Biochemical studies in mice an inherited, progressive muscular dystrophy

Franklin G. Pratt
University of Nebraska Medical Center

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BIOCHEMICAL STUDIES IN MICE WITH AN INHERITED, PROGRESSIVE MUSCULAR DYSTROPHY

by

Franklin Glade Pratt

Submitted in Partial Fulfillment for the Degree of Doctor of Medicine

College of Medicine, University of Nebraska

February 1, 1964

Omaha, Nebraska
PREFACE

I am very grateful to Dr. W. O. Read, Dr. Robert Stratbucker and to the Muscular Dystrophy Association of America, Inc., who made available, in part, the necessary funds.
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I. INTRODUCTION

The distinction between true myopathic disease and functional disorders of skeletal muscle secondary to neuronal degeneration was fully developed by the work of Erb (1891). The disease, known today as muscular dystrophy, was first reported by Meryon (1864). He remarked on what appeared to be a disease restricted to skeletal muscle and suggested the cause to be a nutritional deficiency. Eulenberg (1866), Duchenne (1868) and Sachs (1888) subsequently verified the existence of muscular dystrophy as a clinical entity. Muscular dystrophy is distinguished as a condition of depressed functional capacity secondary to a primary granular and fatty degeneration of skeletal muscle fibers, whose etiology is unknown. The disease is conceded by all to be hereditarily transmitted. Bell (1943), Boyes, Fraser, Lawler and MacKensie (1949) and Tyler and Stephens (1950) have all reported the disease to be inherited as a sex-linked Mendelian dominant characteristic.

As muscular dystrophy is transmitted genetically, it is obvious that the disease cannot be artificially transmitted to an experimental animal. Because of this fact, the data that has accumulated has been of human clinical evaluations, muscle biopsy studies and post-mortem examinations. Therefore, with the discovery of a primary, hereditarily-induced myopathy in an inbred strain of mice (Michelson, Russell and Harman, 1955) new interest in basic research of muscular dystrophy has been generated. The similarities of this mouse dystrophy (called "dystrophia muscularis" by Michelson, Russell and Harman, 1955) and human muscular dystrophy are many.
(see "Review of the Literature"). Because of these similarities, this animal has proved itself to be a valuable research tool in the study of muscular dystrophy.
II. REVIEW OF THE LITERATURE

Mice exhibiting a form of muscular dystrophy were noted in an apparent mutation of breeding stock of inbred strain 129 mice at the Jackson Memorial Laboratory, Bar, Harbor, Maine. According to Michelson, Russell and Harman (1955), the disease is characterized by muscular weakness, gross atrophy of the muscles and progressive ataxia. They described paresis, kyphosis, convulsive nodding of the head, spasmodic flexion and flaccid extension of the hind limbs. There was eventual complete loss of locomotor function and premature death. The affected animals do not live a sufficient length of time to permit breeding. Stevens, Russell and Southard (1957) transplanted the ovaries of dystrophic mice into the ovarian capsules of normal female mice. The normal mouse was then bred and bore young. Part of this litter exhibited dystrophy. By this means, the latter authors were not only able to develop a stock of mice suffering from muscular dystrophy but were able to show that the inheritance of the disease followed the pattern of a single, autosomal recessive gene. Wolfe and Southard (1962) refined these methods, using artificial insemination to produce litters of all dystrophic offspring. Further work lead Russell and others (1962) to a new F1 strain, breeding the 129 Re-dy (dystrophic) mice and C57BC/6J-dy mice. These new animals have been found to possess similar anatomical, physiological and biochemical properties as noted in the 129 Re-dy strain.

After the initial report by Michelson, Russell and Harman (1955), an increasing number of laboratories turned their attention to this strain of mice.
as a valuable research tool for the study of muscular dystrophy. Sandow and Brust (1958) found that the dystrophic muscle of these mice was weaker (directly or in tension developed per gram of muscle), exhibiting less shortening when tension was applied. The contraction times were normal, but the relaxation times were increased threefold. At rest, both dystrophic and normal muscle possessed non-linear elasticity, but the diseased muscle was "stiffer". They also noted that the active state duration (that time in which the muscle is doing work) was shorter. Sandow and Brust (1962) also noted slower relaxation times and concluded that dystrophic muscle develops fatigue more slowly, but Solton (1962), in observing the swimming ability of dystrophic mice noted that these affected mice fatigued more rapidly.

The electromyographic studies of McIntyre, Bennett and Brodkey (1959) indicated that dystrophic muscle possessed a hyperirritability to mechanical stimulation. The muscles exhibited spontaneous high-frequency volleys and fibrillation. Both the high frequency volleys and fibrillary potentials continued undisturbed after nerve block. Similar observations in humans by these same authors led them to conclude that the condition of these mice corresponded closely to clinical electromyographic patterns of human dystrophia myotonica. Kleeman, Partridge and Glaser (1961) also noted these variations and further reported that the resting potentials of the dystrophic muscles were lower than the normals. The observation that alteration of the intra and extra-cellular ion concentrations had no different effect in response to electrical activity of dystrophic muscle as compared to normal control muscle was made by McLennan (1961). The electrical activity of myocardial muscles was explored by Kleinfeld, Magin, Murphy and Stein (1961) who found no change in intra-cellular potential or maximum tension output in dystrophic mice as compared
to their littermate controls.

West and Murphy (1960) studied the histological picture of dystrophic muscle of these mice and reported that the extreme variation of fiber size was the most conspicuous change. They noted that in cross-sections of the tissue the fibers round up and many become hypertrophied. There was a relative and absolute increase in connective tissue. This was due to the thickening of the endomysium and some fatty replacement. There was an increase in the number of nuclei and the nuclei were larger, more vesicular and exhibited internuclear rowing (the nuclei appeared as a string of beads). The sections showed coagulation necrosis, with some subsequent regeneration from isolated muscle nuclei and plasmodial budding. In the opinion of West and Murphy (1960), the existing morphological differences between mouse dystrophy and human dystrophy "are ones of degree rather than kind, and do not rule out the possibility of important similarities in mechanism and etiology." The "rowing" of the nuclei was also reported by Ross, Pappas and Harman (1960), who further noted that the myofibrils broke down by fragmentation and dissolution only in that region of the muscle cell where internuclear rowing was occurring. It was also observed that the mitochondria were less dense and were significantly larger with an irregular shape, probably due to swelling; the endoplasmic reticulum appeared distended or swollen in the dystrophic cells, but the sarcolemma and nuclei appeared to be normal. Kitigakara (1961) stated that the nuclei appeared consistent with those of non-proliferating tissue. His studies, utilizing P32 for radioautography of RNA and DNA, revealed only RNA synthesis. Jasmin and Bajusz (1962), noted myocardial lesions to be present late in the
disease, resembling those found in human muscular dystrophy, thus finding a similarity not noted before.

Brat, Shull, Alfin-Slater and Ershoff (1960) studied the development of the long bones. They found that the dystrophic animal showed a premature closure of the epiphyseal plate. They noted osteoporosis with complete loss of trabeculae in the epiphysis and diaphysis at 12, 16 or 20 weeks of age. It was their hypothesis that these changes were not due to disuse, but possibly due to changes in blood flow. It may be of significance that they could not find any evidence of change in bone in animals of 6 weeks of age. Leonard (1957) had earlier reported that the width of the tibial epiphyseal cartilages in the dystrophic mice was less than normal.

The effect of denervation on the dystrophic muscle has been subjected to investigation. Banker and Denny-Brown (1959) found that, (1) "after denervation, progressive smallness of fibers in genetically determined dystrophy (in mice) was the most evident histological characteristic", (2) the turnover of C\(^{14}\)-creatine was increased in the denervated muscle, (3) the greatest decrease in the creatine index (gm creatine/gm non-collagen nitrogen) was found in denervated dystrophic muscle and, (4) after denervation, simple atrophy of dystrophic fibers was increased. Baker, Wilson, Oldendorf and Blahd (1960) suggested that the muscle of the dystrophic animals, like denervated normal muscle was supersensitive to acetylcholine.

The physiology and biochemistry of the dystrophic muscle has been studied by a number of investigators. Coleman and Ashworth (1959) found
that in mice two months of age, there is an increase of 60% in the content of desoxyribonucleic acid (DNA) and 40% in that of ribonucleic acid (RNA) per gram of muscle wet weight. They reported the uptake of glycine-1-C\textsuperscript{14} was 25-40% faster in dystrophic skeletal muscle. Because the dystrophic mice did not gain weight they concluded that the synthesis of muscle tissue was increased to compensate for the degeneration. Radioautographic studies by Walker (1962) have shown that dystrophic muscle is able to regenerate. Simon, Lessell, Gross and Milhorat (1958) worked with dl-leucine-1-C\textsuperscript{14} and reported a higher incorporation rate into muscle over a 2-24 hour period, while the rate of disappearance of the isotopic carbon over a 20-30 day period from the dystrophic muscle was more rapid than normal. From this data, they postulated, "the loss of muscle protein in this mouse myopathy is not the result of a defect in protein synthesis, but of a more rapid turnover in which the greater rate of synthesis is exceeded by the increased rate of catabolism." This view is also held by Simon, Gross and Lessell (1962). Kruh, Dreyfus, Schapira and Gey (1960) studied the turnover of glycine-2-C\textsuperscript{14} in myosin and water-extractable proteins in normal mice (strain RAP), dystrophic mice and normal littermate mice (strain 129). They noted an increased uptake of the radioactive substance in both myosin and the water-extractable proteins in the dystrophic mice muscles. An increased rate of turnover was also observed in these same animals. According to these authors, "these results can be interpreted as representing a shorter life span of the muscle cell, or of a part of the cell or, more likely, as an acceleration of the turnover of the muscle proteins." Walker (1962) felt that this rapid turnover
reflected an accelerated 'aging', thus the dystrophic muscle becomes 'old' before the rest of the tissues. Weinstock, Epstein and Milhorst (1956) showed that the total nitrogen contents of dystrophic muscle were reduced and the collagen nitrogen was increased when compared to normal muscle on a wet weight basis, but in terms of dry weight the percentage of the nitrogen fractions was unchanged. Baker, Tubis and Blahd (1958) found that the metabolic rate, measured by collecting the expired air and measuring the carbon dioxide production, was normal in dystrophic mice. However, Rosenkrantz and Laforte (1960) measured the oxygen consumption of tissue slices of dystrophic muscle using the Warburg apparatus. They reported an increase in oxygen consumption in a range of 40-122% in their various samples. It was reported by Borgman (1962) that there were no differences noted in oxygen uptake of tissue slices between various dystrophic animal tissues and normal littermate controls. The apparent discrepancies between these studies may be explained on the basis of different techniques. Baker, Tubis and Blahd (1958) also reported that the oxidation of acetate-1-C\textsuperscript{14}, measured as C\textsubscript{14}O\textsubscript{2} in the expired air, was at the same rate in dystrophic mice as in normal mice. Baker, Blahd and Hart (1958) showed that the muscles of dystrophic mice contained less potassium but more sodium than do the normal controls. The values of 12-23\% less potassium and 68-107\% more sodium, based on the fat-free dry weight of skeletal muscle, were reported by Young, Young and Edelman (1959). Burr and McLennan (1961) and Fierles (1961) however, noted that potassium has an increased exchange rate through the membranes of dystrophic skeletal muscle. Kandutsch
and Russell (1958) found a 20% decrease of muscle creatine on a wet weight basis. Using infra-red analysis, Young, Young, and Edelman (1959) observed that dystrophic skeletal muscle showed, (1) 92% increase in total lipids, (2) 168% increase in triglycerides, (3) 140% increase in total cholesterol, (4) 87% increase in non-esterified fatty acids, but (5) a normal phospholipid content. Rabinowitz (1960) noted that homogenized and fortified tissues of dystrophic mice have a greater lipogenesis and cholesterologenesis than their littermate controls. The greatest differences observed by the above author were in brain tissue. The difference in this tissue was closely followed by the differences noted in liver and kidney tissues. Small differences were noted in spleen and skin tissues, while those in muscle tissue were insignificant. He stated, "that these results suggest that definite metabolic differences occur in the various fat-metabolizing centers of the dystrophic mice."

Attention has been brought to bear on the enzyme activities within the dystrophic muscle cell. Tassoni and Harmon (1961) reported that the succinic dehydrogenase varied with the stage of the disease. In the first two to four weeks following birth, the dystrophic and control muscle were much the same in enzymatic activity. When five to seven weeks old, the dystrophic animals showed lower activity while in the seven to twenty week age group, the dystrophic animal showed considerably higher activity than the controls. Weinstock, Epstein and Milherat (1958) stated that the activity of muscle succinoxidase was not affected. Cytochrome oxidase and cathopsin activity were markedly increased in the muscles of dystrophic mice when compared to littermate controls. They also showed that the aldolase activity of dystrophic muscle was
decreased when based on wet or dry weight but was unchanged when based on protein nitrogen or non-collagen protein nitrogen. Zierler (1958; 1961) suggested that aldolase probably diffused faster from dystrophic muscle than normal, which may be a reflection of increased permeability of the muscle. Leonard (1957) found that in the skeletal muscles of the dystrophic mice, the "active" and "total" phosphorylase activities were significantly less than normal, but that the phosphorylase activity ratio were normal. He also stated that the glycogen concentration was higher than normal in the dystrophic muscle. Rulon, Schottelius and Schottelius (1962), in further studies on phosphorylase activity, reported that its activity decreased with age in the dystrophic animal and that its activation under lengthening stimulation was variable, while that of the control animal showed progressive activation. They interpreted their results to show that there is decreased glycogenolysis in dystrophic muscle with advancing age of the animal. McCaman (1960) reported that some of the enzymes requiring triphosphopyridine nucleotide as a cofactor (glucose-6-phosphate dehydrogenase, isocitric dehydrogenase and glutamic reductase) had an increased activity in dystrophic muscle, while some of those requiring diphosphopyridine nucleotide as a cofactor (lactie dehydrogenase and alpha-glycerol-phosphate dehydrogenase) had a decreased activity. Hazzard and Leonard (1959) found that phosphoglucomutase activity was significantly lower in dystrophic leg muscle, determined as total enzyme activity (at infinite coenzyme concentration)or as residual enzyme-coenzyme activity (in absence of coenzyme). The enzyme decreased with the age of the mouse and with the severity of myopathy. They stated that the "defect" seen appeared to be due to the loss of the coenzyme (glucose-1.6 diphosphate). Rabinowitz (1959) studied the ratio of glutamic-oxalacetic
to glutamic-pyruvate transaminase and found that all tissues of the dystrophic mouse exhibited higher activity with the sera showing the greatest difference. McCaman (1960) has suggested that due to the apparent increase in activity of the enzymes requiring TPN as a cofactor and the apparent decrease in activity of those enzymes requiring DPN as a cofactor, that abnormally high levels of reduced TPN or reduced glutathione may bring about an altered intra-cellular metabolism. It has been demonstrated (Rosenkrantz, 1959; Rosenkrantz and Laferte, 1960) that dystrophic mice exhibit an increase in triphenyltetrazolium reducing activity (50% in liver, 43% in kidney and 25% in skeletal muscle). By introducing specific substrates, it was found that muscle showed a 37% increase in glutamic acid dehydrogenase, but no significant change in activity for succinic (see above), alphaketoglutaric, isocitric, beta-hydroxybutyric and malic dehydrogenases. Gould and Coleman (1961) indicated that dystrophic muscle homogenates formed acetoacetate at a much higher rate than normal muscle, but addition of sodium octanoate did not result in formation of acetoacetate in dystrophic muscle as it did in normal homogenates. They suggested that a heat labile factor in dystrophic muscle inhibited acetoacetate formation from octanoate. These authors reported (1962) the existence of an extractable enzyme responsible for acetoacetate formation in normal mice, but that it was not found in dystrophic tissues. Baker, Bloom and Blahd (1959) studied the formation of oxidation products of unsaturated fatty acids in homogenates of skeletal muscle, heart, kidney, brain and liver by means of a thiobarbituric acid assay. Their data suggested that the tissues of dystrophic mice did not
show a regular and significant increase in the formation of oxidation products of "fatty acid peroxides" over that of normal tissue.

Zymaris, Epstein, Saifer, Arousan and Volk (1959) reported that the muscles of the dystrophic and control mice were qualitatively the same in regards to adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), quanosine triphosphate (GTP), quanosine diphosphate (GDP), uridine diphosphate (UDP), enzyme creatine triphosphate (CTP) and inosine diphosphate (IDP). However, they did find that quantitatively the AMP, GTP and GDP were higher and that ATP was lower in the muscles of the dystrophic mice as compared to the normal control animals. In a later study, Fymaris, Saifer and Volk (1960) reported that by utilizing injected p32, they found increased levels of ADP, DPN and GDP and no difference in AMP, ATP and GTP in the dystrophic muscle. In another article (1962), these same authors reported the above results as indicating an increased turnover rate of ADP, DPN and GDP. Pennington (1961) studied mitochondrial enzymes and found there was a 15% increase of ATP in the dystrophic muscle but that the succinate -INT- reductase activity was normal in these tissues.

Bond and Leonard (1959) found that both normal and dystrophic mice had almost identical total blood, red blood cell and plasma volumes per 100 grams of body weight. Rabinowitz (1959) reported that the blood volume per organ is smaller in the dystrophics. Oppenheimer, deLuca and Milhorat (1959) studied paper electrophoretic patterns of sera from dystrophic and normal littermate controls. They found no change in the
relative concentrations of the protein fractions, but the patterns did indicate a shift in distribution, particularly of lipoproteins and to a smaller extent of glycoproteins. They also stated that the concentrations in plasma of protein nitrogen, protein-bound lipids (free and esterified fatty acids) and carbohydrates (hexoses, hexosamines and sialic acid) did not vary significantly from controls. Baker, Tubis and Blahd (1958) indirectly gave some attention to the endocrine glands. Finding that the plasma levels of plasma glucose, cholesterol, protein-bound iodine $^{131}$ and liver cholesterol were normal, they concluded that the pancreas and thyroid must therefore be functioning properly. The data of Kandutsch and Russell (1958) indicated that the dystrophic mice are able to remove injected creatine from the bloodstream more rapidly than the normals.

Several investigators have studied food consumption and urine excretion. Baker, Tubis and Blahd (1958) found that the dystrophic mouse consumes more food per gram of body weight than normal, but may weigh as much as 33% less than their littermate controls as reported by Bond and Leonard (1958). Perkoff and Tyler (1958) demonstrated that the Strain 129 mice excreted more creatine than creatinine, but there was a reduction of urine creatinine in the dystrophic mice. They observed that, "while there was creatinuria, it was less severe than human dystrophy." They also noted that the dystrophic animal excreted the same creatine load as did the controls. McGeer, McGeer, Miller, Derry and Nichol (1962) reported essentially the same results. They interpreted these results to point out a difference between the human and the mouse forms of muscular dystrophy.
Kandutsch and Russell (1958) found that the urine creatine/creatinine ratios were equal and that vitamin E injection did not reduce the creatinuria of dystrophic animals to normal levels. McGaughey (1960) reported that Strain 129 mice excreted more alphaketoglutarate and urea than other species and strains of mice. He showed that the dystrophies excreted significantly higher levels of alpha-ketoglutarate and urea/gm of body weight than did controls, and that these differences increased from weaning to 4 months of age. However, no significant differences were observed in urine excretion of amino acids, either in type or amount. It may be of significance that Rabinowitz (1959) determined that glucose-C\textsuperscript{14} was oxidized at the same rate in dystrophics and normal animals.

Various attempts have been made to halt or reverse the progress of the disease and to prolong the life of dystrophic animals. Baker, Tubis and Blahd (1958) tried administration of vitamins A, D, E, B-complex and ascorbic acid and failed to alter the symptoms of dystrophy. These investigators (Tubis, Baker and Blahd, 1959) also reported that early therapy with vitamins A, D, E, thiamine, riboflavin, pyridoxine, cyanocobalamine, calcium pantothenate, niacinamide, folic acid, biotin, synthetic K, ascorbic acid, unsaturated fatty acids and choline failed to prevent paralysis and abnormal reflexes which are typical of the disease. The treatment had no effect on the skin lesions, the convulsive seizures or the early death. Hall, Hall and Nevis (1959) attempted parabiosis between dystrophic and normal mice of the same sex and strain. Their results showed that survival of dystrophics increased from a normal of 8 - 10 weeks to 24 weeks in males and to 25 weeks in females. The characteristic signs in these animals were often reduced in severity. Survival of the parabiotic pair was limited, due
to the development of cardiovascular disease, apparently as a consequence of parabesisis. However, Pope and Murphy (1960) could not confirm these results. Their data indicated that a soft diet of high quality will increase the life span of the dystrophic mice. Miller, Wood and Gibson (1961) also recognized the importance of diet and the effect of the disease on the animal's ability to feed. They felt that these points must be considered in any drug evaluation program. The administration of 17-alpha-ethyl-19-nortestosterone did not restore the lost function of the dystrophic muscle, but did seem to retard the progress of the disease (Dowben, 1959). The administration of Testosterone propionate and estradiol benzoate was noted by Ershoff, Alfin-Slater and Bernick (1961) to prevent osteoporosis of the hind limbs but not to alter the course of the disease. Borgman (1963) utilized methylandrostenediol dienanthoylacetate to prolong survival time but it did not affect the disease process either.

The location of the initial lesion responsible for the disease state has not been specifically defined by any one study or group of studies. Indeed, it is painfully evident that as yet, no one biochemical locus even appears more promising than others. Rosenkrantz and Laferte (1960) are of the opinion, "that it is possible that the histopathological similarities of nutritional muscular dystrophy, the inherited myopathy of the mouse and human muscular dystrophy result from a biochemical lesion at a different site in the same biochemical pathway or cycle." Tubis, Baker and Blahd (1959) suggests, "that perhaps these animals may have a hereditary induced vitamin deficiency, due to the fact that vitamins
may not be incorporated into some necessary functional structure at the cellular level. Kruh, Dreyfus, Schapira and Gey (1960) stated that, "an acceleration of the turnover of the muscle proteins is probably the most important abnormality of the muscle protein metabolism in dystrophic mice." Walker (1962) felt that the affected tissues undergo accelerated "aging" and thus become "old" before the unaffected tissues. Nichol, McGeer and Miller (1962) felt that the decreased creatine kinase activity noted in dystrophic mouse skeletal muscle might be responsible for early changes that are reflected in further disease. Coleman (1961) linked dietary creatine and serum creatine levels to kidney transamidinase levels and thus indirectly to creatine kinase activity.

Several authors have questioned the relationship between the human and mouse forms of dystrophy. McGeer, McGeer, Miller, Derry and Nichol (1962) stated that the differences noted in creatine and creatinine excretions may point to a basic difference in the diseases. In work on creatine phosphorylkinase content, Read (1962) found that unlike human muscular dystrophy, the dystrophic mouse muscle showed no difference between its control. Golarz and Bourne (1960) stated that human dystrophic muscle showed strong dephosphoralization in connective tissue while dystrophic mouse muscle showed this activity only sporadically in capillary walls. These authors felt that this finding "may mean the two diseases are not comparable."

The fact that it is not possible to question any of the above statements or to regard one as more important than the others, is evidence of our present ignorance of the exact nature of muscular dystrophy.
III. STATEMENT OF THE PROBLEM

From the above data, several facts appear to point to the presence of a biochemical lesion in the carbohydrate metabolism. Baker, Tubis and Blahd (1958) reported that plasma glucose was at a normal level in the dystrophic mice. Rabinowitz (1959) stated that glucose-C\textsuperscript{14} was oxidized at the same rate in dystrophic and normal mice. The rate, as well as the route, of glucose metabolism is, of course, controlled by enzyme activity. Glucose-6-phosphate dehydrogenase was at an increased activity in dystrophic muscle (McCaman, 1960), while phosphoglucomutase was at a decreased activity, due to the loss of its coenzyme, glucose-1, 6 diphosphate, according to Hazzard and Leonard (1959). Earlier, Leonard (1957) reported that the "active" and "total" phosphorylase activities were significantly less than normal, but that the phosphorylase activity ratios of dystrophic skeletal muscle were normal. The activity of aldolase in dystrophic muscle was normal when based on non-collagen protein nitrogen (Weinstock, Epstein and Milhorat, 1958). It can be seen that glucose metabolism is "pointed" toward its being utilized for energy, rather than storage. McCaman (1960) reported that alpha-glycerol-phosphate dehydrogenase was at a decreased activity, as was lactic dehydrogenase. This latter enzyme is of importance in anaerobic glycolysis, and its decreased activity would seem to indicate that dystrophic muscle relies mainly on aerobic glycolysis.

A number of enzymes important in aerobic glycolysis have been studied. Rosenkrantz and Laferte (1960) found no significant increase in the activity of isocitric dehydrogenase by their technique. However,
using another method, McCaman (1960) did report an increase in the activity of this enzyme. Rosenkrantz and Laferte (1960) reported that alpha-ketoglutaric acid dehydrogenase activity was not significantly varied in dystrophic muscle, nor was the activity of succinic dehydrogenase altered. The activity of muscle succinoxidase was not affected, according to Weinstock, Epstein and Milhorat (1958). Malic dehydrogenase did not vary in muscles of dystrophic mice from that activity of normal muscle (Rosenkrantz and Laferte, 1960). Zymaris, Epstein, Saifer, Arousan and Volk (1959) reported on the muscle content (dystrophic and normal mice) of many of the coenzymes and cofactors necessary for metabolism. They found that dystrophic muscle had quantitatively higher amounts of adenosine monophosphate, guanosine diphosphate and guanosine triphosphate than did normal muscle. However, the dystrophic muscles were quantitatively lower in their content of adenosine triphosphate, the substance thought to provide immediate energy for work by all cells of the body. These above mentioned enzymes and cofactors all point to the fact that the tricarboxylic acid cycle is functioning properly, if not at an increased rate. Further evidence of this fact seems to be evident. Cytochrome oxidase is at an increased activity, according to Weinstock, Epstein and Milhorst (1958). This indicates that metabolism is accelerated, as does the reported finding of Rosenkrantz and Laferte (1960) that muscle oxygen uptake is increased. The report that cathepsin activity in muscle is greatly increased (cathepsins are those enzymes responsible for protein breakdown) by Weinstock, Epstein and Milhorat (1958) and that there is an increased muscle protein turnover (Simon, Lessell, Gross and Milhorat, 1958; Coleman
and Ashworth, 1959; Kruh, Dreyfus, Schapira and Gey, 1960) all indicate that carbohydrate metabolism must be increased in order to provide the energy necessary for the increased rate of muscle protein synthesis.

It becomes obvious that if there is an increased carbohydrate metabolism, there must be an increased source of the raw material. Baker, Tubis and Blahd (1958) found that the dystrophic mouse consumes more food per gram of body weight than does its littermate control. However, Bond and Leonard (1958) found that the dystrophic mouse may weigh as much as 33% less than its littermate control. Thus, it is seen, that dietary intake cannot supply the now increased demand for carbohydrate. When proteins break down, they are reverted back to their original form as amino acids. These amino acids, in the course of their metabolism, are first deaminated. Rabinowitz (1959) found that all tissues of the dystrophic mouse have increased transaminase activity. The function of a transaminase is to take the amino group from one compound and place it on another. If these enzymes are at an increased activity, then the deaminated amino acids would be at a greater concentration. These deaminated amino acids may be easily incorporated into the tricarboxylic acid cycle. Thus, with an increased diet and the availability of deaminated amino acids, the tricarboxylic acid cycle would have more substrate available for the production of more energy. Also, because of the increased rate of deamination of amino acids, more urea would be formed from the increased amount of amino groups liberated by the deamination. An increased urea excretion by littermate control and dystrophic mice of Strain 129 had been reported by McGaughey (1960). This would
seem to indicate that there is an increase in amino acid deamination. However, McGaughey (1960) also reported an increase of alpha-ketoglutaric acid in the urine of these same animals. His findings indicated that the dystrophic mouse excreted a significantly higher amount than did the littermate controls, and that this difference increased from weaning to 4 months of age (normal time of death). If the tricarboxylic acid cycle is operating properly, this substrate should not be found in the urine. It could come from several sources. If alpha-ketoglutarate dehydrogenase were at a decreased activity, the increased glucose metabolism would "flood" the enzyme and the excess would spill out into the urine. However, Rosenkrantz and Laferte (1960) reported it to be at normal activity. The kidneys could play a part also in the excretion of alpha-ketoglutaric acid. It is known that deamination of glutamic acid yields alpha-ketoglutaric acid. Glutamic reductase is at an increased activity (McCaman, 1960) as is glutamic acid dehydrogenase (Rosenkrantz and Laferte, 1960). These enzymes then could be responsible for the alpha-ketoglutaric acid excess found in the urine of Strain 129 mice (McGaughey, 1960).

It became the object of this study to determine if alphaketoglutarate dehydrogenase is responsible for this excretion of its substrate.
IV. EXPERIMENTAL METHODS

The mice studied were dystrophic (dydy) and heterozygous "carriers" (Dydy from Strain 129/Re+dy. The notation 129/Re+dy designates the subline of the 129 inbred strain in which the dystrophic (dydy) mutation rose. The mice were obtained from the Russell B. Jackson Memorial Laboratories, Bar Harbor, Maine, and were approximately 2 months when received. They were kept in 12" x 12" wire screen cages in well ventilated, air conditioned animal quarters. They were fed Purina laboratory chow and allowed water ad libitum.

The animals were given an intra-peritoneal injection of sodium pentobarbital (40 mg/kg). The gastrocnemius muscles were quickly excised and placed in a covered weighing bottle set in cracked ice.

Motor end plates were stained using the vital methylene blue technique of Coers and Woolf (1959). The injection of the dye into the un-fixed muscle and the subsequent oxygenation formed a stable dye-lipid complex which selectively deposited the blue dye on the bare nerve endings. Although the exact mechanism of this selectivity is unknown, the procedure constitutes an excellent means of studying gross morphology of nerve terminal arborization.

The Coers and Woolf (1959) histochemical technique for staining cholinesterase was utilized for the study of post-synaptic membranes. This technique was originally outlined by Koelle and Friedenwald (1949) and modified by Couteaux (1951). In this method, cholinesterase, concentrated in the lamellae of the post-synaptic membrane, is complexed by a disulfide bond. This complex appears as fine black granules on the
prepared slide, and thus presents a picture of the post-synaptic membrane.

Cholinesterase activity was determined by the method of Augustinsson (1948). This method measures the carbon dioxide evolved from liberated acetic acid when acetylcholine as a substrate is hydrolyzed by cholinesterase.

The determination of the non-collagen protein nitrogen was made using the technique of Lilenthal, Zierler, Folk, Buka and Riley (1950). The method of Lanni, Dillon and Beard (1950) was used for wet combustion followed by direct nesslerization using the Nessler's reagent of Koch and McMeekin (1924). These methods provided a suitable and accurate reference basis for the comparison of muscles in which one group (dystrophic) differed significantly from the other (littermate control) in fat, collagen and protein content.

Alpha-ketogluterate dehydrogenase activity was determined by the procedure of Kaufman, Gilvary, Cori and Ochoa (1953). The Warburg apparatus was set to oscillate at 90 cycles per minute, with a shaking amplitude of approximately 7 cm. The water bath was maintained at 25°C. This method determined the activity of the enzyme by measuring the release of carbon dioxide from alpha-ketoglutaric acid, according to the metabolic scheme as summarized by Cantarow and Schepartz (1957). The results are expressed as microliters of carbon dioxide released per mg of fat-free, non-collagon protein nitrogen in the tissue sample.

Creatine phosphokinase was isolated by the first two steps of procedure B of Kuby, Noda and Lardy (1954). The enzyme activity was measured using the technique of Read and Nehorayan (1959). Phosphate was deter-
mined by the method of Fiske and Subbarow (1925). This method followed the enzyme activity by measuring the amount of phosphorus enzymatically transferred from ATP to creatine.

Alpha-ketoglutaric acid was measured in the muscle by the following procedure, modified from McGaughey (1960). The gastrocnemius muscles were removed and homogenized in 5 times their weight in distilled water. Two ml of 0.1% 2,4-dinitrophenylhydrazine (DNPH) in 2N HCl were added to the homogenate and allowed to stand for 15 minutes. The homogenate-DNPH mixture was then extracted with 2 ml of ethyl acetate. Two ml of 1N Na₂CO₃ was added to the ethyl acetate extract, the solution acidified with 2N HCl and the Na₂CO₃ extracted with 5 ml of ethyl acetate. The optical density of the ethyl acetate was measured at 480 μm in a spectrophotometer (Bausch and Lomb, Spectronic 505). A reagent blank was run on 1 ml of redistilled water simultaneously with the sample. Standards of 1, 3 and 5 mg of alpha-ketoglutaric acid in 1 ml of redistilled water were extracted in the same manner and similarly analyzed. The method eliminates most of the contaminants and excess DNPH and converts alpha-ketoglutaric acid to the yellow hydrazone salt.

The metabolism of pyruvate was studied in order to determine the efficacy of the tricarboxylic acid cycle. Radioactive labeled pyruvate was utilized. Injections of 0.5 ml of saline containing 10 microcuries of activity of pyruvate-2-Cl⁴ were made into the intraperitoneal cavity of dystrophic and littermate control mice. The mice were isolated and separated in all-glass metabolism cages (Roth, Leifer, Hogness and Langham, 1948) in which urine, food and feces were individually separated. The exhaled carbon dioxide was collected in KOH and BaCl₂. One half of
the 24 hour urine volume was extracted for alpha-ketoglutaric acid as stated above, with the exception that the last ethyl acetate fraction was 2 ml. The other half of the 24 hour urine volume was dried on a planchet. The extracted urine, in the form of the ethyl acetate fraction, was also dried on another planchet. The collected $\text{C}^{14}\text{O}_2$ now in the form of $\text{BaC}^{14}\text{O}_3$ was plated on another planchet (150 mg $\text{BaC}^{14}\text{O}_3$). The planchets were counted in a Geiger tube, model D47.
V. EXPERIMENTAL RESULTS

Motor End Plates

The terminal end plates and subterminal fibers of dystrophic and non-dystrophic littermate control mice are compared in figures 1 and 2. The terminal end plates are stained with methylene blue. This method employs methylene blue as a non-specific stain for the lipoidal substance of nerve terminals and gives information on only the gross morphology of the end plates and branching of the subterminal fibers.

In figure 1 is shown a single motor end plate of non-dystrophic control stained with methylene blue. In longitudinal sections of whole muscles (as in figure 1) the motor end plates are concentrated in narrow zones (innervation terminal band) situated at the mid-point of the muscle (Coers, 1953). Figure 1 represents such a motor end plate. It has been selected because it represents a typical end plate found in mice skeletal muscle. It can be seen that the subterminal nerve fibers (black wavy lines

Figure 1. Motor-End Plate From A Littermate Control Gastrocnemius Muscle. Methylene Blue Technique. 1150X
in upper right of figure 1) become increasingly separated from one another as they proceed distally. They weave in front of or behind the muscle fibers, crossing them approximately perpendicular. The terminal fibers run only a short course, ending in a terminal arborization on the surface of a muscle fiber. The terminal arborization, in methylene blue preparations, show some branching with swellings on the branches. The branches end, not in a tapered point, but in a terminal enlargement. Functionally, these endings make contact with the post-synaptic membrane, a specialized portion of the muscle membrane.

In figure 2 is shown a single motor end plate on a muscle fiber taken from a dystrophic mouse. While it is somewhat smaller and exhibits less branching than does the motor end plate from the normal mouse, it is still within normal limits. The terminal arborization shows branching,

Figure 2. Motor-End Plate From A Dystrophic Gastrocnemius Muscle. Methylene Blue Technique. 1150X

with swellings on these branches. Adams, Denny-Brown and Pearson (1953) described motor end plates in human dystrophic muscle. They found that these structures were smaller and some showed "clubbing"
of the entire unit. Figure 2 has no such "clubbing" but it is smaller and appears less developed than the normal unit. These differences that are noted are not of a significant nature. They represent functional changes due to the disease present in the muscle cell. Adams, Denny-Brown and Pearson (1953) ascribed the differences noted in human muscles to the fact that the muscle fiber which the unit innervates has undergone degeneration, and perhaps complete dissolution. The nerves to these fibers undergo atrophy more slowly, and thus they are seen to be smaller and less developed.

Post-Synaptic Membranes

The post-synaptic membrane taken from the skeletal muscle of a littermate control mouse is shown in figure 3. It is typical of the appearance of these structures as found in these mice and stained with this procedure (Augustinsson, 1946). The black precipitate is located within the lamellae of the membrane and thus presents a picture of the

Figure 3. Post-Synaptic Membrane From A Littermate Control Gastrocnemius Muscle. Cholinesterase Stain. Koelle & Friedenwald (1949) Technique. 1150X
structure. This specialized area in the muscle membrane is functionally able to receive nerve impulses from the nerve terminals which make functional contact with this membrane. The post-synaptic membrane follows the pattern of the motor end plate, as shown in figure 3.

The post-synaptic membrane of the dystrophic mouse skeletal muscle is seen in figure 4. The thickness of the sections necessary for this staining procedure make it difficult to obtain photographs. The membrane may be situated at a slight angle to the objective of the microscope. Thus, the examiner must focus in several points along the membrane in order to study the structure in its entirety. However, the camera cannot change focus in this manner and the resulting photograph is a composite view of the object being viewed. This is seen in figure 4. This figure was included because it showed typical morphology when seen under the microscope. Microscopically, the post-synaptic membranes conformed closely to the morphology of their respective motor end plates and there appeared to be no significant

Figure 4. Post-Synaptic Membrane From A Dystrophic Gastrocnemius Muscle. Cholinesterase Stains. Koelle & Friedenwald (1949) Technique. 1150X
gross difference between dystrophic muscle and littermate controls.

Quantitative Cholinesterase Determination

The histochemical demonstration of cholinesterase by the Koelle and Friedenwald technique cannot be considered as quantitative. In a series of slides prepared for demonstration of the post-synaptic membranes by this method, the dystrophic animals gave a faint staining reaction when compared to the bold staining reaction of the littermate controls. It was therefore decided to determine the cholinesterase activity in a quantative manner. Cholinesterase was determined by the method of Augustinsson (1948). The results are shown in table 1. The test is a measurement of the carbon dioxide evolved from liberated acetic acid when acetylcholine as a substrate.

Table 1.

Quantitative Cholinesterase Determination of Skeletal Muscle of Dystrophic and Littermate Control Mice

<table>
<thead>
<tr>
<th>Animal</th>
<th>Microliters of CO₂ released/mg fat-free, non-collagen nitrogen/40 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>LITTERMATE CONTROL</td>
<td>DYSTROPHIC</td>
</tr>
<tr>
<td>(1)</td>
<td>32.5</td>
</tr>
<tr>
<td>(2)</td>
<td>34.1</td>
</tr>
<tr>
<td>(3)</td>
<td>35.5</td>
</tr>
<tr>
<td>(4)</td>
<td>33.7</td>
</tr>
<tr>
<td>Average</td>
<td>33.95±0.62*</td>
</tr>
</tbody>
</table>

\[ \sqrt{\frac{\xi_d^2}{n(n-1)}} \]

is hydrolyzed by cholinesterase. The volume of carbon dioxide (in microliters) is then referred to milligrams of non-collagen nitrogen as a reference base. The experiment was run for 40 minutes. The results show that there is no significant difference between control and dystrophic animals. Thus,
the difference noted in the staining intensity between the two groups may have been due to the fact that the smaller dystrophic muscle simply did not contain as much cholinesterase per unit area and therefore gave a less intense staining reaction.

Fat-Free, Non-Collagen Protein Nitrogen

It is known that there is an increase in fat in the dystrophic muscles (West and Murphy, 1960). Therefore, the wet weight as a reference base would not be satisfactory for quantitative work because the two muscle groups differ in their fat content and any comparison on this basis would not reflect a true quantitative comparative value. The collagen content is also increased in dystrophic muscle (West and Murphy, 1960) and therefore the fat-free, dry weight base is also inadequate for the same reason as given above. Lilenthal, Zierler, Folk, Buka and Riley (1950) have shown the validity of the fat-free, non-collagen protein nitrogen as a quantitative reference base. Therefore, the fat-free, non-collagen protein nitrogen was selected as a reference base for the comparison between the two muscle groups. Table 2 shows the variation in the fat-free, non-collagen protein nitrogen content of skeletal muscle of dystrophic and littermate control mice. The dry weight of both groups were the same, averaging 23-24% of the wet weight. However, the fat content of the dystrophic muscles was 9% as compared to 4% in the littermate control muscles. These results then, seem to agree with the work of West and Murphy (1960), who reported histological evidence of fatty replacement in dystrophic muscle. Weinstock, Epstein and Milhorat (1958) found 18.9 mg of non-collagen nitrogen in littermate control muscle and 16.3 mg in dystrophic muscle. The results expressed in table 2 are in
good agreement with their results. The reduction of protein nitrogen in Table 2.

The Fat-Gree, Non-Collagen Protein Nitrogen Content of Dystrophic and Littermate Control Mice Skeletal Muscle

<table>
<thead>
<tr>
<th>Littermate Control (1)</th>
<th>Dry Wgt. % of Wet.</th>
<th>Fat-Free Dry Wgt. % of Wgt.</th>
<th>Non-collagen nitrogen mg/gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2)</td>
<td>24</td>
<td>21</td>
<td>20.2</td>
</tr>
<tr>
<td>(3)</td>
<td>23</td>
<td>19</td>
<td>19.4</td>
</tr>
<tr>
<td>(4)</td>
<td>22</td>
<td>22</td>
<td>16.6</td>
</tr>
<tr>
<td>(5)</td>
<td>26</td>
<td>19</td>
<td>18.3</td>
</tr>
<tr>
<td>(6)</td>
<td>23</td>
<td>19</td>
<td>16.4</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>23.8±0.48</strong></td>
<td><strong>19.8±0.54</strong></td>
<td><strong>18.3±0.62</strong></td>
</tr>
</tbody>
</table>

| Dystrophic (1)         | 23                  | 14                         | 17.8                        |
| (2)                    | 23                  | 15                         | 14.4                        |
| (3)                    | 23                  | 17                         | 13.7                        |
| (4)                    | 22                  | 14                         | 16.3                        |
| (5)                    | 23                  | 10                         | 15.4                        |
| (6)                    | 23                  | 14                         | 14.8                        |
| **Average**            | **22.8±0.17**       | **14.0±0.85**              | **15.4±0.16**               |

\[ \sqrt{\frac{\sum d^2}{n(n-1)}} \]

dystrophic muscle is approximately 15%, which is significant. Therefore, because the non-collagen nitrogen is reduced in dystrophic muscle, it is necessary to recognize this fact when comparing dystrophic to littermate control muscle. Thus, the fat-free, non-collagen protein nitrogen reference base provides a suitable and reasonably accurate reference base for comparing dystrophic and littermate control muscle.

Creatine Phosphokinase

The results are presented in table 3. It can be seen that dystrophic and littermate control muscles do not vary significantly in their levels.
of activity. Thus, the dystrophic muscle is able to store high-energy phosphate bonds as readily as the littermate control muscle.

Table 3.

Creatine Phosphokinase Activity of Skeletal Muscle of Dystrophic and Littermate Control Mice

<table>
<thead>
<tr>
<th>Littermate Control</th>
<th>Dystrophic</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu \text{N P transferred from ATP to creatine/gm fat-free, non-collagen protein nitrogen/2-1/2 minutes} )</td>
<td>( \mu \text{N P transferred from ATP to creatine/gm fat-free, non-collagen protein nitrogen/2-1/2 minutes} )</td>
</tr>
<tr>
<td>Animal (1)</td>
<td>12.3</td>
</tr>
<tr>
<td>(2)</td>
<td>10.0</td>
</tr>
<tr>
<td>(3)</td>
<td>10.4</td>
</tr>
<tr>
<td>(4)</td>
<td>9.2</td>
</tr>
<tr>
<td>Average</td>
<td>10.5( \pm )1.1*</td>
</tr>
</tbody>
</table>

* \( \sqrt{\frac{\sum d^2}{n(n-1)}} \)

Alpha-Ketoglutarate Content of Muscle

McGaughey (1960) reported that both dystrophic and littermate control of Strain 129 mice excreted more alpha-ketoglutarate and urea in their urine than did other strains of mice. Before approaching an enzymatic study, it seemed desirable to check the content of skeletal muscle as a means of determining if simple accumulation of alpha-ketoglutarate in muscle could account for the increased urinary excretion.

The alpha-ketoglutarate content of the skeletal muscle of dystrophic and littermate control animals is shown in table 4. The results are expressed as mg of alpha-ketoglutarate per gram of fat-free, non-collagen nitrogen.
Table 4.

Alpha-Ketoglutarate Content of Skeletal Muscle from Dystrophic, Littermate Control Mice and Normal White Mice

<table>
<thead>
<tr>
<th></th>
<th>Littermate Control</th>
<th>Dystrophic</th>
<th>Normal White</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg alpha-ketoglutarate/gm fat-free, non-collagen nitrogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td>150</td>
<td>(1) 0</td>
<td>(1) 160</td>
</tr>
<tr>
<td>(2)</td>
<td>110</td>
<td>(2) 0</td>
<td></td>
</tr>
</tbody>
</table>

The alpha-ketoglutarate in the littermate control averaged 130 mg/gm fat-free, non-collagen nitrogen while a normal white mouse gave a figure of 160, which is well within the range of the littermate control. The dystrophic muscle had no alpha-ketoglutarate, at least it was below the minimum detectable by the test procedure. As the purpose of the test was to determine if a significant increase in muscle alpha-ketoglutarate could account for the increased urinary excretion, it was felt that a limited number of animals would answer this question. The inability to detect alpha-ketoglutarate in dystrophic muscle would indicate against the concept that the alpha-ketoglutarate found in the urine is a result of this compound accumulating within the substance of dystrophic muscles.

Alpha-Ketoglutarate Dehydrogenase

In a further search for the biochemical lesion responsible for the increased urinary excretion of alpha-ketoglutarate, the alpha-ketoglutarate dehydrogenase activity was measured. This enzyme is responsible for the first step in the metabolism of alpha-ketoglutarate. A reduction in the enzyme level would result in a lowered ability to metabolize alpha-ketoglutarate and result in its excretion.
The alpha-ketoglutarate dehydrogenase activity of skeletal muscle from dystrophic, littermate controls and normal white mice is shown in Table 5. The normal white mice are included to record a strain difference in the mice. The results show that the littermate control mice have a dehydrogenase activity of 6.05 microliters per mg fat-free, non-collagen protein, while the dystrophic muscle gave a figure 65% above this. The difference of the two means is statistically significant \((P \leq 0.01)\). These results are consistent with the low alphaketoglutarate content of dystrophic muscle, whereby the high level of enzymatic activity is sufficient to keep the alpha-ketoglutarate content low. It is also consistent with the adequate and normal level, when compared to normal white mice, of alpha-ketoglutarate content of the littermate control skeletal muscle. The depressed alpha-ketoglutarate dehydrogenase activity of littermate controls, 6.05 as compared to 8.42 for

<table>
<thead>
<tr>
<th></th>
<th>Littermate Control</th>
<th>Dystrophic</th>
<th>Normal White</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{\text{CO}_2}{\text{gim}} ) released per gm fat-free, non-collagen nitrogen per 4 minutes.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal (1)</td>
<td>5.87</td>
<td>10.61**</td>
<td>8.43</td>
</tr>
<tr>
<td>(2)</td>
<td>4.82</td>
<td>11.38</td>
<td>8.22</td>
</tr>
<tr>
<td>(3)</td>
<td>5.87</td>
<td>9.09</td>
<td>8.82</td>
</tr>
<tr>
<td>(4)</td>
<td>7.63</td>
<td>9.09</td>
<td>8.22</td>
</tr>
<tr>
<td>Average</td>
<td>6.05±0.62*</td>
<td>10.04±0.57</td>
<td>8.42±0.14</td>
</tr>
</tbody>
</table>

* \( \sqrt{\frac{\xi_{d^2}}{n(n-1)}} \)

** Each run represents tissue pooled from 2 animals

Each animal represents tissue pooled from 2 animals.
normal white mice, at least is consistent with the alpha-ketoglutarate content of these muscles.

Pyruvate-$2-C^{14}$ Metabolism

The results of this study are shown in table 6. To check the possibility of the tricarboxylic acid cycle as a possible source of the excess alpha-ketoglutarate found in the urine, the metabolism of radio-active pyruvate was studied. It was felt that there was sufficient evidence that the tricarboxylic acid cycle was functioning and therefore, even a very limited study would be sufficient to clarify this point. Both the dystrophic and littermate control mice were able to metabolize pyruvate. Radio-active labeled carbon dioxide was detected in the exhaled air of these animals. The urine revealed the presence of $C^{14}$, of which a part was in the form of alpha-ketoglutaric acid. These results may be interpreted

Table 6.

Pyruvate-$2-C^{14}$ Metabolism of Dystrophic and Littermate Control Mice

<table>
<thead>
<tr>
<th></th>
<th>Urine Counts per Minute</th>
<th>Exhaled CO$_2$***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated*</td>
<td>Extracted**</td>
</tr>
<tr>
<td>Littermate Control*</td>
<td>18,191</td>
<td>4,060</td>
</tr>
<tr>
<td>Dystrophic</td>
<td>12,418</td>
<td>2,630</td>
</tr>
</tbody>
</table>

* Both groups received 10 microcuries of $C^{14}$ labeled pyruvate
* One-half of the 24 hour urine volume
** The ethyl acetate fraction of the extracted 24 hour urine volume
*** Counted as 150 mg of BaC$_{14}$O$_3$

as showing that a part of the alpha-ketoglutaric acid found in the urine of Strain 129 mice is originating from pyruvate metabolism. As pyruvate
is a product of glucose metabolism, it may be further postulated that glucose metabolism contributes to the alpha-ketoglutaric acid found in the urine of these animals. It is also possible that rapid conversion of alanine to pyruvate is the pathway to the excess alpha-ketoglutarate.
VI. DISCUSSION

Michelson, Russell and Harman (1955) reported that there was an absence of apparent lesions in the nervous system of the dystrophic mice. They did not, however, include studies on the motor end plated and post-synaptic membranes. The present report is consistent with the view that the muscular dystrophy of mice is a disease of muscle and does not involve the nerve terminals.

A consistent observation in all studies of muscular dystrophy is the increased excretion of urinary creatine, decreased excretion of creatinine and a decreased ability to retain injected creatine (Milhorat and Wolff, 1937). The work of Benedict, Kalinsky, Scarrone, Wertheim and Stetten (1955) indicated, at least in human patients, that no defect existed in creatine synthesis but that the excess urinary creatine was due to an inability of creatine to enter the muscle cell. Heinrich and Mattill (1949) concluded that the increased urinary excretion of creatine of vitamin E-deficient rabbits was due to defective phosphorylation of creatine. Hummel (1948) agreed with the latter viewpoint, for, in his experiments with tissue homogenates, skeletal muscle from vitamin E-deficient rabbits had a low ability to phosphorylate creatine. It had been reported that muscle creatine is the precursor of urinary creatinine (Block, Shoenheimer and Rittenberg, 1941), a reaction which proceeds through a preliminary phosphorylation of creatine within the muscle cell (Ennor, Rosenberg and Armstrong, 1955). The low activity of the enzyme, creatine phosphorylkinase, was interpreted to mean that the non-phosphorylated creatine was unable to be transferred into the muscle cell and hence
was excreted. However, Dinning and Fitch (1958) showed that the turnover rate of creatine was elevated in skeletal muscle of vitamin E-deficient rabbits. This implied that, rather than not being able to penetrate the muscle cell, the creatine molecule was able to penetrate at even a greater than normal rate. Read and Nehorayan (1959) showed that in early vitamin E-deficient rabbits, at a time when the urinary creatine excretion was rising, the enzyme, creatine phosphorylkinase, was higher than normal. The latter authors then rejected defective phosphorylation as causing the urinary excretion, in conformity to the results of Dinning and Fitch (1958).

The above discussion concerning vitamin E-deficient animals is not intended to imply a similarity between the vitamin deficiency and muscular dystrophy. It is intended to call attention to hypotheses concerning the origin of the increased urinary creatine.

To carry the analysis further, Feuer and Wollemann (1952) reported that creatine was bound to the actin molecule. They further suggested that creatine phosphorylkinase, bound to actin, was necessary for actin polymerization. Strohman (1959) recently reported that the creatine phosphorylkinase system is intimately concerned in actin polymerization by reversible phosphorylation-dephosphorylation of the actin bound nucleotide. Read (1960) submitted tentative evidence for the participation of creatine in actin polymerization which suggested that creatine was bound to actin and participated in actin polymerization by serving as the phosphate acceptor from actin bound nucleotide during polymerization.

This reaction is enzymatically catalyzed by creatine phosphorylkinase. Hence, by inference, deficiency of creatine phosphorylkinase, leads to a deficiency of actin polymerization and an inability to contract. Indeed,
Read and Johnson (1959) reported data on foetal rabbits, which showed that, in skeletal muscle, the appearance of the enzyme, creatine phosphorylkinase, occurs coincident in time of foetal development with the appearance of contractility. Hence, in any study of the specific biochemical lesion of muscular dystrophy, the demonstration of depressed activity of creatine phosphorylkinase is of importance and could explain most of the symptoms. Pertinent to the present discussion, is the report of Ronzoni, Wald, Berg and Ramsey (1958) who stated that the creatine kinase activity of skeletal muscle of human muscular dystrophy is only 20 to 40 percent of normal control muscle. This would indeed suggest at least the possibility of deficient phosphorylation as the cause of the inability of muscle to incorporate and retain creatine and explain many of the dystrophic symptoms. While some criticism of this work can be made on the grounds of a rather crude enzyme preparation, the necessity of excessively small samples from humans precludes extensive purification. Their data is suggestive and should be explored further. The data reported in the present work indicates that the enzyme levels of dystrophic and normal mouse muscle do not differ. This discrepancy between human and mouse dystrophic muscle is important to resolve, for it may indicate a fundamental difference between human and mouse dystrophy.

It is readily apparent that the activity of alpha-ketoglutarate dehydrogenase is greatly increased in the skeletal muscle of dystrophic mice. This increase in activity may be due to an increase in the amount of alpha-ketoglutaric acid "delivered" to the enzyme for metabolism.
Protein anabolism and catabolism are increased in the dystrophic mice (Simon, Lessell, Gross and Milhorat, 1958; Coleman and Ashworth, 1959; Kruh, Dreyfus, Schapira and Gey, 1960). The amino acids thus liberated from protein breakdown will be available for re-synthesis, or may be metabolized. Deamination is the first step in the metabolism of amino acids. It has been reported (Rubinowitz, 1959) that the ratio of glutamic-oxalacetic transaminase to glutamic-pyruvate transaminase is increased in all tissues of the dystrophic mice, with the sera showing the greatest difference. Chowdhury, Pearson, Fowler and Griffith (1960) have reported increased serum transaminase levels in human patients with progressive muscular dystrophy. Thus, if the transaminases are increased in this disease, it can be seen that the deamination of the now increased production of amino acids from the increased protein breakdown will result in more substrate for the tricarboxylic acid cycle and an increase in amino groups. These amino groups may then be transferred to alpha-ketoglutaric acid forming glutamic acid. As glutamic acid dehydrogenase activity is increased in dystrophic muscle (Rosenkrantz, 1959) this new load of amino groups can be passed to the ornithine-citrulline-urea cycle without "backing up" at the glutamic acid site. If this portion of protein metabolism is functioning at an increased activity, it can be seen that the high amounts of urea found in the urine of the dystrophic mice (McGaughhey, 1960) is an expected finding.

When glutamic acid passes its amino group to the urea cycle, alpha-ketoglutaric acid is regenerated and made available for further transamination of amino acids, and the eventual production of urea. An increase in oxygen
consumption in dystrophic muscle has been reported by Rosenkrantz and Laferte (1960); cytochrome oxidase is reportedly increased, also (Weinstock, Epstein and Milhorat, 1958). These increases may be interpreted as indicating an overall increase in the activity of the tricarboxylic acid cycle. Thus, additional alpha-ketoglutaric acid may be formed to help in the deamination of amino acids. However, McGaughey (1960) reported an increase in alpha-ketoglutaric acid in the urine of Strain 129 mice, with the dystrophic mice having a significantly higher excretion than the normals. This study has shown that alpha-ketoglutarate dehydrogenase is at an increased activity in the dystrophic leg muscles. This finding, then, does not explain the data of McGaughey (1960). The preliminary study in this paper seems to indicate that pyruvate metabolism through the tricarboxylic acid cycle to alpha-ketoglutaric acid is functioning, and that a portion of the alpha-ketoglutaric acid found in the urine of the dystrophic mice may come by way of this route. In view of these findings, it appears as if an excess of alpha-ketoglutaric acid is formed through the increased protein breakdown, resulting in increased amino acids (in particular, glutamic acid which may be deaminated by glutamic acid dehydrogenase and thereby directly form alpha-ketoglutaric acid) and an increased activity of the tricarboxylic acid cycle. This excess production, resulting in the presence of alpha-ketoglutaric acid in the urine of Strain 129 mice, may then exceed the metabolism of alpha-ketoglutaric acid by the tricarboxylic acid cycle, or it may be a function of the kidneys, or a combination of these two factors.

The littermate control mice present somewhat of a different problem. This strain of mice presents two distinct genetic groups, represented as 129/Re+ly subline and 129/J subline (communication from Russell B. Jackson

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Memorial Laboratory, Bar Harbor, Maine). The 129/J subline is referred to as DyDy (homozygous normal controls) in which dystrophy has not been reported. The subline 129/Re dy has the given genetic description of Dydy (heterozygote, or littermate control), Dy? (control) and dydy (dystrophic). The animals used as controls in this study were of the Dydy and Dy? groups. It is apparent that these animals may have a predisposition toward dystrophy and that their label of "normal" is based on the absence of clinical signs of the disease. Thus, though they differ from normal white mice and the dystrophic mice activity levels, an attempt to say that the difference is of etiological importance is rather presumptuous. However, this fact cannot be completely disregarded. The explanation, of the lowered activity in these animals, must await more refined techniques in the correlation of disease with heredity.
VII. SUMMARY

1. Gastrocnemius muscles of dystrophic mice present essentially the same pattern as normals in regard to motor end plates and postsynaptic membranes.

2. Alpha-ketoglutarate dehydrogenase activity is increased in dystrophic leg muscle as compared to littermate controls. Normal white mice leg muscles have an activity that is intermediate between littermate control and dystrophic leg muscles. This increase in dystrophic muscle may be expected, due to an increase in activity of the tricarboxylic acid cycle and an increase in protein breakdown.

3. Creatine phosphokinase activity does not vary significantly between dystrophic leg muscle and littermate control leg muscle.
BIBLIOGRAPHY


