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Enzymes of the glycogen cycle and glycolysis in various human neuromuscular disorders[1]

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Previous investigations in several laboratories (Cooper and Miller, 1962; Schapira and Dreyfus, 1963; Pennington, 1964; Laudahn and Heyck, 1965) have shown that the activity of glycolytic enzymes is decreased, whereas that of mitochondrial enzymes is essentially unchanged in skeletal muscle from patients with progressive muscular dystrophy. It is still uncertain whether the above biochemical abnormalities reflect peculiarities in the metabolism of carbohydrates by the muscle fibres before the onset of degenerative changes, or whether they are merely the consequence of these changes.

Further, the activities of enzymes more directly concerned with glycogen metabolism have been little investigated in human dystrophic muscle. It is recorded only that the activity of phosphorylase is considerably decreased at advanced stages of the disease (Dreyfus, Schapira, and Schapira, 1954). Investigations of U.D.P.G.-glycogen synthetase activity in muscular dystrophy have not yet been reported, though this enzyme is believed to be an important regulatory factor in glycogen synthesis (Leloir, 1961).

In order to investigate aspects of carbohydrate metabolism in human dystrophic muscle for specific abnormalities, it was decided, therefore, to examine the relative tissue activities of phosphorylase, U.D.P.G.-glycogen synthetase, and of three sequential enzymes of the Embden-Meyerhof pathway, in muscles from patients with progressive muscular dystrophy and with neurogenic muscular wasting.

With the above objects in mind, it seemed proper to restrict our investigation to earlier enzymic changes which may precede the appearance of gross dystrophic manifestations and can, therefore, be more directly related to the primary pathological lesion.

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Substrates and cofactors for enzymic assays were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Crystalline enzymes from C. F. Boehringer & Soehne, Mannheim, Germany. Other products were commercial grade reagents.

METHODS

Under local anaesthesia muscle was taken either from the palmaris longus or the vastus medialis in patients with progressive muscular dystrophy and with neurogenic muscular wasting. Additional muscle specimens, obtained either by biopsy from healthy subjects or in the course of routine orthopaedic operations, were used as controls.

A fragment of tissue was fixed in formalin-mercuric chloride fluid. Blocks were embedded in paraffin, cut at 8 μ, stained with haematoxylin-eosin, and used for light microscopy. The unfixed remainder was freed from any obvious connective tissue, rapidly frozen, and stored at –25°C. until assay.

At the time of analysis thawed and blotted aliquots of about 200 mg. were weighed out in an analytical balance. Homogenates (final concentration, 10%) were prepared from the tissue with a Potter-Elvehjem apparatus, in 5 mM Tris-1 mM E.D.T.A., *p*H 7·4, or in 20 mM K-phosphate buffer, *p*H 8·0. Tris-E.D.T.A. homogenates were used for assay of U.D.P.G.-glycogen synthetase (U.D.P. glucose: glycogen α-4-glucosyltransferase, E.C. 2.4.1.11) and phosphorylase (phosphorylase phosphohydrolase, E.C. 3.1.3.17). For the latter purpose homogenates were further diluted with three volumes of 20 mM NaF, 1 mM E.D.T.A., *p*H 6·0. The 10,000 g. × 10 min. supernatant fraction of 10% homogenates in phosphate buffer was used for the assay of the glycolytic enzymes.

The U.D.P.G.-glycogen synthetase was measured in the presence of 10 mM glucose-6-phosphate, in a reaction mixture having the same composition as the standard medium of Leloir and Goldemberg (1960), except that it was buffered with Tris-acetate, *p*H 7·5 (Gallucci, Novello, Margreth, and Aloisi, 1966).

Phosphorylase activity was measured in the presence of 1 mM AMP at *p*H 6·0, according to the procedure of Cori, Illingworth, and Keller (1955). P_i was determined on appropriate aliquots of the reaction mixture by the method of Lindberg and Ernster (1966).

Phosphofructokinase (A.T.P.: D-fructose-6-phosphate 1-phosphotransferase, E.C. 2.7.1.11) activity was determined spectrophotometrically by the method of Ling, Byrne, and Lardy (1955), in a reaction medium of the following composition: 33·3 mM Tris, *p*H 8·0; 6·0 mM

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$MgCl_2$; 2·0 mM A.T.P.; 0·1 mM NADH; 1·0 mM fructose-6-phosphate, and an excess of crystalline aldolase, triose-phosphate isomerase, and α-glycerophosphate dehydrogenase.

Aldolase (fructose-1, 6-diphosphate: D-glyceraldehyde-3-phosphatelyase, E.C. 4.1.2.13) activity was determined by the oxidation of N.A.D.H. at *p*H 7·4, in the presence of fructose-1, 6-diphosphate, triose-phosphate isomerase, and α-glycerophosphate dehydrogenase (Wu and Racker, 1959); lactate dehydrogenase (D-lactate: N.A.D. oxidoreductase, E.C. 1.1.1.28) by the oxidation of N.A.D.H. by pyruvate at *p*H 7·4 (Kornberg, 1955).

Serum aldolase activity was measured by the colorimetric method of Sibley and Lehninger (1949a), expressed as μlitres of fructose-1, 6-diphosphate split per hour by 1 ml. of serum at 38°C. The normal ranges are 3-10 units (Sibley and Lehninger, 1949b).

Protein measurements in T.C.A. precipitates of whole tissue homogenates and tissue extracts were made by the Lowry, Rosebrough, Farr, and Randall (1951) procedure.

CLINICAL MATERIAL AND HISTOLOGICAL CLASSIFICATION

Muscle specimens were made available to us from 41 patients: 21 were cases of progressive muscular dystrophy (Table I) and 20 were cases of neurogenic muscular wasting (Table II).

All cases of progressive muscular dystrophy were classified according to the criteria of Walton and Nattrass (1954). Serum aldolase activities were measured in most cases. In agreement with earlier data in the literature (Thomson, 1962; Heyck and Laudahn, 1965), these were found most elevated in the Duchenne type of dystrophy, and less so in the limb-girdle type and in dystrophia myotonica. Patients with the Duchenne type of dystrophy were at an early stage of the disease: all patients were ambulant, although they showed a more or less marked rolling gait and muscular weakness.

In order to make possible a comparison between enzymic activities in progressive muscular dystrophy and neurogenic muscular wasting at similar stages of each process, and also at different stages of the same disease, affected muscles were grouped on the basis of histological criteria, irrespective of the specific clinical disease. Histological classification was based on the type and extension of elementary changes in the muscle fibres, such as simple atrophy, hypertrophy, increase in the number and centralization of sarcolemmal nuclei, degenerative changes, and increase of interstitial fat and connective tissue. These elementary changes are present in both progressive muscular dystrophy and neurogenic muscular diseases, the variable proportion of atrophic and degenerative features depending essentially on the rate of progression of the disease (Aloisi, 1965).

Two stages, corresponding to the very early and to the more advanced manifestations of the pathological process were identified. Group 1 included cases showing histological appearances characterized by simple atrophy, hypertrophy, increase in number and centralization of sarcolemmal nuclei. Degenerative changes, when present, were early and involved either a large number of fibres, as in some cases of progressive muscular dystrophy, or, as was more frequently observed with neurogenic muscular wasting, were limited to a few scattered fibres. There was little evidence of adipose or connective tissue infiltration. On the other hand, the cases included in group 2 showed marked atrophy, greatly increased numbers of subsarcolemmal nuclei, advanced and/or widespread degenerative changes, and appreciable connective tissue infiltration.

TABLE I

PROGRESSIVE MUSCULAR DYSTROPHY

Case No.	*Type of Muscular Dystrophy*	*Age (yr.)*	*Sex*	*Histological Group*	*Serum Aldolase (units per ml.)*
1	Duchenne	4	M	1	—
2	Duchenne	8	M	1	32
3[1]	Duchenne	3	M	1	—
4[1]	Duchenne	6	M	1	74
5	Duchenne	16	M	1	24
6	Duchenne	5	M	2	170
7	Duchenne	4	M	2	—
8	Duchenne	6	M	2	118
9[2]	Duchenne	4	M	2	91
10[2]	Duchenne	7	M	2	50
11	Duchenne	6	M	2	—
12	Duchenne	6	M	2	140
13	Limb-girdle	36	M	1	38
14	Limb-girdle	42	M	2	—
15	Limb-girdle	18	M	2	25
16	Facio-scapulo-humeral	14	F	1	29
17	Facio-scapulo-humeral	49	F	1	9
18	Facio-scapulo-humeral	11	F	2	29
19	Dystrophia myotonica	38	F	1	—
20[3]	Dystrophia myotonica	34	F	1	25
21[3]	Dystrophia myotonica	29	F	1	21

[1,2,3] Siblings

TABLE II

NEUROGENIC MUSCULAR DISEASE

Case No.	*Condition*	*Age*	*Sex*	*Histological Group*
1	Chronic peripheral neuritis	45 yr.	M	1
2	Chronic peripheral neuritis	35 yr.	F	1
3	Chronic peripheral neuritis	39 yr.	F	1
4	Chronic peripheral neuritis	52 yr.	M	1
5	Chronic polyneuritis	28 yr.	F	1
6	Chronic polyneuritis	50 yr.	F	1
7	Chronic polyneuritis	18 yr.	F	2
8	Acute polyneuritis (Guillain-Barré)	43 yr.	F	1
9	Acute polyneuritis	62 yr.	F	1
10	Amyotrophic lateral sclerosis	54 yr.	F	1
11	Amyotrophic lateral sclerosis	58 yr.	F	1
12	Amyotrophic lateral sclerosis	70 yr.	F	1
13	Amyotrophic lateral sclerosis	66 yr.	F	1
14	Amyotrophic lateral sclerosis	43 yr.	F	2
15	Werdnig-Hoffmann disease	7 mth.	F	2
16	Werdnig-Hoffmann disease	18 mth.	F	2
17	Werdnig-Hoffmann disease	6 yr.	M	2
18	Werdnig-Hoffmann disease	8 mth.	M	2
19	Werdnig-Hoffmann disease	3 mth.	F	2
20	Werdnig-Hoffmann disease	2 yr.	M	2

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TABLE III

ACTIVITIES OF PHOSPHOFRUCTOKINASE, ALDOLASE, AND LACTATE DEHYDROGENASE IN NORMAL AND DYSTROPHIC HUMAN MUSCLE[1]

Muscle	*Histological Group*	*Phosphofructokinase (μMoles F.D.P. formed /mg. pr./min.)*	*Percentage of Normal*	*Aldolase (μMoles N.A.D.H. ox/mg. pr./min.)*	*Percentage of Normal*	*Lactate Dehydrogenase (μMoles N.A.D.H. ox/mg. pr./min.)*	*Percentage of Normal*
Control	—	0·370 ± 0·030 (11)	—	0·840 ± 0·051 (11)	—	2·25 ± 0·28 (10)	—
Neurogenic muscular wasting	1	0·307 ± 0·011 (9)	83	0·706 ± 0·024 (9)	84	1·60 ± 0·19 (6)	71
Progressive muscular dystrophy	1	0·273 ± 0·034 (11)	74	0·709 ± 0·078 (11)	84	1·89 ± 0·18 (11)	84
Neurogenic muscular wasting	2	0·217 ± 0·067 (4)	58	0·350 ± 0·095 (4)	42	0·69 ± 0·14 (4)	31
Progressive muscular dystrophy	2	0·177 ± 0·026 (10)	48	0·430 ± 0·072 (9)	51	1·17 ± 0·16 (8)	52

[1]Values are given ± S.E.M. and number of analysis in parentheses.

TABLE IV

ACTIVITIES OF GLYCOGEN SYNTHETASE AND PHOSPHORYLASE IN NORMAL AND DYSTROPHIC HUMAN MUSCLE[1]

Muscle	*Histological Group*	*Glycogen Synthetase (μMoles U.D.P. formed/mg. pr./hr.)*	*Percentage of Normal*	*Phosphorylase (μMoles Pi formed/mg. pr/10min.)*	*Percentage of Normal*
Control	—	2·30 ± 0·21 (10)	—	7·40 ± 0·37 (10)	—
Neurogenic muscular wasting	1	2·28 ± 0·24 (9)	99	6·31 ± 0·66 (9)	85
Progressive muscular dystrophy	1	2·30 ± 0·15 (10)	100	3·65 ± 0·39 (10)	49
Neurogenic muscular wasting	2	1·95 ± 0·39 (6)	85	3·33 ± 0·95 (5)	45
Progressive muscular dystrophy	2	1·43 ± 0·13 (7)	62	1·86 ± 0·25 (8)	25

[1]Values are given ± S.E.M. and number of analysis in parentheses.

RESULTS

The data in Table III show that in progressive muscular dystrophy there is a considerable decrease in the activity of the three glycolytic enzymes studied, namely, phosphofructokinase, aldolase, and lactate dehydrogenase, but no greater than that found in neurogenic muscular wasting at comparable early histological stages of the process. It may also be noted from Table III that the decrease in activity was of the same order for each of the glycolytic enzymes, so that their reportedly constant ratios in several mammalian skeletal muscles (Pette and Bücher, 1963) were largely unaffected in the course of both progressive muscular dystrophy and neurogenic muscular diseases.

Likewise, the changes in U.D.P.G.-glycogen synthetase activity (Table IV) were independent of the type of muscular disease, although they occurred later than those of glycolytic enzymes. A definite decrease of U.D.P.G.-glycogen synthetase became apparent only during the late stages of the disease.

In contrast, however, Table IV shows particular differences in phosphorylase activity in progressive muscular dystrophy as compared with neurogenic muscular diseases. In progressive muscular dystrophy a marked decrease of phosphorylase activity is apparent in the early stages of the disease and progresses further with time. The relationship of the changes in muscle phosphorylase activity with the clinical progress of progressive muscular dystrophy is also supported by the comparative data on affected siblings at various stages of the disease (Table V). In neurogenic muscular wasting, however, muscle phosphorylase activity diminishes significantly only in the late stages, and even then it remains about twice as high as in progressive muscular dystrophy.

DISCUSSION

The decreased activity of glycolytic enzymes in human dystrophic muscle has received considerable attention in recent years, particularly since their excessive leakage into the bloodstream here, but not in neurogenic wasting, provides important diagnostic evidence for the presence of the disease (see, for discussion, Pennington, 1964). However, the data presented here show that, despite these substantial differences between the serum activities of glycolytic enzymes in progressive muscular

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TABLE V

ACTIVITY OF MUSCLE PHOSPHORYLASE IN SIBLINGS AFFECTED WITH DUCHENNE PROGRESSIVE MUSCULAR DYSTROPHY

Case No. (see Table I)	*Age (yr.)*	*Phosphorylase activity (μMoles Pi/10 min./mg. protein)*	*Percentage of Normal*	*Histological Group*
3	3	5·35	72	1
4	6	3·13	42	1
9	4	2·65	36	2
10	7	1·68	23	2

dystrophy and in neurogenic muscular disorders (Thomson, 1962; Heyck and Laudahn, 1965), the decrease of their activities in muscle is of the same order of magnitude in both conditions at comparable gross histological stages, *i.e.*, it is apparently secondary to the muscular wasting itself.

Because of the similarities in the behaviour of glycolytic enzymes and also U.D.P.G.-glycogen synthetase in both progressive muscular dystrophy and in neurogenic muscular wasting, our finding that muscle phosphorylase activity decreases earlier and more markedly in progressive muscular dystrophy is of considerable interest as a possibly specific biochemical feature of the dystrophic process.

While the above data support reports that the utilization of glucose is less affected than that of glycogen, at least in animal hereditary muscular dystrophy (Fink, 1961; Dale, 1965), the actual mechanism(s) diminishing glycogenolysis more in progressive muscular dystrophy than in neurogenic muscular wasting are still obscure.

Two alternative explanations may be entertained. One is that progressive muscular dystrophy, unlike neurogenic muscular disorders, involves a selective loss of a special type of muscle fibre with high phosphorylase activity. The other is that dystrophic muscle reverts to a less differentiated, embryonic type with low phosphorylase activity.

The first hypothesis rests on the fact that adult human muscle, like that of other vertebrates, contains fibres with different enzymic contents (Dubowitz and Pearse, 1961). Though their proportions are variable, two main types of fibres are generally described, which differ widely in their content of phosphorylase as well as of a number of glycolytic and oxidative enzymes (for review, see Beckett, 1962). In the present context, it is of interest that phosphorylase-rich fibres, which are more numerous in 'white' muscles, appear to be particularly affected in dystrophic muscles of the domestic fowl (Cosmos, 1966). This first hypothesis, moreover, does not conflict with our findings that U.D.P.G.-glycogen synthetase, unlike phosphorylase, is affected to the same degree in both progressive muscular dystrophy and in neurogenic muscular diseases. Measurements carried out in this laboratory on homogenates of different muscles from several species have so far shown that total U.D.P.G.-glycogen synthetase is quite uniformly distributed in 'white' and 'red' muscles (Margreth *et al.*, unpublished data). Thus a loss of U.D.P.G.-glycogen synthetase activity would depend more on the number than on the type of fibres involved.

The second hypothesis accords with the histochemical and biochemical evidence that phosphorylase activity is low in immature fibres both from embryonic avian muscle (Cosmos, 1966) and from regenerating rat muscle (Gallucci *et al.*, 1966), and increases with differentiation. However, both mechanisms may in fact be involved in progressive muscular dystrophy since the degeneration of phosyphorylase-rich 'white' fibres may be combined with dedifferentiation changes toward less mature 'red' fibres (see, for discussion, Muscatello, Margreth, and Aloisi, 1965).

The above considerations relate to the early stages of the dystrophic process, since our data show that, despite considerable enzymic differences between progressive muscular dystrophy and neurogenic muscular wasting, the latter may also eventually exhibit a marked decrease of phosphorylase at advanced stages of the disease.

The metabolic and functional similarities between dystrophic and embryonic muscle has suggested to several authors (Dreyfus, Demos, Schapira, and Schapira 1962; Brust, 1966) the possibility that the normal differentiation of skeletal muscle might be impaired in progressive muscular dystrophy.

Although the failure in dystrophy to maintain a normal content of muscle phosphorylase would be consistent with the above hypothesis, it is at present difficult to assign to impaired phosphorylase activity a prominent role in the onset of the degenerative changes of the muscle fibres. Absence of muscle phosphorylase, as in McArdle's disease (McArdle, 1951), is not regularly accompanied by gross dystrophic manifestations, although these were reported in some cases (Rowland, Fahn, and Schotland, 1963). It cannot be excluded that changes of phosphorylase activity in dystrophic muscle may be secondary to more specific enzymic alterations (Hooton and Watts, 1966).

A decrease in muscle phosphorylase activity thus appears to be a biochemical characteristic of muscular dystrophy, which may even prove of additional diagnostic use in differentiating early muscular dystrophy from neurogenic muscular disease.

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SUMMARY

The activities in skeletal muscle of phosphofructokinase, aldolase, lactate dehydrogenase, U.D.P.G.-glycogen synthetase and phosphorylase, have been compared in progressive muscular dystrophy and in neurogenic muscular wasting.

Both myogenic and neurogenic cases have been classified in two main groups, according to the severity and/or extent of histological changes, in order to make possible a comparison of the enzyme pattern in early and advanced stages of each type of disease.

A comparable decrease in the activities of the three glycolytic enzymes and of U.D.P.G.-glycogen synthetase was found in both progressive muscular dystrophy and neurogenic muscular wasting and according to the stage of the disease.

In contrast phosphorylase showed a loss of activity which was earlier and far more marked in progressive muscular dystrophy than in neurogenic diseases. The possible role of this unusual behaviour of phosphorylase in the pathogenesis of progressive muscular dystrophy is discussed.

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