PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 1225–1231 (1974)

Tricarboxylic Acid Cycle Intermediates in Muscular Dystrophic Mice (Strain 129) (37986)

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One feature observed in experimental studies on muscular dystrophy is a change in the lipid metabolism of skeletal muscle (1–3). Differences in the relative rates of incorporation of two specifically labeled acetates have been reported for normal and pathological groups of mice, both in *in vitro* studies (4) and in our own *in vivo* studies (5–7). While there was no marked uptake of a carboxyl-labeled acetate into tissues of pathological mice (5–7) both *in vitro* and *in vivo* studies showed a significantly increased uptake of methyl-labeled precursors (4–7). These data led us to postulate that the difference was one of intermediary metabolism, possibly changes in the TCA cycle, rather than one of cholesterol metabolism.

From the results of others there is evidence for believing that mitochondria may be malfunctioning in dystrophic animals, and this may account for the measured metabolic changes noted. Taussky *et al.* (8) for example, have reported an increase of citric acid in the tissues of dystrophic rabbits. Studies by Lin *et al.* (9, 10) and Jacobson *et al.* (11) on mitochondria in dystrophic muscle also have revealed metabolic defects. The reduced oxidative activity observed per unit weight of mitochondrial protein is an indication that mitochondria may be functioning poorly.

In order to augment our original observations further, we measured the levels of stable (nonketo) Krebs-cycle acids as a reflection of mitochondrial metabolism in groups of normal and dystrophic mice at different ages by means of gas–liquid chromatography (GLC). In this study tissues other than muscle were examined since there was reason to believe that a general biochemical dysfunction exists in affected mice (5–7).

Materials and Methods. Strain 129 dystrophic mice and their heterozygous and homozygous littermates were obtained from the Jackson Memorial Laboratory, Bar Harbor, ME. Animals 6, 8, 10, 12, and 14 wk old were represented in each of our three genetic study groups which are designated as (a) Normal (Dy Dy), (b) Carrier (Dy dy), and (c) Dystrophic (dy dy). For each weekly data point and for each group two mice at each age mentioned above were anesthetized with ether. They were killed by decapitation for the removal of brain, kidney, liver, intestinal, and hind limb muscle tissue samples. The tissue samples were washed in saline, blotted dry, and quick frozen in a dry-ice–acetone mixture. Frozen tissues were stored in a deep freeze ($-30°$) until ready to be used for analysis.

The isolation method for TCA acids was essentially the same as that described by Bligh and Dyer (12). In our hands, this particular technique gave a more complete extraction than did other available methods. Homogenized mouse tissues were extracted in four solvents: (a) 1.0 vol of water; (b) 3.7 vol of MeOH:$CHCl_3$; (c) in 1.2 vol of water; and (d) in 1.2 vol $CHCl_3$. Each was centrifuged at 18,000 rpm for 15 min and the supernatant of each was saved. The extraction was repeated twice with each solvent. All the collected supernatant frac-

tions were then combined and centrifuged at 18,000 rpm for another 5 min. The methanol–water layer alone was carefully drawn off and this was evaporated on a water bath at 85°C. The residue contained the short-chain fatty acids.

The efficiency of this method was determined by using fumaric acid as a standard. The extraction efficiency for this particular unsaturated fatty acid was believed to be a critical value, for, during extraction, isomeration as well as oxidation of this acid takes place. If it could be recovered efficiently, then all the other acids in the sample ought to be equally possible to recover by this method (13).

Prior to gas–liquid chromatography of the TCA cycle intermediates, the acids had to be converted to their more stable and volatile ester derivative. Several methods of esterification were tried: low-temperature esterification (14), trimethylsilylation (15), and treatment of acids with methyl alcohol, using sulfuric acid as a catalyst (13 and references therein). In our hands, the last procedure gave the most consistent yield. In particular, the acid (100 mg) was dissolved in 15 ml absolute methyl alcohol and refluxed in the presence of 1.0 ml concentrated H_2SO_4. To test for decomposition and for hydrolysis the methyl ester of fumaric acid as then employed in the same way with our extracted fatty acid residue from our heated tissue samples to obtain their ester.

The isolated esters were dissolved in ether and injected into a gas chromatograph (Packard Instrument Co. series 7800). The operating conditions are detailed in Table I. The accuracy and precision of separation was again first determined by using fumaric acid as a test standard.

Fatty acid standards were run for individual peak height measurements. Data values, the mean of two animals, are expressed as μg/g wet tissue. Conversion of the acids to their molar equivalent was not necessary since each acid value was compared with itself; i.e., the value of any one of the six acids identified in dystrophic tissue was compared with its counterpart value in carrier and normal tissue.

Results. The rate of esterification for the individual nonketo tissue acids were determined. Results indicated that a 3-hr reflux was necessary to achieve maximum yield (>94%). Using the extraction and glc method described, the standard acid (fumaric) was recovered and measured with an efficiency of 95 ± 3% (n = 6). In column 5 of Table II, the satisfactory elution of all six, stable, nonketo, Krebs-cycle acids determined in this study can be compared with the results of others (16, 17).

The TCA content of liver, kidney, intestine, muscle, and brain was measured for each genetic group at five time intervals (6, 8, 10, 12, and 14 weeks of age). In order to make comparisons more obvious, nonketonic acid data for an individual tissue is presented in a single graph. The pattern, rather than the level of a specific fatty acid, was considered to be important.

The pattern exhibited by the six TCA

TABLE I. Glc Operating Conditions.

Stationary phase		14.5% EGSS-X on Gas Chrom P (an acid alcoholic-base washed celatom) 100–120 mesh
Column	Dimension	4 mm. i.d. × 6 ft coiled glass
	Temperature	100–200°C at 5.5° min
	Gas flow (argon)	45 ml/min
Director	Temperature	210°C
Combustion	Hydrogen	30 ml/min
	Air	300 ml/min
	Voltage	100 V
Sensitivity		1×10^{-9} A
Recorder chart		Speed 6 in./hr
Injection port		Temperature 300°C

TABLE II. Retention Times of Methyl Esters of TCA Cycle Acids Stationary Phase.

Methyl esters	LAC 728[16]	25% Castorwax[17] Gas Chrom W	"Silicone"[16] grease	15% EGSS-X on Gas Chrom Q
Succinate	(1.00)	(1.00)	(1.00)	(1.00)
Malate	2.06	2.40	1.10	2.19
Aconitate	2.46	—[a]	2.88	2.69
Fumarate	—[a]	—[a]	1.07	0.78
Citrate	3.00	4.49	2.88	3.30
Isocitrate	—[a]	5.79	3.24	5.24

[a] Values not reported.

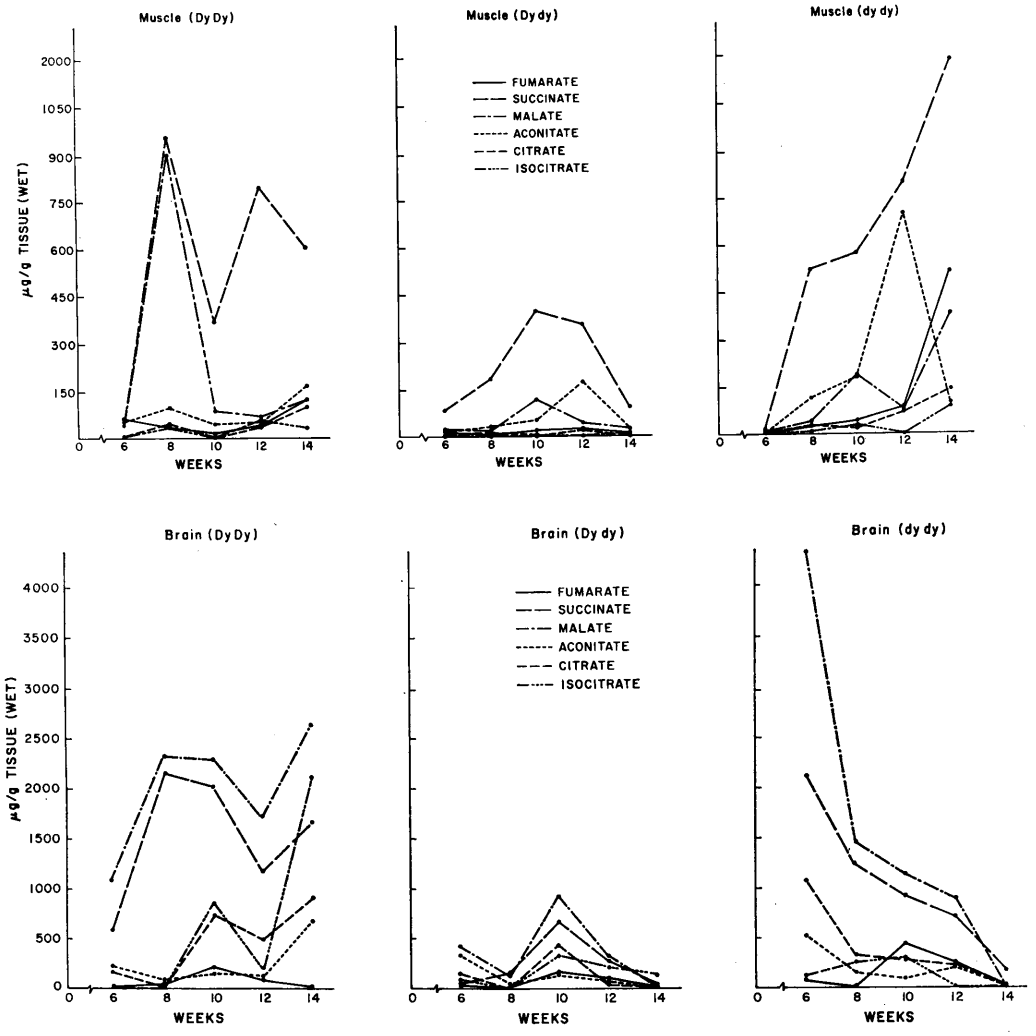

FIG. 1. Muscle and brain tissue values obtained by gas–liquid chromatography. Data are presented for nonketonic TCA intermediates in three genetic mouse strains: Normal (DyDy); carrier (Dydy), and dystrophic (dydy).

acids extracted from muscle indicates marked differences in all three genetic groups of mice (Fig. 1). Although all groups showed low initial values (6 wk), at the end of 14 wk the upward trend of malate, and especially succinate, were higher in the dystrophic group as compared to the normal. Except for aconitate, the changes in the other fatty acids were less dramatic. Values for the carrier (Dy dy) group were generally midway between the extremes of the other two genetic groups.

The most consistent difference between genetic groups found in our experiments were for fatty acids extracted from the brain. In the normal mouse, initial TCA intermediate had values which were low but increased with time. On the other hand, dystrophic brain values were higher than normal and became lower with time. In the carrier groups (Dy dy) the values were low and, except for high values at 10 wk, continued to exhibit low values for all six fatty acids.

Data were obtained for liver, intestine, and kidneys. For the sake of brevity, only the data for liver and intestine are presented (Fig. 2). Kidney fatty acid content, while

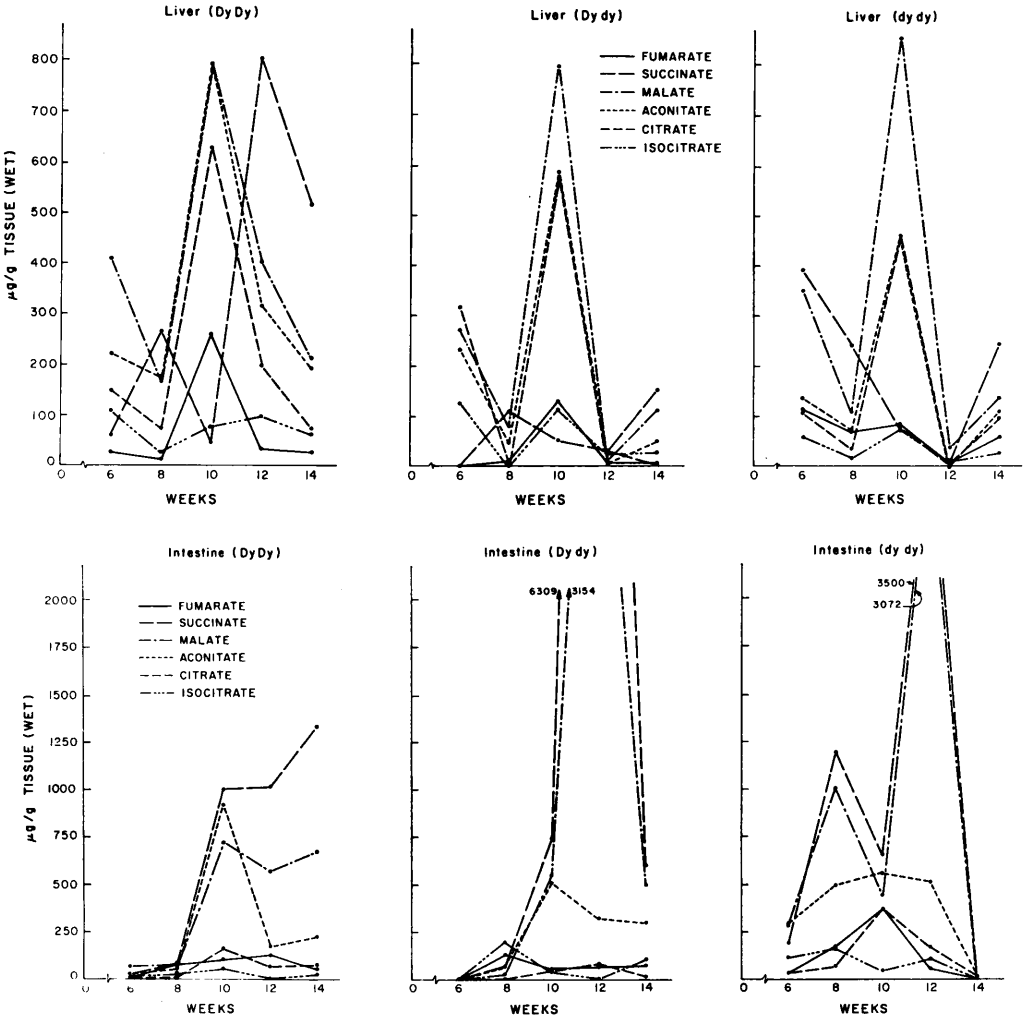

FIG. 2. Liver and intestine tissue values obtained by gas–liquid chromatography, data is presented for nonketonic TCA intermediates in three genetic mouse strains: Normal (DyDy); carrier (Dydy), and dystrophic (dydy).

higher than liver or even intestine, was similar to pattern to the latter tissue for all three groups. Comments made for intestine and kidney are identical.

The liver data are representative of pattern comparison that can be made rather than comparing levels of specific TCA intermediates (Fig. 2). Such a visual comparison shows that data for all three genetic groups to be identical. In this instance, both the quantitative value for the TCA intermediate, as well as the profile of change in this tissue of each group, were parallel.

The intestine values represent variations between the three groups studied. In general, and except for values at 14 wk in the Dy dy and dy dy groups, higher than normal values are measured.

The extremely low values for dystrophic mice at 14 wk may be more of an indication of a premorbid condition rather than a reflection of a specific pathology. Mice with muscular dystrophy begin to die at 14 wk of age and have a 50% survival level at 18 wk.

Discussion. While a number of analytical procedures for the isolation and assay of TCA intermediates were available (13, and references therein), the techniques had limited application to biological material. One of the more difficult problems faced was the extraction of the TCA components. A number of procedures were tried and the most satisfactory method was that of Bligh and Dyer (12). Early attempts to separate fatty acids from the crude extract by use of thin-layer chromatography were unsuccessful. While model nonketonic acids could be separated in ethanol:saline:25% ammonium hydroxide (100:12:16), the same systems applied to biological extract yielded single but heterogenous spots.

The analytical method of choice was gas–liquid chromatography. This technique allowed both the separation and quantification of crude tissue acid extracts without further purification.

At the time these studies were initiated only a few methods were available for comparisons. During and since that time many methods have been described in the literature for the determination of intermediates in the citric acid cycle (18 and references therein). Because of the difficulty in esterifying keto acids and because of the exploratory nature of the problem, we only analyzed for the nonketonic acids. We chose fumaric acid as our standard because of previous difficulties with this acid (18). Also, Rosenquist *et al.* (18) indicated that recoveries for this acid were low (55–65%). Using the method described, our high efficiency (>92%) gave confidence to the analytical data in this report.

Prior workers used columns which gave poor separation of methyl ester (15, 17). Even the use of two distinct columns were not satisfactory (15). In our hands 15% EGSS-X on Gas Chrom Q and temperature programming gave satisfactory results. Table II gives a comparison of our retention times with that of other workers. Retention times obtained were reasonable; the earliest, that of fumarate, was recorded after 18 min and the latest, isocitrate, 1 hr and 20 min later (Fig. 2).

During these studies, two compounds identified only as compound 4 and compound 8 with retention times of 31.5 and 90.0 min, respectively, were detectable but not identified. Since $CHCl_3$ was one of the solvents used in the extraction process, inclusion of some long-chained fatty acids might have been inevitable. Gas–liquid chromatographic analyses of long-chained fatty acids were run for comparative purposes. Palmitoleic, a long-chained (16:1) fatty acid, was found to have the same retention time as compound 4. Minor peaks in the glc of the crude extract might correspond to some other long-chained fatty acids. No definite answer could be given as to why palmitoleic, rather than some other acid, was extracted in such great quantities as compared to the rest of the long-chained fatty acid constituents. It might be possible that the presence of the double bond enhances its attraction for the more polar MeOH–H_2O layer. Peak 8 was not identified.

As far as we know, citric acid cycle metabolites in dystrophic animals have not been determined earlier by gas chromatog-

raphy. Indeed, most of our knowledge connected with this area of research on muscular dystrophy is conflicting.

While some workers (19, 20) have claimed the intactness of the TCA cycle in dystrophic animals, still many others (21–24) have reported alterations in the activity of the enzymes associated with the metabolic pathways. These latter studies suggest a change from normal.

Specifically, Mayers and Epstein (20), by measurement of lactic acid production in the homogenates of dystrophic mouse muscle concluded the intactness of the glycolytic pathway. Similarly, the TCA cycle was studied by measurement of O_2 consumption of mitochondrial and microsomal suspensions derived from normal pathological mouse muscle (20). Since no significant differences were found in those measurements, they also suggested that the citric acid cycle was unimpaired in the dystrophic mice. The intactness of the TCA cycle was further attested to by Baker *et al.* (19) whose experiments show that the cumulative rates of $^{14}CO_2$ formation from intravenously injected acetate-1-^{14}C are virtually identical for normal and dystrophic mice.

There are, however, indications that specific components and enzymes necessary to the cycle may be altered. As previously reported, phosphorylase was changed (25), elevation of succinic dehydrogenase took place and myosin ATPase was markedly increased (26, 27).

The single enzyme in the TCA cycle which requires NADP as a cofactor, isocitric dehydrogenase, was also shown to be increased (26). However, it is not clear whether this increase is sufficient to account for the increased excretion of 2-ketoglutarate in the urine of dystrophic mice (28). Rosenkratz and LaFerte (29) reported no change in activity of isocitric dehydrogenase in homogenates of dystrophic muscle when triphenyltetrazolium chloride was used as electron acceptor instead of NADP.

In view of this contradiction, we reevaluated the problem using a more specific analytical technique. Where previous workers attempted to draw conclusions about the cycle through investigations of the end product of metabolism, CO_2 (19) or lactic acid production (20), the present study concerned itself with direct extraction of the TCA cycle acids themselves. Acids were analyzed both qualitatively and quantitatively by glc.

Our results suggest that the pattern, rather than the level of change in various tissues, to be important. Also, the change in the profile of TCA intermediate in one tissue is not predictable from changes in another tissue. It was of interest that tissue patterns other than muscle were abnormal. The profile of Krebs cycle acids isolated from brain showed the most deviation from normal. This supports our earlier conclusion (5–7) concerning the involvement of the nervous system in this pathological condition.

It should be kept in mind that the widespread disturbance in a biochemical parameter (s), as measured by TCA intermediate in our experiments, may be a result rather than a cause of the myopathic process. Also, as E. Bajusz has suggested (30), "the nervous system exerts an important influence on the functional activity of enzymes in muscle cells (. . . tissues) and thereby contributes to the changes measured. . . ." Possible, more relevant to the problem at hand is the increasing number of chronic myopathies which are associated with structural and functional abnormal mitochondria (31 and reference therein).

It is our working hypothesis that muscular dystrophy is not a primary muscle disease. Whether the factor(s) involved is humoral or neutral remains to be clarified. What does seem certain is the presence of biochemical changes in tissues other than muscle. Our findings of changes in brain tissue continue to prejudice our thinking of the involvement, either primary or secondary, of the central nervous system in this disease process.

While the present data do not allow us to draw any specific conclusions concerning the kinetic formation or blockage of TCA intermediates in dystrophic mice, evidence is presented which indicates an altered cycle does exist in muscle as well as other

body organs of a pathological animal, particularly brain. Information concerning rates of metabolism, rather than levels of metabolites, is desirable if we are to understand underlying basic mechanisms in this disease.

Summary. Three genetic groups of mice (DyDy, Dydy, and dydy) were studied at intervals of 6–14 wk. TCA intermediates were extracted, separated, and measured. Data indicate that mice with genetically induced muscular dystrophy (Strain 129) show alteration in several body tissues as well as in muscle itself. Of particular interest were changes measured in the brain of dystrophic mice. Support, but not proof, is given to the neurogenic concept of mouse muscular dystrophy.

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Received Oct. 19, 1973. P.S.E.B.M., 1974, Vol. 145.