pH 4 was greater in older mice.

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