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Biochemical Studies Related To Lipid Metabolism In Muscular Dystrophy

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BIOCHEMICAL STUDIES RELATED TO LIPID METABOLISM IN MUSCULAR DYSTROPHY

by

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ABSTRACT

The incorporation of radioactivity from palmitate-1-^{14}C into the expired CO_2 and into the fatty acid and cholesterol from liver, kidney, brain and muscle of normal and dystrophic mice Bar Harbour strain 129) was determined 60 minutes after intraperitoneal injection of the isotope. A decreased incorporation into the cholesterol from kidney, liver and muscle, together with an increased incorporation into the fatty acids from muscle and a reduction into the expired CO_2 were observed in the dystrophic animals. Similar experiments carried out using glucose-U-^{14}C as isotope showed (as the most significant change) an increased incorporation of radioactivity into the neutral lipids of muscle. The increase was seen in both the fatty acid and glycerol moieties. No differences were observed in the phospholipid content of muscle or in the radioactivity incorporated into this fraction.

The activity of the hexose-monophosphate shunt for the metabolism of glucose was determined using, as enzyme source, high-speed (90,000 x g) supernatant obtained from muscle homogenates of both normal and dystrophic mice. In separate experiments the fprmation of pentoses and $^{14}CO_2$ from glucose-U-^{14}C and the utilization of ribose-5-phosphate were studied. In the former experiments the use of phenazine methosulfate stimulated the reaction nine-fold. Both metabolic pathways were found to be elevated in dystrophic animals.

The metabolism of triglyceride, (both anabolic and catabolic), was studied in homogenates obtained from normal and dystrophic mice. The incorporation of palmitate-1-^{14}C was found to be increased in the dystrophic muscle but no differences were observed using sn-^{14}C-glycerol-3-phosphate as substrate. The cofactor requirements were found to be different for both substrates: the former is stimulated by glucose, NAD and inhibited by F^- (1.2×10^{-3} M), while the latter is not affected by these compounds. The results suggested that the incorporation of sn-^{14}C-glycerol-3-phosphate may represent the de novo synthesis of triglyceride while the incorporation of palmitate-1-^{14}C may be more indicative of exchange reactions between the fatty acids of the preformed triglyceride, with the exchanges being mediated by action of lipases. The hydrolysis of triglycerides by muscle homogenates was studied manometrically or by an isotopic method, using as substrates, tributyrin or tripalmitin, respectively. The enzyme systems responsible for the hydrolysis of one or other of these substrates appear to be different. Thus, the "long-chain triglyceride lipase" was released by treatment of muscle homogenates with the detergent "Cutscum". The enzyme showed a maximum of activity at pH 6.8, was not stimulated by epinephrine, Na-taurocholate or albumin and was inhibited by F^-, DTNB, EDTA and N-ethyl-maleimide. On the other hand the short-chain triglyceride lipase was inhibited by F^- and Mg^{++} and not affected by eserine, Nu 683, Nu 1250, EDTA or N-ethyl-maleimide.

The analysis of the lipids from muscle of normal and dystrophic muscle showed an increase in triglyceride and free fatty acids in the affected muscle, but no differences in the total phospholipid

content. Analysis of the fatty acid composition of different lipid fractions was made by gas-liquid chromatography. No differences were observed in the fatty acid distribution of the triglycerides, phospholipids or free fatty acid pool.

The activities of different enzymes have been determined in isolated muscle mitochondria and muscle homogenates from both normal and dystrophic mice. In mitochondria no differences were found for the enzymes of the tricarboxylic acid cycle or for the enzymatic systems of the electron transport chain but a reduction of about 35% was found for the enzyme, carnitine acetyltransferase, in dystrophic mice together with an increased activity for monoaminoxidase. The latter enzyme was also found to be increased in the isolated "microsomal" fraction (pellet obtained after centrifugation at 90,000 x g for 100 minutes) and in the whole homogenate. Succinate dehydrogenase was found to be elevated in homogenates from muscle of dystrophic mice, indicating an increased mitochondrial content.

The most relevant finding was a reduction of about 60% in the intramitochondrial pool of acid-soluble CoA (free CoA plus acetyl CoA) in the isolated muscle mitochondria from dystrophic mice. No differences were found for the carnitine content between normal and dystrophic muscle.

The activities of monoaminoxidase and short-chain triglyceride lipase were determined in muscle from mice undergoing atrophy after denervation. Monoaminoxidase remained unaltered in activity up to the 14th day after denervation. Lipase activity increased by 30% one day after denervation, and reached 175% after 7 days.

Cytochrome oxidase, monoaminoxidase and short-chain triglyceride lipase were also determined in muscle biopsies obtained from patients with different myopathies and in some normal controls. Decreases in the values of monoaminoxidase were found in 8 patients affected with Duchenne Muscular Dystrophy, in two with congenital myopathia and in two with limb-girdle muscular dystrophy, but normal values were observed in two patients with a myopathy of late onset and in one carrier of Duchenne Muscular Dystrophy. No differences in the activity of cytochrome oxidase were observed. In regard to short-chain triglyceride lipase some tendency towards an increased activity was seen in the cases of Duchenne facioscapulohumeral and limb-girdle MD and congenital myopathia and normal values were obtained in one carrier of Duchenne MD and in two patients with myopathy of late onset.

From the results obtained it seems that one of the most logical explanations for the phenomenon of lipid accumulation in dystrophic muscle is that of a decreased utilization of fatty acid by muscle mitochondria. It was concluded that the reduced content of CoA observed in mitochondria from dystrophic muscle could contribute to or be the primary factor responsible for such a deficient utilization of fatty acids.

TO MY MOTHER AND THE MEMORY OF MY FATHER

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"...Esta faena es la ciencia; como se ve consiste en dos operaciones distintas. Una puramente imaginativa, creadora, que el hombre pone de su propia y libérrima substancia; otra confrontadora con lo que no es el hombre, con lo que le rodea, con los hechos, con los datos. La realidad no es dato, algo dado, regaladosino que es construcción que el hombre hace con el material dado...."

J. ORTEGA Y GASSET

(In: <u>En torno a Galileo</u>)

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GLOSSARY

ADP	=	adenosine-5'-diphosphate
ATP	=	adenosine-5'-triphosphate
ATPase	=	adenosine triphosphatase
CoA	=	coenzyme A
CAT	=	carnitine acetyltransferase
CP	=	creatine phosphate
CPT	=	carnitine palmityltransferase
DNA	=	deoxyribonucleic acid
EDTA	=	ethylenediaminetetraacetic acid
EM	=	electron microscopy
g	=	acceleration due to gravity
NADP	=	nicotinamide adenine dinucleotide phosphate
NADPH	=	reduced NADP
P	=	probability of an event due to chance alone
PMD	=	progressive muscular dystrophy
PPO	=	2,5-diphenyloxazole
POPOP	=	1,4-bis-2-(5-phenyloxazolyl)-Benzene
S.D.	=	standard deviation
S.E.M.	=	standard error of estimation of mean values
Tris	=	2-amino-2-hydroxymethylpropane-1,3-diol
t	=	Student's test
μc	=	microcurie
μg	=	microgram

CHAPTER I

INTRODUCTION

The Muscular Dystrophies are defined as a group of genetically determined, progressive myopathic diseases. From the time of Duchenne, who first described this disease over a century ago (1, 2) an enormous amount of research has been carried out in order to elucidate its cause and pathogenesis. Unfortunately, most of the data obtained, rather than helping, have often cast further doubts on the definition of the disease in respect to its myopathic characteristics. At present it is still not clear whether the muscular dystrophies are a group of primary muscular diseases or if the muscle alterations are secondary to defects elsewhere.

Of the many investigations undertaken a number of observations are of practical interest. These include the increases noted in certain serum enzymes of muscular origin and the accumulation of fat in the dystrophic muscle. Whether any interrelationship exists between these changes is uncertain. However, studies from this laboratory have begun to provide some insight into the problem of fat accumulation. Recently Lin et al. (135) have shown that muscle mitochondria isolated from dystrophic mice exhibit a decreased capacity to oxidize palmitic-1-^{14}C acid.

Because of these findings several aspects of fatty acid metabolism have been investigated using dystrophic mice and their litter-

mates controls (Strain 129), Jackson Memorial Laboratory). Initially, in vivo studies were carried out in order to elucidate the metabolism of palmitic-1-^{14}C acid and glucose-U-^{14}C. Radioactivity incorporated into the lipids of brain, kidney, liver and muscle and into the expired CO_2 were determined and a comparison was made between the normal and the myopathic animal.

Since the main bulk of fat accumulated in the muscle from dystrophic patients or animals is in the form of triglyceride, its synthesis and hydrolysis have been studied in vitro, using muscle homogenates. Synthesis has been determined by observing the incorporation of palmitic-1-^{14}C acid and sn-^{14}C-glycerol-3-phosphate into both triglycerides and phospholipids. At the same time the activities of two types of triglyceride lipase were studied, the first using tributyrin as substrate and the second using tripalmitin.

The hexose-monophosphate shunt for glucose metabolism has been evaluated mainly because of its potential relationship to the fatty acid synthesis pathway as a source of NADPH. In this study the formation of pentoses and $^{14}CO_2$ from glucose-U-^{14}C, when incubated with a high-speed supernatant of muscle homogenate were determined, as well as the disappearance of ribose-5-phosphate from the medium.

Studies carried out in this laboratory during the past several years have concentrated on analyzing in more detail the metabolism of palmitic-1-^{14}C acid by muscle mitochondria isolated from dystrophic muscle in order to localize the primary defect responsible for its low utilization. As a result of these studies it was found that the defect mentioned above is not specific for palmitic-1-^{14}C acid since similar decreases also occurred for pyruvate (135) and acetylcarnitine (253).

Because of these findings the main emphasis in our work has been the investigation of several factors that are common to the metabolism of the above three substrates. In this respect, several enzymes have been studied in isolated muscle mitochondria from normal and dystrophic mice. These include the determination of the activities of the enzymes of the tricarboxylic acid cycle, the electron transport chain and acetylcarnitine transferase. In addition the intramitochondrial pool of acid-soluble CoA (acetyl CoA plus free CoA) has been determined. At the same time determinations of carnitine in homogenates of muscle have been carried out.

Some of the techniques used in the work carried out on mice were also applied to the study of some enzymes from muscle of patients with different types of myopathy, and also in the muscle of mice undergoing atrophy after denervation. These enzymes include short-chain triglyceride lipase, monoaminoxidase and cytochrome oxidase activities in homogenates of the denervated muscle and non-denervated contralateral muscle.

CHAPTER II

REVIEW OF LITERATURE

A. Muscular Dystrophy - Definition of the Disease

In 1868 Duchenne (1, 2) described a muscle disease that was characterized by weakness of movements which gradually worsened and spread in an ascending way over the affected muscles. An increase in volume of all, or part, of the affected muscles was associated with these changes. Duchenne proposed to call this disease "Pseudohypertrophic Muscular Paralysis". With the help of the "histological punch", a forerunner of the more modern methods of muscle biopsy, Duchenne performed an histological study of the affected muscles and concluded that the fundamental lesion in the muscle is hyperplasia of the interstitial connective tissue with the development of numerous fatty vesicles. These features, he explained, would produce both the weakness and the increase in muscle volume.

In the century that has elapsed since the publication of these papers by Duchenne a large number of observations have been made on this disease but the pathogenesis is not yet known. The "Pseudohypertrophic Muscular Paralysis" of Duchenne forms part of a larger group of genetically determined muscle diseases now listed under the name, proposed first by Erb, of Muscular Dystrophy (MD). The word "Dystrophy" as applied to muscle indicates some defect in the nutrition of muscle of unknown origin or etiology. Because of our lack of understanding of the primary cause of muscle disease of this kind, the term (Muscular

Dystrophy), even though unsatisfactory, has remained. At present the clinical appearance, genetics, and course of the disease form the only bases for both definition and classification.

The muscular dystrophies are now defined as a group of genetically determined, progressive myopathic diseases. "Myopathic" disease includes all diseases of muscle, exclusive of those that are secondary to diseases of the nervous system (i.e. neurogenic atrophy). This definition is somewhat paradoxical since, in any of the muscular dystrophies, it is not known whether the primary defect is muscular. Indeed, any one of these disorders may be a more generalized disease with manifestations mainly in muscle. Since the description of the disease by Duchenne, Aran and Charcot, controversy has continued between supporters of a myogenic and a neurogenic origin for the Muscular Dystrophies. Furthermore many authors have pointed to the involvement of other organs, especially in the Duchenne type.

Some authors (3), including Duchenne (1, 2) have reported mental abnormality in patients with MD, but there is disagreement on whether the mental deficiency arises from some basic metabolic defect responsible for the dystrophic process (4) or from environmental deprivation (5). Neuropathological findings in several patients have shown extensive demyelinative lesions (6) or microscopic abnormalities in the cerebrum which are interpreted to result from the arrest of cortical migration of neurons during early development of the brain (7), but the results are not conclusive inasmuch as the findings are not present in all cases. Electroencephalographic (EEG) studies have also yielded variable results. Borderline or frankly abnormal EEG patterns have been described in about 50% of the patients with Duchenne dystrophy (8), but

the significance of these findings is largely invalidated by similar findings being observed in children with roughly the same motor handicap following denervation atrophy (9).

Other systems or organs also have been reported to be involved in Duchenne dystrophy. Because of the similarities between skeletal muscle and cardiac muscle, this organ has been among the first to be examined and several reports suggest that there is a constant involvement of the heart in Duchenne dystrophy. The main electrocardiographic findings are sinus arrhythmia, abnormally tall R-waves in the right precordial leads, and depression of the ST-segment with flat or negative T-waves in the right precordial leads (10). Also an analysis of the QRS vector loop shows an increase in the velocity of depolarization in the heart of muscular dystrophy patients suggesting either pathological changes in the myocardium or ventricular hypertrophy (11). Some defects in intestinal absorption have also been observed in the Duchenne type of muscular dystrophy due possibly to involvement of the smooth muscle of the intestine by the dystrophic process (12).

The classification of the muscular dystrophies based on clinical-anatomical data has proved to be unsatisfactory and to-day they are classified upon clinical and genetic criteria (13), as shown in Table I.

Basic biochemical research such as that undertaken in this study is difficult to do on human muscular dystrophy primarily because of the difficulty of obtaining good comparable biopsy material from both normal and affected muscle. Research on muscular dystrophy has been greatly aided during the last fifteen years by the occurrence of myopathy, either as an inherited or induced condition, in several animals (e.g.

TABLE I

CLASSIFICATION OF MUSCULAR DYSTROPHIES

I Duchenne-type muscular dystrophy

- a) Sex-linked recessive variety
  - 1) Severe
  - 2) Benign
- b) Autosomal recessive variety

II Limb-girdle muscular dystrophy

- a) Autosomal recessive
- b) Sporadic

III Facioscapulo-humeral muscular dystrophy

Autosomal dominant

IV Distal muscular dystrophy

V Ocular myopathy, including oculo pharyngeal

VI Congenital muscular dystrophy

mouse, Syrian hamster, white Pekin duck and chicken). In the studies reported in this thesis, mice suffering from muscular dystrophy are the main experimental animals used.

Mice with muscular dystrophy (strain 129/Re) first became available in 1955 (14). Three genetic types occur in litters of the dystrophic (dy) stock: affected animals (dydy), non-affected heterzygous carriers (Dydy) and non-affected homozygous animals (DyDy). The affected mice show a number of clinical, histological, biochemical and physiological similarities to the Duchenne type of muscular dystrophy, but there are significant differences (for example, in mice the disease is autosomal recessive whereas in Duchenne dystrophy it is sex-linked).

B. Skeletal Muscle Involvement. Morphological, Physiological and Biochemical Aspects.

a) Introduction

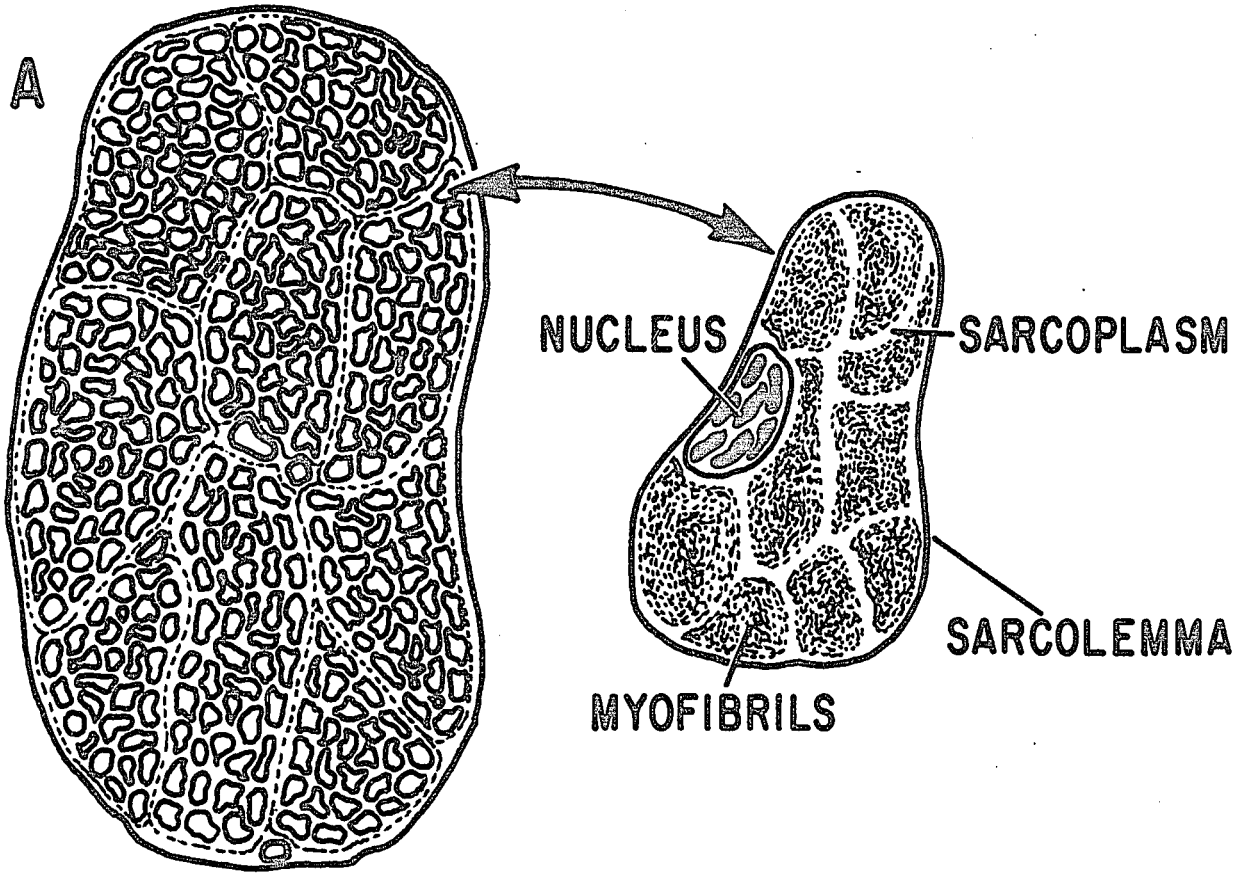
Skeletal muscle constitutes two fifths of the total body weight and over half of the weight of the soft body tissues. The main property of muscle is its contractibility which allows it to function as the moving force throughout the animal kingdom. Muscle also helps to carry on other functions such as that of participating in thermoregulation in warm-blooded animals. In studying the role of skeletal muscle in the body it is necessary to consider that, because of its bulk and the constant exchange of substances between different body compartments, any change in the ability of muscle to function could cause considerable alteration in the dynamic equilibrium of the body.

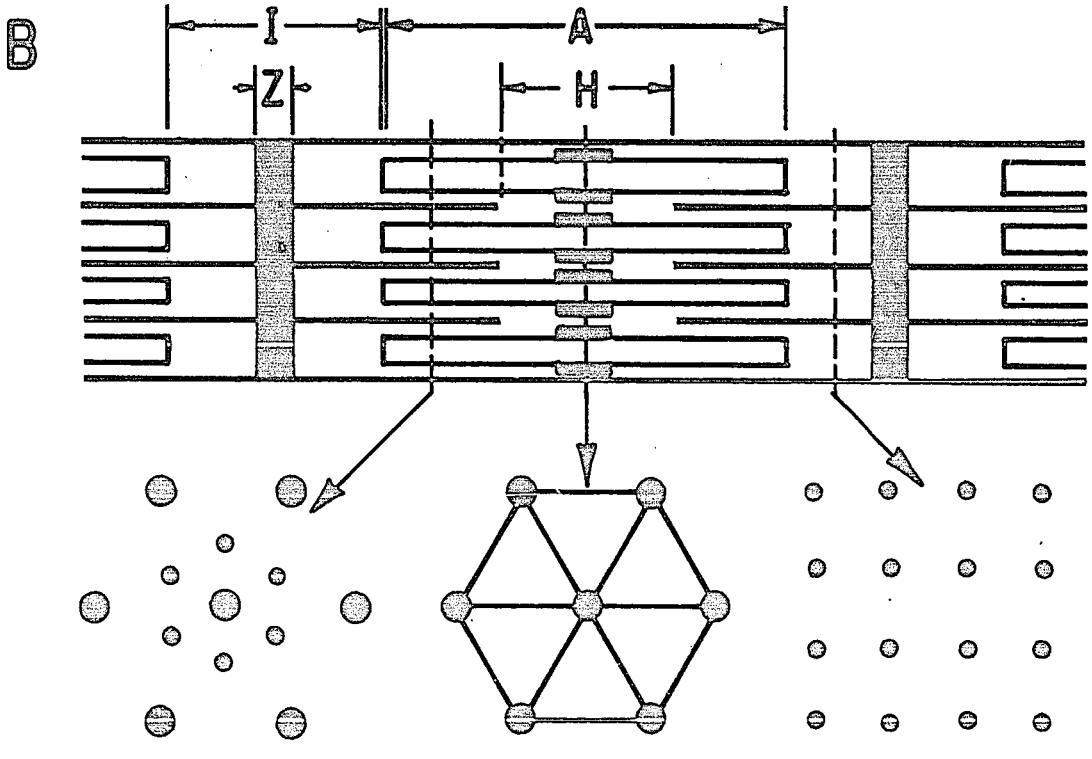
The main characteristic of muscle structure is its myofibrillar nature which is responsible for the transverse striations of the fiber. Seen under the light microscope in a transverse section (Figure 1, A)

Figure 1 - Skeletal muscle structure.

A = transverse section as seen under the light microscope: (1) Bundles of myofibrils are separated from one another by a layer of connective tissue. (2) shows a larger magnification of an isolated myofibril.

B = scheme showing both longitudinal and transverse arrangements of the different filaments of the myofibril.


A
NUCLEUS
SARCOPLASM
SARCOLEMMA
MYOFIBRILS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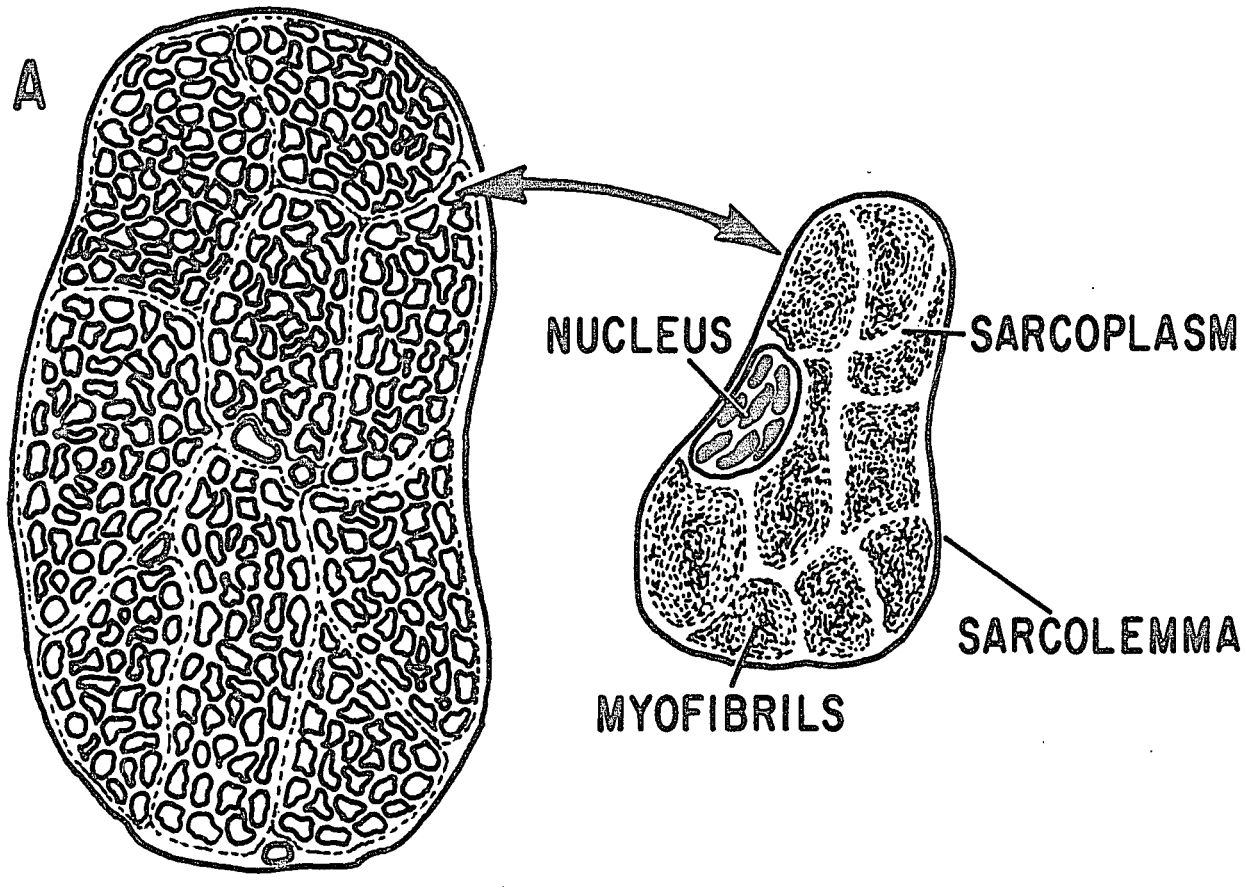
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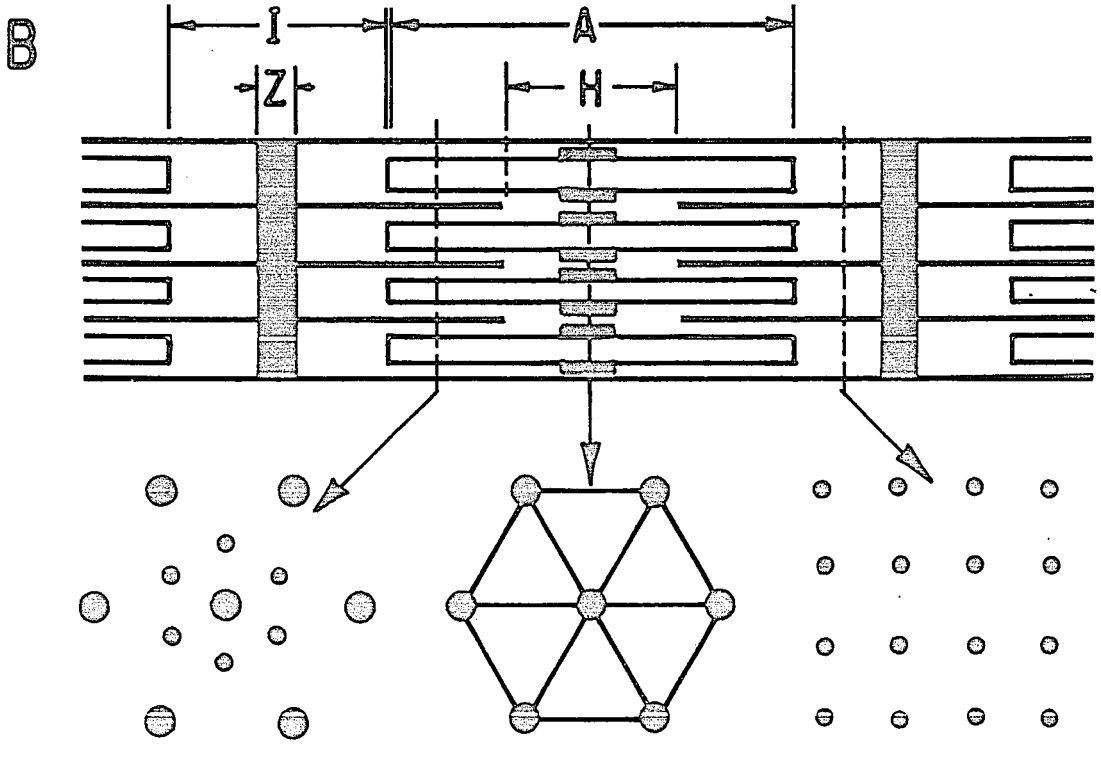
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muscle appears as a package of primary units called myofibrils with a nearby polygonal outline, separated one from another by a thin layer of sarcoplasm. These myofibrils are arranged in groups very close to one another with each group clearly separated from another group by a thicker sarcoplasmic layer.

Amongst each of these groups several additional structures can be seen. The nucleus usually is in a peripheral position and the nucleolus is readily visible. Small granules, which are the mitochondria or sarcosomes, can be seen between the myofibrils. Also it is possible to see small lines that form the sarcoplasmic reticulum. In the following pages the present knowledge available on these different subcellular structures will be reviewed. Brief consideration will be given to the morphological and physiological aspects. The biochemical aspects, particularly those relevant to the study reported here, will be described in greater detail. The main changes discovered in patients will be commented upon in the appropriate sections.

b) The Sarcolemma

The sarcolemma or muscle membrane can be resolved into two components: a plasma membrane, and a basement membrane. There is also a delicate net of collagen filaments which some authors consider as the third membrane. The plasma membrane is about 100 Å thick and in muscle fixed with osmium tetroxide it can be resolved into three layers. The basement membrane is a uniform, structureless lamellae of 300-500 Å in width and gives a positive periodic acid-Schiff reaction suggestive of mucopolysaccharides (15).

It has been suggested that the basement membrane plays an important role in determining the shapes of individual cells. The resist-

ance of this membrane to trauma and ischemia allows it to survive muscle injury and form an intact "sarcolemmal tube" that guides the growth of the regenerating fiber. Because the new fiber develops with its own basement membrane, it will form a fiber with two basement membranes (16).

Several functions are attributed to the muscle membrane in addition to its role in fiber regeneration. The most important are its participation in the conduction of the electrical impulses and its role as a permeability barrier between the intracellular and extracellular spaces. It is because of this latter function that many substances are not lost from or accumulate in muscle and that differences in concentration of electrolytes can exist between both spaces.

Even though dystrophic muscle (Duchenne) does not appear to show any deficiency in the conduction of electrical impulses (17), its permeability properties may be impaired. The first evidence of such impairment came from studies in which it was shown that increases in certain serum enzymes (particularly those peculiar to skeletal muscle) occur in patients with muscular dystrophy. Further support has come from observed changes in the concentration of Na^+ and K^+ ions.

Na^+ and K^+ are among the ions found in greater quantity in muscle tissue. The proper maintenance of intracellular concentration in relation to extracellular concentration is most important in maintaining the membrane electrical potential. Potassium ion is also particularly important because of its relation to protein metabolism (18) and to the contractile mechanism in skeletal muscle (19).

The first studies done to determine the concentration of these ions in muscle from dystrophic patients have shown a decreased intracellular K^+ (20). This finding which has been related to an increase

in the chronaxie observed in these muscles (21), has been later confirmed in studies using ^{42}K (22). These results have been found for Duchenne muscular dystrophy and myotonia dystrophica (23), and also in dystrophic mice (24).

Biochemical studies with isolated membranes from muscle have been carried out in order to elucidate the state of the components concerned in the active transport of these ions. The characteristic response of membrane-bound ATPase to ouabain is a lowered rate of ATP hydrolysis. Instead, in membranes from muscle of dystrophic mice ATPase was found to be stimulated by ouabain, suggesting some defect in the membrane-transport mechanism (25).

In addition to the genetics and clinical description of the disease the most useful tool in the diagnosis of Muscular Dystrophy is the biochemical determination of certain serum enzymes. Transaminases (GOT and GPT) (26, 27, 28), aldolase (26, 27, 28, 29), lactic dehydrogenase (26, 27), malate dehydrogenase (26, 27) and creatine phosphokinase (CPK) (27, 28, 30) have been found to be elevated in the serum of patients affected with MD. The measurement of CPK (which is widely used today) not only assists in the diagnosis of the disease (important in the differential diagnosis with myopathies of neural origin where only transient elevation occurs(30)) but also in the detection of carriers and subsequent eugenic counselling (28). The elevation in serum enzymes is more clearly manifested in young patients. As the disease progresses the elevations observed may decrease to normal values. This is often evident when the patient ceases to be ambulatory (31) and probably reflects the associated diminution of muscle mass. A number of studied (32-35) point

to the fact that the above elevation of serum enzymes and an associated increase in sensitivity to acetylcholine result from an increase in the membrane permeability of affected muscles.

Despite the physiological and biochemical signs pointing to an abnormality of the sarcolemma, in patients with Duchenne type MD no alteration in ultrastructure either in the plasma or basement membrane has been found when studied by electron microscopy (17). Only in advanced stages of the disease has sarcolemma breakdown been reported to occur (36). On the contrary, studies done on dystrophic mice have shown an absence of intact sarcolemma around affected fibers even in a preclinical stage, suggesting that this defect could be the initial alteration or injury to the muscle cell (37). However these results have not been confirmed and other authors have not been able to find any defect in the sarcolemma of the dystrophic mice during its clinical phase (38, 39).

c) Sarcoplasmic Reticulum (SR) and Transverse Tubular System (T)

Among the different kinds of structures that are contained in the interfibrillar space, are the sarcoplasmic reticulum (SR) and the transverse tubular (T) system.

The T system can be considered to be part of the sarcolemmal membrane. In a longitudinal section of muscle it appears as a transverse tubule with a localization that changes from species to species. Evidence for the continuity of the T system with the sarcolemma has been provided by immersing the muscle in a solution of ferritin and observing its penetration into the muscle. Penetration was confined to the interior space of the T system (40) and showed that no connection exists between the T system and the SR system even though some bridge-

like structures seem to exist (41).

The available evidence strongly suggests that the T system is the structure responsible for the conduction of the impulses inwards (42). With the use of microelectrodes (43) it was possible to demonstrate that striated muscle from pig muscles could be activated only in the region of the Z band. This observation has been interpreted to indicate propagation of the excitation through the T system (44). The relationship between the T system and propagation of the excitation is supported by the fact that slow "tonus" fibers of frog skeletal muscle do not have the T system (45) and that muscles with fast contraction are rich in elements of the T system and also of the SR system (46).

The SR system is located along the sarcomere. Elements of the SR system surround the myofibrillar component, and fill the interfibrillar space giving it a complex structure. The SR forms in conjunctionwith the T system the so-called "triad" (41). Studies carried out on frog striated muscle suggest that the T system and SR system are different since the former, together with the myofibrils , is glycerol stable while the SR system is glycerol extractable (47).

Several functions have been attributed to the SR system. Because of its close relationship with mitochondria, lipid droplets and glycogen, the SR probably participates in the exchange of metabolites between the myofibrils and the sarcoplasm (48). About twelve years ago another function was assigned to it, namely, that of coupling excitation to the contraction mechanism which is now briefly described.

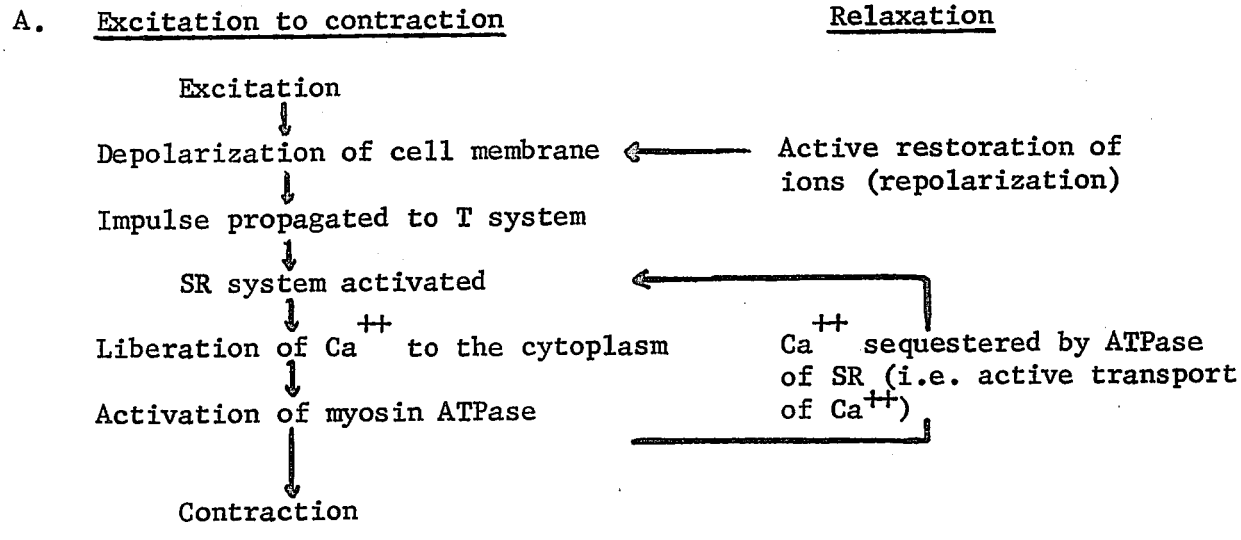
A relaxing factor has been described that can prevent the ATP-induced contraction of actin and myosin and can even produce relaxation in a previously contracted muscle. This factor has been localized to the sarcotubular system (49). A relationship was very soon established between Ca^{++} and the relaxing factor; it was already known that Ca^{++} was able to originate contraction of the skeletal muscle and that the phenomenon of superprecipitation and ATPase activity of actomyosin were maximum in the presence of Ca^{++}. Furthermore, a correlation does exist between the abilities of chelating agents to bind Ca^{++} in the presence of Mg^{++} and their function as relaxing agents. When the Ca^{++}-binding capacity of the vesicles obtained from the SR system was studied, they were found to have an affinity comparable to that of chelating agents (50). This property was shown to be dependent on ATP, since in the presence of Ca^{++} (10^{-6} M) phosphate was liberated from ATP (51).

At present the process of transition from contraction to relaxation is thought to be originated by a shift in the equilibrium Ca^{++}-Actomyosin $\rightarrow$ Actomyosin + Ca^{++}, followed by a dropping of the concentration of Ca^{++} in the cytoplasm during relaxation through active accumulation into the sarcoplasmic reticulum (52). In Figure 2, are shown the main events taking place in the transition from excitation to contraction.

Kinetic studies on muscular contraction carried out in normal and dystrophic mice have shown the existence in dystrophic animals of a longer relaxation period (53). Because of the relatively normal contraction period, attention was directed to the study of the relaxation system. Fragments of sarcoplasmic reticulum from dystrophic and normal

FIGURE 2

SUMMARY OF EVENTS IN MUSCLE CONTRACTION



mice were examined and a decrease in Ca^{++} uptake was found without any change in ATPase activity (54). Even though no differences were found in the amino acid and phospholipid composition of the fragmented sarcoplasmic reticulum preparation from dystrophic mice when compared with that of normal mice however, the rates of hydrolysis on treatment with phospholipase C or protease were increased for dystrophic muscle (55). Also an abnormal structure of the isolated sarcoplasmic reticulum from dystrophic mice has been observed with the electron microscope using the negative staining technique (56).

The sarcoplasmic reticulum of children affected with Duchenne MD has also been investigated. Both Ca^{++} uptake and ATPase activity have been found to be reduced: normal values were found in cases of myotonic dystrophy studied at the same time (57). No differences were found in the lipid composition of the sarcoplasmic reticulum from patients with MD. As observed for normal children, 54% of the lipids were phospholipids. Fifty per cent of the latter consisted of phosphatidylcholine (58). These authors also confirmed the decrease in Ca^{++} uptake and in ATPase activity mentioned previously (57).

Several abnormalities in the ultrastructure of the sarcoplasmic reticulum of children affected with MD (Duchenne) have been observed, however no agreement exists regarding their pathogenetic importance: while some authors (59) consider these changes are a primary manifestation of the disease, others consider them to be secondary to mitochondrial changes (17) or to alterations in the myofibrils (36).

In dystrophic mice a marked swelling and vacuolization in the cells of dystrophic muscle has been observed (38) but the outstanding

degenerative change appears to be a loss of the contractile elements within the affected cell. One possible important finding that has been reported in dystrophic mice is the fact that in parts of the muscle where the phenomenon of "nuclear rowing" (long chain of nuclei centrally located within the cell) is very prominent, there is also prominence of granular endoplasmic reticulum together with an increase of free ribosomes. This suggests an activation of protein synthesis related to the regenerative process (39). This increase in free ribosomes might prove to be one criterion of help in the difficult problem of differentiating between degenerating and regenerating regions. Both processes must go on at the same time in the affected muscle, with the relative contribution of each dependent upon the course of the disease.

d) Myofibrils (Contractile proteins and contraction)

The structures directly responsible for muscular contraction are the filaments. As shown in Figure 1, B, two different kinds of filaments are observed. The thick filaments (160 Å diameter) in the center of the sarcomere form the A band and the thin filaments (50-70 Å diameter), which interdigitate with the thick filaments and are inserted in the Z line, form the I band. This interdigitation gives rise (a) to the bands (A and I), zones (H) and lines (Z) shown, and (b) the regular arrays seen in transverse sections.

It was considered for a long time that both filaments were composed of the same substance and the word "myosin" was used to designate the globulin extracted from muscle by slightly alkaline salt solutions. However, in 1941 it was demonstrated that the properties of "myosin" changed according to the length of extraction; the longer the

extraction the more viscous the preparation became. It was found that this effect is due to the presence of another protein called actin (19). Now at least six main protein fractions have been found related to the filaments: myosin, actin, tropomyosin, troponin, α-actinin and β-actinin.

The thick filaments of skeletal muscle are composed mainly of myosin. It has been demonstrated that when myosin is selectively extracted the A band disappears (61) and also that on polymerization myosin forms filaments of identical shape to the thick filaments in the A band (62). Two main properties of the myosin molecule are its ATPase activity and its ability to bind actin. After controlled tryptic digestion of myosin two products appear that have been called meromyosins: a slow sedimenting protein or light-meromyosin (LMM) that is responsible for the solubility properties of myosin and a fast sedimenting protein or heavy-meromyosin (HMM) that possesses the ATPase activity and actin-binding capacity (63, 64) (see Figure 3, A).

Actin has been identified as the main component of the thin filaments on the basis that its extraction produces a disappearance of the I band (61). Actin exists in two forms: monomeric or G-actin (globular) and polymerized or F-actin (fibrous). The G$\longrightarrow$F transformation takes place on the addition of salts and is accompanied by an increased viscosity of the solution (65). ATP is specifically required for the polymerization of G-actin (66, 67, 68) to which it is loosely bound since it can be easily extracted by charcoal; however during polymerization there is a release of phosphate (69) after which charcoal treatment of F-actin has no effect in removing the bound ADP (70). The

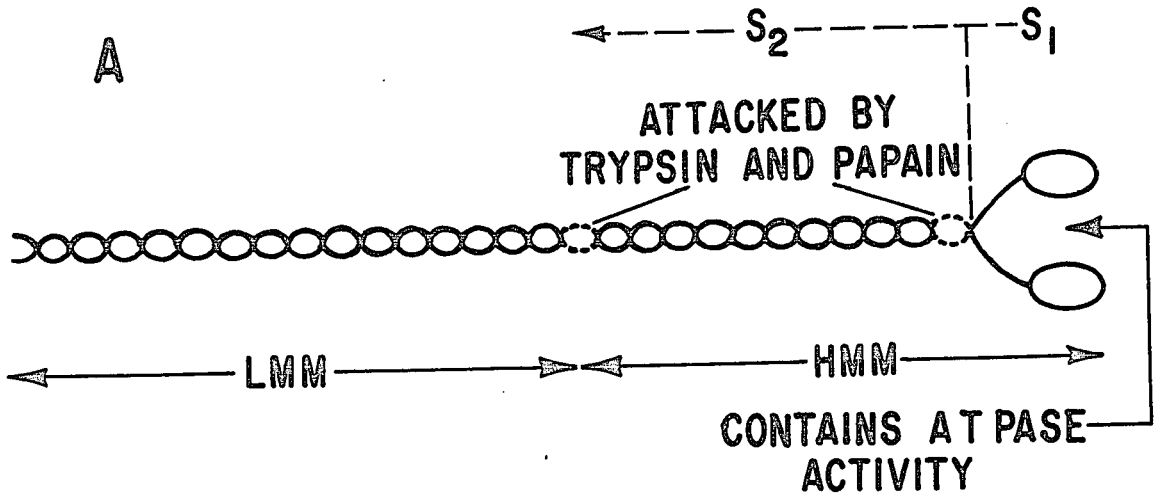
Figure 3-A. Diagrammatic representation of the molecule of myosin.

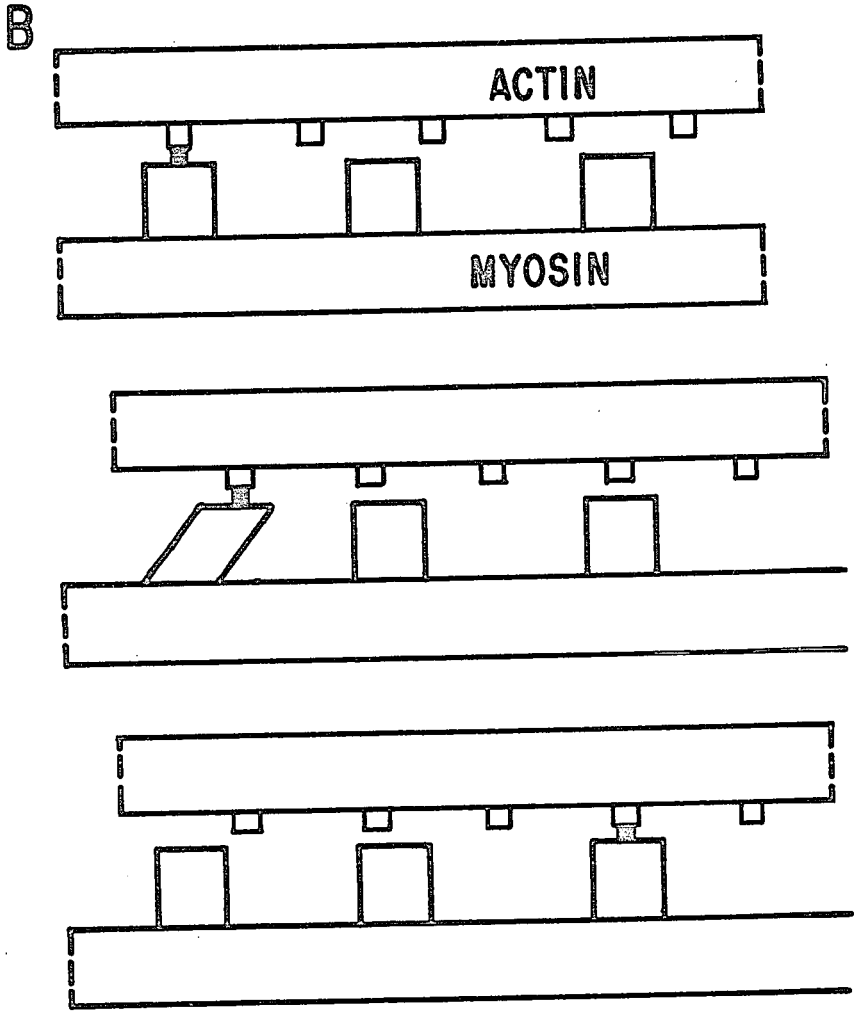
LMN, light meromyosin

HMN, heavy meromyosin

B. Scheme showing the action of cross-bridges in the sliding theory of muscle contraction.

(After: H. E. Huxley - Proc. Roy. Soc. London B, 178, 131-149 (197))


A
S2
S1
ATTACKED BY
TRYPSIN AND PAPAIN
LMM
HMM
CONTAINS ATPASE
ACTIVITY


B
ACTIN
MYOSIN

following scheme for the transformation G⟶F⟶G has been suggested (71):

$$G + ATP \longrightarrow G\text{-}ATP$$

$$G\text{-}ATP \longrightarrow F\text{-}ADP + Pi$$

$$F\text{-}ADP \longrightarrow G\text{-}ADP$$

$$G\text{-}ADP + ATP \longrightarrow G\text{-}ATP + ADP$$

Tropomyosin is a fibrous protein first isolated in 1948 by Bailey (72). The similarity of its structure to the appearance of the Z line suggests that it is a main constituent of this structure (62). If tropomyosin labelled with a fluorescent dye is added to myofibrils partially digested with trypsin, a very weak fluorescence appears in the A band and strong fluorescence in the I band while the Z band is only partially stained. Similar results obtained using antibodies point to its localization in the thin filaments and partially in the Z line (73).

Another protein related to the filaments is troponin. It has been shown that this protein enhances the interaction of actin and myosin in the presence of Ca^{++} during the excitation-contraction coupling in living muscle. This property is related to its very high affinity for Ca^{++} at concentrations comparable to the physiological concentration of Ca^{++} during contraction (74). Recently it has been reported that troponin consists of two proteins: troponin A which inhibits the Mg^{++} - activated ATPase of synthetic actomyosin, and troponin B which appears to be associated with the Ca^{++} sensitivity (75). Studies done with fluorescent dye marked-troponin or with antibodies have localized the troponin along the whole thin filament but not at the Z band (76).

α-Actinin is a protein recently isolated from the crude extracts of tropomyosin (76). If a certain amount of α-actinin is added to F-actin it gelatinizes immediately at room temperature. Its action seems to be to facilitate latent association of F-actin filaments. Its localization has been made with the use of antibodies and it appears to be confined to the Z-line region (77).

The last of the structural proteins of the filaments studied so far is β-actinin, isolated by Maruyama in 1965 (76). It is an actin-dispersing factor, that regulates the length of the polymers of F-actin and inhibits the formation of a net-like structure (78).

Even though many mysteries remain to-day regarding the mechanism of muscle contraction, it is explained on the basis that the thin filaments slide inwards over the thick filaments (see scheme in Figure 2, B). The main evidence to support this hypothesis has been provided through measurements made on contracted and relaxed muscle using the contrast and interference microscope (79, 80). When muscle contracts, the length of the A band remains constant, while the I band diminishes until it disappears completely. At this stage (i.e., the limit of physiological contraction) the fiber reaches 65% of the resting length. Independently of the dynamic state of the fiber the distance from the Z line to the edge of H remains constant. It has been found that each thick filament is made up of a group of long L-shaped myosin molecules whose side pieces can interact and form temporary cross-linkages with adjacent actin filaments (62). During activity these cross-linkages appear to be broken and remade in rapid succession. Figure 3,B shows a diagram of the possible action of these cross-bridges. The

specific spatial structure and/or conformation changes of the proteins are considered to allow and bring about the movement of one filament towards the other which results in shortening or in the development of active tension.

The interaction between myosin and actin occurs in the presence of ATP. One of the most important discoveries in muscle function was the finding by Engelhardt and Ljubimova (81) that myosin is an active ATPase. In this way a link was established between the structural basis of contraction and its energy requirements. So important is this finding, that it has been said to mark the beginning of molecular physiology (82). Also, this function of myosin links contraction with the function of the SR system because the myosin-ATPase is strongly stimulated by Ca^{++} ions; MG^{++} ions have an inhibitory effect (83).

In cases of Duchenne MD, abnormalities in the myofibrils have been reported even in the early stages of the disease, but very often myofibrils still existed in places where there were no traces of sarcoplasmic reticulum. In the more advanced cases disruption of the myofibrils appeared first at the A-I band "boundary" and the thick filaments were found to be more separated than the thin ones, with a resultant increase in the interfibrillary space (59). Other authors have observed that the myofibrillar disruption is preceded by changes in the mitochondria (84).

Also in mice with muscular dystrophy, a general loss of myofibrils has been considered to be the most prominent feature of the diseased muscle when seen by electron microscopy. The remaining space is occupied by a number of structures that could represent residues of

the Z lines from pre-existing myofibrils (38). This loss of myofibrils is at first localized in determined points of the cell but it progresses over the entire cell. These results are open to at least two interpretations: (a) they reflect the primary pathological change in dystrophic muscle, in which case the myofibrils are degenerating, or they reflect attempts by the muscle to regenerate, in which case the myofibrils are in a stage of new formation or regeneration.

Not too much work has been done so far on the properties of the different contractile proteins isolated from muscle of dystrophic patients or mice. The amount of myosin has been found to be greatly reduced in the muscle of Duchenne dystrophic patients (85) and in mice affected with muscular dystrophy (86) whereas the amount of actin has been reported to be normal. This decrease in myosin can have its origin in a defective synthesis, or an increased catabolism due to extrinsic factors to the myosin molecule, such as increased proteolytic enzymes, or to intrinsic factors, such as some alteration in the molecule which makes it less resistant to attack. Protein synthesis has been found to be increased in muscle from dystrophic mice as shown by studies on the incorporation of glycine-1-^{14}C (87) or leucine-^{14}C (88) into the protein fraction. On the other hand proteolytic activity over neutral and alkaline ranges have been found to be very much increased in dystrophic animals using proteins of normal and dystrophic muscle from mice as substrate (89). Also depots with acid phosphatase activity were shown by histochemical methods to be grouped in regions where the breakdown of myofibrils occurred (90). It is important to notice here that a protease acting specifically on myofibrils has been reported to exist in

normal rat muscle and it was found to be greatly increased in muscle undergoing atrophy after denervation (91).

The various properties of myosin from dystrophic muscle of mice have been studied. These were normal with respect to ATPase activity, ability to combine with F-actin and pattern of behaviour in the ultracentrifuge. However, light-scattering measurements seemed to indicate a greater polydispersity for myosin from dystrophic muscle (86). The same authors have correlated the loss of myosin with differences found in the distribution of lactic dehydrogenase isoenzymes and alterations of myoglobin in these muscles. They interpret these results to mean that there is a regression of muscle to the embryonic state in myopathies.

Using slightly younger mice other authors have reported a similar myosin content in dystrophic mice (92). However, they found some differences in amino acid composition (higher content of alanine, valine, isoleucine and lower content of glycine and histidine) and a lower availability of SH groups to SH reagents. Myosin from dystrophic muscle was found to be less stable on storage. About 25% of it appeared as aggregates in conditions where it should have been monomeric.

Summarizing what has been said so far, it appears that the decrease in myosin is not due to a decrease in its synthesis, but mainly to an increase in its catabolism caused by an increased proteolytic activity.

e) Extramitochondrial and Mitochondrial Reactions of Cellular Metabolism

(i) General Aspects

For many years it has been accepted that skeletal muscle depends largely upon carbohydrate utilization as the primary "fuel" for

contraction. However, when it was demonstrated in 1913 (93) that circulating lipids were removed from blood by skeletal muscle it was suspected that lipids could also serve as energy source for muscle. This became more evident when determinations of the respiratory quotient (RQ) of muscle in rest revealed that only 1/5 of the oxygen uptake could be attributed to the oxidation of carbohydrate. However, early attempts to demonstrate an in vitro oxidation of long-chain fatty acids by skeletal muscle were unsuccessful until the cofactor carnitine was discovered (94). Carnitine has been shown to stimulate the oxidation of long-chain fatty acids in all tissues studied. In skeletal muscle this dependance also applies since it has been shown that this tissue is unable to catabolize long-chain fatty acids unless carnitine is present (95). With the realization that long-chain fatty acids could be oxidized by skeletal muscle, the former concept that fatty acids could be utilized by muscle only after they had been previously converted to ketone bodies by liver, was rejected.

Even though uncertainty exists concerning the relative utilization of each foodstuff, it is now known that both lipids and carbohydrates can be used by muscle. This has reached the point where doubt has been expressed as to whether dissimilation of muscle glycogen occurs in intact man at rest. There are several factors that make the resolution of this problem difficult. The first is the existence of, at least, three different types of muscle fibers of which two, type I or C (red and slow) and type II or A (white and fast) represent the main types; the third type is intermediate in its properties (type B). The criteria used to distinguish the fibres are morphological, based on

differences in the mitochondrial content, shape of the sarcoplasmic reticulum, size and shape of the myofibrils, and also cytochemical criteria such as mitochondrial enzymatic activity, phosphorylase activity, glycogen content and triglyceride content (96). These differences point to a possible heterogeneity of fibres with respect to substrate requirements.

Another factor which could determine preferences in the utilization of one or another substrate is the stage of development of muscle and the influence of the nervous system on muscle. Changes in the distribution of fibers I and II have been observed when muscle passes through different stages of development, and also after it is deprived of its innervation (97, 98). Increased amounts of lactic acid in denervated muscle together with fat accumulation, decreased glycogen content and several enzymatic changes suggest that the role of carbohydrate as substrate for energy increases after denervation (99, 100).

The third consideration concerns the differences in muscle at rest and work. During exercise the RQ of muscle approximates 1 until the glycogen content falls. This is followed by a decrease in the RQ, indicative of utilization of other substrates (101). At rest there is a decrease in the RQ with an increase in the glycogen content indicating a greater utilization of fatty acid. It is possible that with prolonged periods of work, fat is used as indicated by the disappearance of lipid from fat depots of migratory birds in response to the considerable energy demands of continuous flight (102).

When it was found that muscle was able to contract after being poisoned by iodoacetic acid (103) (interferes with the formation

of lactic acid) it was considered that contraction and energy were directly related through the breakdown of creatine phosphate, a high-energy compound that had been discovered in muscle (104). Confirmation was provided when a relationship between the tension developed by muscle and the breakdown of creatine phosphate was demonstrated and also by the fact that much of the lactic acid production occurs during the post-contraction period (105). With these findings lipid and carbohydrates were considered to serve as suppliers of energy for the resynthesis of creatine phosphate.

Creatine, the precursor of creatine-phosphate is formed mainly in the liver. The synthetic pathway requires as first step the condensation of glycine and arginine to form glycocyamine (106) followed by the transfer of a methyl group from methionine to glycocyamine to give creatine (107). Creatine is stored mainly in skeletal muscle (over 90%) either as the free form or as creatine phosphate, and it is excreted in the urine under normal conditions only in the form of its anhydride creatinine. The only source of creatinine is the creatine from muscle and the rate of conversion is relatively constant.

After the discovery of ATP in muscle (108) and the observation that myosin has ATPase activity (81), the idea that creatine phosphate was the immediate source of energy for muscle contraction was changed in favor of one where carbohydrates, lipids and creatine phosphate supply energy for the build up of ATP which on hydrolysis by the action of the myosin ATPase could liberate the required energy for contraction. Also it was found that ADP was necessary for the breakdown of creatine phosphate, according to the reaction sequence:

$$ATP \longrightarrow ADP + Pi \quad (1)$$

$$\text{Creatine phosphate} + ADP \xrightarrow[\text{kinase}]{\text{Creatine Phospho-}} \text{Creatine} + ATP \quad (2)$$

The last reaction, called Lohmann's reaction (109) is reversible, and it can be used to synthesize creatine phosphate. The latter is stored as part of the immediately available energy store for muscle. Another pathway for the formation of creatine phosphate, without ATP being involved, was later described (110). This pathway involves a reaction between creatine and 1,3-diphosphoglyceric acid:

$$\text{Creatine} + \text{1,3-diphosphoglycerate} \xrightarrow{Mg^{++}} \text{3, phosphoglycerate} + \text{creatine phosphate} \quad (3)$$

Summarizing, from a physiological point of view, the mechanism leading to the release of energy in muscle can be divided into three phases (a) the phase of energy liberation, including the metabolism of fatty acid, glucose, pyruvate, etc....; (b) the phase of energy conservation in the form of creatine phosphate and ATP; and (c) the phase of energy utilization which is related to the interaction between actomyosin and ATP. From a structural point of view all these reactions can be divided into extramitochondrial and mitochondrial reactions. Glycolysis and creatine phosphate metabolism are extramitochondrial reactions, and fatty acid oxidation, citric acid cycle oxidations and oxidative phosphorylation are mitochondrial.

(ii) Creatine and Creatinine

One of the first biochemical abnormalities observed in patients affected with muscular dystrophy was creatinuria which occurred

together with a reduction in the excretion of creatinine in the urine. Although the amount of creatinine in urine may be no greater than that found in other diseases like myasthenia gravis or secondary to a lesion in the nervous system, it appeared that the ability of muscle to retain creatine was usually more impaired in the dystrophic patient as deduced by a more diminished creatine tolerance test. Also, after feeding glycine to dystrophic patients an increase in the excretion of creatine output and the abnormality in the creatine tolerance test would appear to be expressions of an improperly functioning muscle mass per se, and not an index of the amount of wasted muscle, or the primary lesion of the disease (111).

More recent experiments were performed feeding patients with ^{15}N-glycine and investigating the ^{15}N in urinary creatine and creatinine. Initially a far greater concentration of isotope was found in creatine than in creatinine and the decline in isotope abundance with time was more rapid in creatine than in creatinine. The conclusion made from this experiment is that urinary creatine does not originate in muscle but represents freshly synthesized creatine which has been denied access to muscle (112). However, similar experiments carried out with ^{14}C-creatine have shown that the rate of disappearance of radioactivity as urinary creatinine was more rapid in patients with muscular dystrophy. Parallel decreases in specific activities of urinary creatine and creatinine were observed, pointing to a defect in the muscle of patients with muscular dystrophy to retain the creatine rather than a decreased rate of entry (113). It is possible that both mechanisms, (that is a defect in uptake of creatine by muscle due to the decrease in functional

muscle, and a defect in retaining the creatine due to some abnormality in the sarcolemma or in some intramuscular mechanism) may be acting together.

An impaired ability to retain creatine was also observed in muscle from dystrophic mice as shown by a decreased turnover time of both total body creatine and skeletal muscle creatine, determined after injection of ^{14}C-creatinine. The excretion of creatine plus creatinine by dystrophic mice is similar to that observed for normal mice (114).

Besides the possible defect in the active transport mechanism of creatine, another factor that could contribute to the lack of retention of creatine by dystrophic muscle is a decrease in creatine phosphokinase (85, 115). This defect could be very important considering the relatively rapid reaction catalyzed by this enzyme and the impermeability of the cell membranes to high-energy-phosphorus compounds.

(iii) Glycolysis - Extramitochondrial

Glycolysis is an extramitochondrial process in which the end product is lactic acid. The glycolytic enzymes appear to be located in the soluble part of the cell and (at least in part) in the sarcoplasmic reticulum, an example of the latter being phospho-fructokinase (116). The main characteristics of glycolysis in skeletal muscle are the lack of the enzyme glucose-6-phosphatase, the almost complete absence of free glucose in the muscle cell and the failure to utilize lactic acid.

Glycogen, an important constituent of muscle, can be seen with electron microscopy as small granules (α and β granules) in the interfibrillar space. The amount of glycogen changes according to the state of muscle. The enzyme responsible for its degradation is phosphorylase, an enzyme, believed for many years to catalyze the reverse reaction

until Leloir and associates demonstrated the existence of the glycogen synthetase system.

In patients affected with MD (117) or in dystrophic mice (118) a decrease in the activity of both a and b phosphorylases was observed, with a normal ratio of a/b. This decrease in activity has been found in dystrophic mice not only to occur in the thigh muscle where the pathological process is more visible, but also in the fore-limb muscle and abdominal muscle. The normal a/b ratio is in contrast to what happens in muscle undergoing atrophy after denervation where there is a decrease in phosphorylase activity, together with a decrease in the a/b ratio (119).

The level of glycogen in muscle from dystrophic mice was found to be only 72% of that in the normal control (118).

Glycolysis has been studied in vitro by determining the rate of formation of lactic acid by muscle homogenates incubated in the presence of glycogen (120) as substrate. A marked reduction in the ability to carry out glycolysis was observed in muscle from Duchenne MD patients. In dystrophic mice, glycolysis has been assessed in a similar way using fructose-1,6-diphosphate as substrate, and a small, but statistically non-significant increase was observed when the results were expressed in mg lactic acid formed/gm wet tissue (121).

It is not surprising to find a reduction in glycolysis in dystrophic muscle when we consider that all the individual enzymes studied so far, with the exception of hexokinase, have been found to be reduced, as can be seen in Table II. Several authors have also reported the existence in patients affected with Duchenne MD of an abnormal behaviour

TABLE II

Muscular enzymes related to glucose metabolism in Muscular Dystrophy (human Duchenne MD and/or Murine MD) compared with normal controls.

Enzyme	Found in Dystrophy	Type of Dystrophy	Reference	Observations
PHOSPHORYLASE	↓	Duchenne	117	a/b normal.
		Mice	118	a/b normal. Assayed by the reaction: G-6-P ⟹ Glycogen.
PHOSPHOGLUCOMUTASE	↓	Duchenne	115	
		Mice	122	Determined with or without Glucose-1,-6-diphosphate as acceptor.
HEXOKINASE	↑	Mice	123	Same pattern in denervated muscle.
PHOSPHOHEXOSE-ISOMERASE	↓	Duchenne	115	Activity/gm. fresh tissue.
ALDOLASE	↓	Duchenne	115	
		Mice	124, 125	Decreased if expressed as activity/gm. wet weight
PHOSPHOTRIOSE-ISOMERASE	↓	Duchenne	115	
PHOSPHOGLYCERALDEHYDE-DEHYDROGENASE	↓	Duchenne	115	
		Mice	125	
α-GP-DEHYDROGENASE	↓	Mice	125	Observed only after 1 1/2 weeks of age (135)

TABLE II Continued...

Enzyme	Found in Dystrophy	Type of Dystrophy	Reference	Observations
ENOLASE	↓	Duchenne	115	
LACTIC ACID DEHYDROGENASE	↓	Duchenne Mice	115 123	Observed also in denervated muscle.
GLUCOSE-6-PHOSPHATE-DEHYDROGENASE	↑	Mice	123	Also in denervated muscle.
6-PHOSPHOGLUCONIC ACID DEHYDROGENASE	↑	Mice	123	
PHOSPHOENOL-PYRUVATE KINASE	=	Mice	123	

in blood glucose regulation as indicated by an abnormal response to the glucose tolerance test, to the glucose-insulin test, or to the tolbutamide test (126, 127, 128).

(iv) Lipid Metabolism: Extramitochondrial and Mitochondrial

As we have seen before, lipids can be an important "fuel" for muscle contraction and most of the energy derived from them comes from the oxidation of the fatty acid moiety. The catabolism of long chain fatty acids takes place in two different cellular compartments: an activation reaction that is extramitochondrial, and the β-oxidation reactions that are mitochondrial. In between these two reactions the transport of fatty acids through the mitochondrial membranes takes place and this process offers the special characteristic in muscle of being completely carnitine-dependent. The enzyme carnitine-palmitylCoA transferase plays a fundamental role in this transport. Another function of carnitine is to mediate in the transport of acetyl groups out of the mitochondria, this reaction is catalyzed by the enzyme carnitine acetyl CoA transferase. It has been suggested (129) that two different pools of acetyl CoA exist in the mitochondria, and that exchange between the two compartments may be facilitated by carnitine.

One of the more visible characteristics of dystrophic muscle is its fat accumulation. In patients affected with Duchenne MD this accumulation is mainly due to an increase in cholesterol, cholesterol-ester, free fatty acids and triglycerides (130) which can reach up to 10 times the amount in the normal muscle (131). A small decrease in the phospholipid fraction has been reported (130). With regard to the distribution of the different fatty acids, only slight differences were

reported, the main one being in the phospholipid fraction where decreases in palmitic, stearic and linoleic acid and increases in palmitic and oleic acid were observed (130). Basically the same pattern was observed in dystrophic mice, that is, an increase in cholesterol (132), fatty acid and triglycerides (133), but no differences were observed in the phospholipid fraction as a whole. However, recently some differences in the phospholipid composition of different subcellular structures of muscle from dystrophic mice have been found, such as an increased percentage of phosphatidyl ethanolamine in the mitochondria, and less lecithin but more phosphatidylethanolamine and sphingomyelin in the microsomes (134).

Mainly because of this excess of lipid found in dystrophic muscle, studies have been carried out in this laboratory to determine the ability of muscle mitochondria to oxidize long-chain fatty acid. A marked reduction in the release of CO_2 from long-chain fatty acid was observed, and this together with an increase in the synthesis of fatty acid from acetate by high-speed supernatant of dystrophic muscle from mice could be contributing factors to the accumulation of fat in these muscles (135). The exact localization of the defect in the degradation of fatty acid has not been found. Some enzymes such as fatty acyl CoA synthetase (136) and carnitine-palmitylCoA transferase (137) have been reported to be normal.

In humans, cases of myopathy have been reported to be associated with a possible defect in lipid metabolism, but in these cases there was no family history of myopathy (138, 139, 140). In patients with Duchenne MD also there appears to be evidence of a marked decrease

in fatty acid oxidation by muscle mitochondria (141).

The main part of the energy utilized by muscle is formed in the mitochondria. In muscle the main localization of mitochondria is between the contractile elements, the perinuclear region and in the region of the motor end plate. Basically the mitochondria (or sarcosomes) of muscle are similar in structure to those of other tissues. Each mitochondrian has an outer membrane surrounding a folded internal membrane. Structure and numbers of mitochondria are related to the activity of the tissue; the greater the activity of the muscle the more numerous are the cristae and the number of mitochondria. The mitochondria contain the tricarboxylic acid cycle enzymes, enzymes for the β-oxidation of fatty acids, the electron-transport chain system, and the coupling process of oxidative phosphorylation. All these reactions are interrelated and their main role is the synthesis of ATP.

Mitochondrial alterations have been reported in various myopathic states. These alterations range from simple differences in size and electron-density of the cristae (142, 143) to the appearance of vacuoles, concentric lamellae resembling cristae, inclusions of glycogen-like granules surrounded by a membrane (144), quadruplicate lamellar structure in the outer mitochondrial chamber (145) or mitochondria forming aggregations (146). A review of these cases shows: (a) that no family history of myopathy was associated with the appearance of inclusions; (b) that in the hereditary myopathies (including Duchenne) only changes in size (swelling and/or hypertophy) and cristae attenuation have been observed and even these alterations have focal localization (144); (c) the relation between these alterations and the etiology of the dystrophic

process can not be established. Thus, while the above abnormalities in the mitochondria could originate serious metabolic defects affecting the whole muscle, it may be that the abnormalities found are the consequence and not the cause of the disease. Furthermore, a lack of specifity of these alterations has been demonstrated since similar changes in the mitochondria occur after denervation of muscle (147) or after acute ischemia by supression of local blood supply. Thus, it seems that abnormalities in mitochondria are only signs of an imbalance in the degenerative-regenerative processes which can arise from multiple factors and are completely non-specific for the Muscular Dystrophies.

In dystrophic mice the same kind of alterations have been reported, that is the swelling and differences in density of the cristae. An important observation is the appearance of histological alterations in a preclinical state of the disease (37).

No differences have been found in the concentration of various mitochondrial enzymes in the dystrophic muscle. Although cytochrome oxidase, "succinoxidase", fumarase, aconitase (120) and succinic dehydrogenase (85) activities have been reported to be slightly decreased in the muscle of patients affected with MD, the values given can be considered normal if the wasting of muscle and its infiltration by non-muscular components are taken into consideration. Studies carried out on muscle homogenates of patients with Duchenne MD have shown that they have a normal oxidative phosphorylation (indicated by a normal P : O ratio), but with a depressed respiratory control, characteristic of a loose coupling of oxidative phosphorylation (148). These results have not been obtained in dystrophic mice where the different parameters of

oxidative phosphorylation have been found to be normal (149). In children affected with Duchenne MD an important reduction has been found in the concentration of the end product of oxidative phosphorylation, ATP (150). This reduction could result from an increase in ATPase activity or a defect in ATP synthesis. The real importance of this reduction in ATP should be studied together with the concentration of the other nucleotides forming the adenylate system (AMP, ADP, ATP) recently discussed under the concept of "Energy Charge" (151).

Oxidative phosphorylation is an aerobic process. One of the important factors in giving an adequate supply of oxygen to the mitochondria is the myoglobin concentration inside the muscle cell. The role of myoglobin is to facilitate the inward diffusion of oxygen into muscle. This myoglobin-facilitated oxygen flux results from the movement of oxymyoglobin molecules from the periphery to the center of the fiber (152).

After the discovery by Pauling (153) of the alteration in the molecule of hemoglobin as the cause of sickle cell anemia, a discovery that marks the beginning of molecular pathology, it was thought that a similar situation might occur in Muscular Dystrophy involving some alteration in the myoglobin molecule. Studies in this field have shown that the amount of myoglobin is decreased in the muscle of these patients (154). More recently (155) changes in the UV spectrum of the myoglobin of affected children has been reported. An abnormal chromatographic, electrophonetic and spectral behaviour has been found in the myoglobin from heterozygote carriers of the Duchenne type MD (156); this behaviour being intermediate between that of normal subjects and that of persons with severe myodystrophy. Although some authors have reported normal

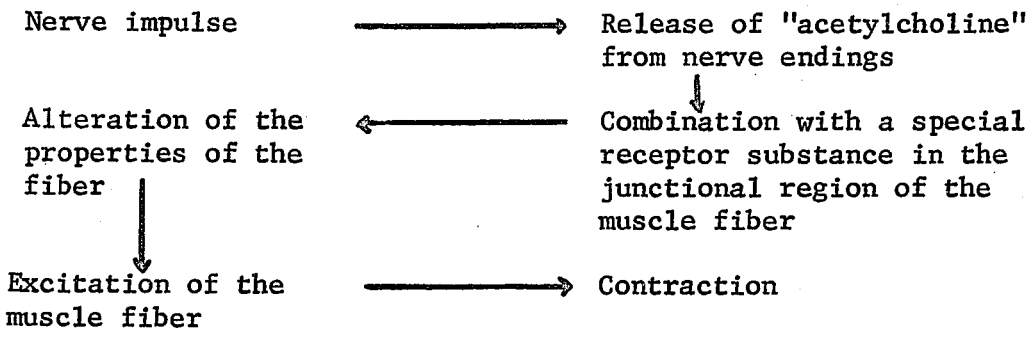
"fingerprint" patterns and chromatographic, electrophoretic and spectral behaviour identical with normal myoglobin, a decrease in adult myoglobin and an increased amount of fetal myoglobin were found suggesting some defect in its maturation (157). An analogy has been traced between the etio-pathogenesis of progressive muscular dystrophy and that of obstructive cardiomyopathy leading to the hypothesis that altered myoglobin plays a fundamental pathogenetic role in both diseases (158).

(f) The Myoneural Junction

The myoneural junction is represented by a highly specialized structure responsible for the transmission of impulses from nerve to muscle. This structure is formed by the motor end plate and a modification of the sarcolemma that constitutes the post-synaptic membrane or receptor site. The neural portion or motor end plate, covered by cytoplasm of the Schwann cells as a roof, lies in a depression of the muscle fiber surface called the primary synaptic cleft. The sarcolemma of this region extends inward to form a complicated system of infoldings that are the secondary synaptic cleft. The number of the latter can vary in different types of fiber. The plasma membranes bounding the axon and muscle fiber are separated at all points along the primary synaptic cleft by a single basal lamina which presumably represents a fusion of basal lamina from the two cells. An extension of this structure enters each secondary synaptic cleft as a single layer and continues along each wall of the cleft.

The physiological concept of the motor end plate arose in 1850 with Claude Bernard as a result of his experiments with curare. He observed that curare had no effect on muscle excitability or nerve conductibility, but it blocked the passage of stimulus from nerve to

muscle. Later, it was shown that the transmission of the impulse from nerve to muscle results from the release of an active substance that has been identified as acetylcholine (AcCh) or an "acetylcholine-like" substance. The whole process is believed to occur as follows:



The role of the motor end plate as transmitter of impulses responsible for muscle contraction has been extensively studied and there are recent reviews on this subject (159).

Another function related to the motor end plate about which little is known is the "neurotrophic effect" which presumably has a major role in the regulation of the metabolism and differentiation of muscle. Although there are many facts in favor of a neurotrophic influence on muscle no such factor has been found to date. The phenomenon of amphibian limb regeneration gives the best model for study of the trophic function of the neuron. It has been shown that the presence of nerves in the amputation site in the salamander is a necessary condition for limb regeneration (160) with the nerves not only initiating the process but also maintaining it (161).

As a special case of Cannon's law of denervation (162) it is known that the muscle cell becomes more sensitive to acetylcholine once it has been denervated, becoming in this respect like fetal muscle

in which the entire length of the cell is sensitive to acetylcholine. If this denervated muscle is reinervated the acetylcholine-sensitive area is reduced until confined to the region of the motor end plate (163).

The influence of nerve on muscle differentiation has also been demonstrated. As we have seen before (96), muscle fibers have been classified into several groups. One of the characteristics of each muscle is its distribution of different fibers (e.g. slow versus fast). It has been demonstrated that after operative cross-union of nerves between fast and slow muscles a change in the muscle fibers takes place (164). Also it has been shown that in the morphological and cytochemical maturation of infantile muscle the intact nerves are important in regulating the transformation from myotubes to the adult differentiated fiber (165).

It is of general knowledge that muscle after denervation undergoes inactivity and atrophy. This atrophy is in part a consequence of disuse, but also there is an intrinsic component in the denervation itself. As already noted it has not been resolved whether atrophy is due to the cessation of neurotransmitter released at the myoneural junction or to some other factor. There is evidence in favor of the latter possibility. For example, it has been found that the length of the muscle fiber sensitive to acetyl-choline returns to normal after reinnervation, but before transmission has been restored (163). It is noteworthy that hypersensitivity to acetylcholine was not abolished by prolonged application of acetylcholine (166). After a chronic complete block of the sciatic nerve by lidocaine the EMG of the muscle affected showed a complete electrical silence but the surgically denervated leg

showed abnormal EMG potentials on the third day and widespread fibrillation on the fourth (167). These findings support the theory of an independence between trophic neural influence on muscle and acetylcholine release at the myoneural junction. Flow of material down the axon has been reported (168) and it could be related to this effect.

Normal muscle contains different types of fibers in a mosaic-like structure. A pattern contrasting with this, consisting of large groups of I and II fiber, called "type grouping", has been found to be correlated with chronic denervation diseases in humans. A possible new approach to the human "neuromuscular" diseases is that based on the study of selective or non-selective susceptibility of muscle fiber types. The proposition is that in humans there are types I and II lower motor neurons (inervating uniform units of type I and II muscle fibers, respectively) with different metabolic and physiological properties (169). Several muscle diseases have been reported where there is a predominant involvement of one of the fiber types, i.e., central core disease or rod myopathy (169). In Duchenne muscular dystrophy, on the contrary, there is no evidence of preferential fiber type involvement but both types are affected and it is less easy to distinguish fiber types than in normal muscle (170).

In mice affected with muscular dystrophy it was found that some alterations, like the presence of "fibrillation potentials", closely resemble those found in muscle of chronically denervated animals (171). Pharmacological studies have shown that the isolated nerve-muscle preparations of dystrophic mice are hypersensitive to inhibitors of acetylcholinesterases; these preparations respond with a violent twitch to physostigmine (172), and they show an increased resistance to drugs

that compete with AcCh for receptor sites such as d-tubocurarine (173). These experiments are suggestive of a defect existing at the myoneural junction of dystrophic mice.

With electron microscopy it was found that some changes in the intramuscular nerve endings are fairly constant in Duchenne MD, depending upon the degree of atrophy and fibrosis of the muscle. In slightly affected muscles the only observed changes were abnormal variations in size of the motor end plate, corresponding to an abnormally large spectrum of muscle fiber size. The main changes observed are: 1) fine beaded fibers wandering in a thickened endomysium, 2) reduced end-plates, 3) expanded irregular end-plates. However these changes could be secondary to a muscular defect. But in some cases, the pictures observed have suggested the possibility that a primary neural involvement should play a part in the clinical picture, or that some disease processes could affect both nervous and contractile elements (174).

The possibility of an alteration of some trophic effect of nerve on muscle in the etiopathogenesis of Muscular Dystrophy cannot yet be ruled out, and in general the statement of Erb in 1890 might still be valid: "I cannot avoid the suspicion that after all the affection may be dependent upon the nervous system. It is tempting to suppose, as I formerly expressed it, that we have to do with a kind of trophoneurosis having its origin in the trophic centres of the cord - a disturbance of the function of these centres which finds its expression in the very complicated muscle changes of the disease".

CHAPTER III

METHODS

A. Tissue Sources

1) Animals

The experimental animals used in these experiments were dystrophic mice (dydy) of strain 129 and littermate controls (either non-affected heterozygous carriers (Dydy) or non-affected homozygous animals (DyDy)) obtained from the Jackson Memorial Laboratory, Bar Harbour, Maine. In most of the experiments the animals were fed ad libitum with standard Purina laboratory chow and allowed free access to water. In some experiments they were fed a high-carbohydrate diet of the composition indicated in Table III. Tissues (skeletal muscle, liver, kidney and brain) were removed and used in a number of studies as described in later sections. The age of mice ranged from 6-11 weeks.

2) Human muscle biopsies

Muscle biopsies from gastrocnemius muscle were obtained under anaesthesia with pentothal and halothane by the attending surgeon. The biopsies came from the following groups: 3 from normal controls; 9 from patients affected with Duchenne MD; 1 from a Duchenne MD carrier; 2 from patients with congenital myopathia; 2 from patients with facio-scapulo-humeral MD; 2 from patients affected with limb-girdle MD and 2 from patients with a late onset myopathy.

TABLE III

Composition of High-Carbohydrate Diet for Mice

Casein:	15.8 %
Vitaminized casein:*	5.0 %
Sucrose:	73.7 %
Fat sol. vitamins mix:**	1.5 %
Salt mixture:***	4.0 %

*

	gm/ 4000 gm casein
Choline chloride	80
Inositol	8
Niacin	4
Calcium pantothenate	4
Thiamine-HCl	0.4
Riboflavin	0.4
Pyridoxine-HCl	0.4
Biotin	0.04
Folic acid	0.04
p-aminobenzoic acid	8.0
Menadione	0.4

**

Corn oil (Mazola)	950 ml
α-Tocopherol	6 ml
Ostogen (Frosst) (Vit. A-D)	50 ml

***

	gm / 1 kg
$CaCO_3$	300
$CaHPO_4 .2\ H_2O$	75
$CoCl_2 .\ 6\ H_2O$	0.05
$CuSO_4 .\ 5\ H_2O$	0.30
$Fe(C_6H_5O_7)_2 .\ 6\ H_2O$	27.5
$MgSO_4 .\ 7\ H_2O$	102
$MnSO_4 .\ \ H_2O$	3.82
KI	0.8
K_2HPO_4	322
NaCl	167
$ZnCl_2$	0.25

B. Reagents

1) Labelled compounds

D-glucose-U-^{14}C (2.9 mc per mmole), and palmitic acid-1-^{14}C (44.3 mc per mmole) were purchased from the Radiochemical Centre, Amersham, Bucks. (England). Tryptamine-2-^{14}C-bisuccinate (8.9 mc per mmole) was obtained from the New England Nuclear Corporation, Boston, Mass. Glycerol-tri-(Palmitate-1-^{14}C) and Glycerol-1-^{14}C were obtained from Amersham-Searle Corporation, Arlington Heights, Illinois.

2) Other reagents and materials

The following chemicals were obtained commercially, as follows: ribose-5-phosphate (Na^+), phenazine methosulphate, hexokinase from yeast (type III), cytochrome c (from horse heart type II), D, L-carnitine, Na-succinate, Na-alpha-ketoglutarate, Na-deoxycholate, malate dehydrogenase (from pig heart), citrate synthetase, glycerokinase, phosphotransacetylase, NADH, palmitic acid, D, 1-α-glycerophosphate (Na^+), standard cholesterol, acetyl CoA, L-ascorbic acid, albumin (BSA), N-ethyl maleimide, and gum arabic were purchased from the SIGMA Chemical Company, St. Louis, Missouri. CoA, ATP, ADP, NADP and NAD were obtained from P-L Biochemicals, Inc., Milwaukee, Wisconsin. Malic acid and oxaloacetic acid came from Nutritional Biochemical Corporation, Cleveland, Ohio. Na-citrate was supplied by the Mallinckrodt Chemical Works, Montreal. Na-EDTA, florisil, glucose, digitonin, dioxane, anhydrous ethyl ether, tripalmitin, tributyrin, propylene glycol, were purchased from Fisher Scientific Company, Fairlawn, New Jersey. D, L-isocitric acid and Na-fumarate were supplied by Calbiochem, Los Angeles, California, and cysteine hydrochloride by Eastman Chemicals, Rochester, New York.

Acetyl chloride, phenol reagent, petroleum ether, and Nonidet P-42 were obtained from the British Drug Houses, Ltd., Poole, England. Rotenone was kindly provided by Dr. Chefurka from the Agriculture Research Institute, London, Ontario.

C. Chemical Preparation of Substrates

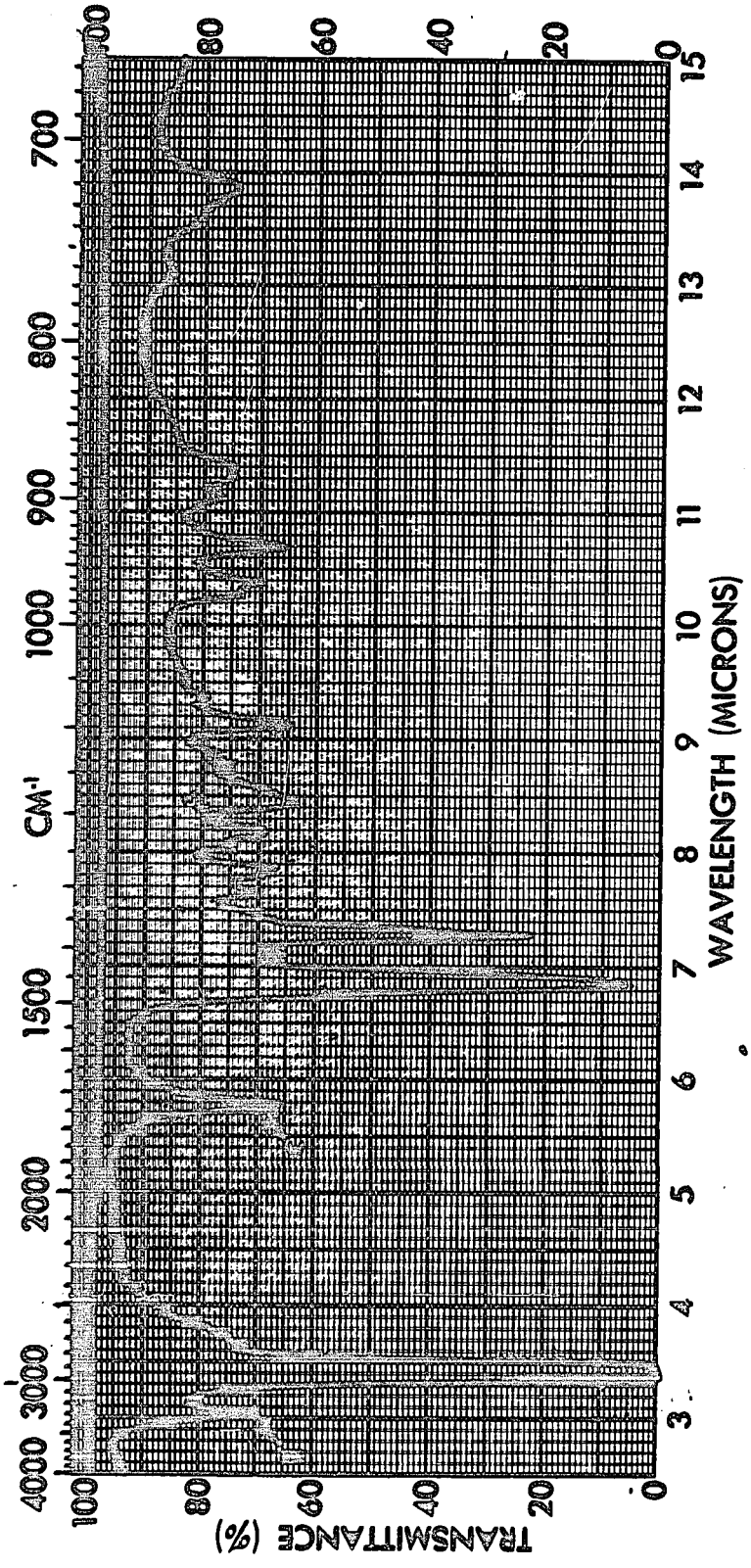
1) Albumin-bound Palmitate-1-^{14}C

The method of Milstein and Driscoll (175) was used in the preparation of albumin-bound palmitate-1-^{14}C. The molar ratio of albumin to palmitate was 0.14.

2) D,L-acetylcarnitine

D,L-Acetylcarnitine was synthesized by a slight modification of the method of Fraenkel and Friedman (176): D,L-carnitine hydrochloride was dissolved in the minimum amount of warm acetic acid required and left in contact with 10 volumes of acetyl chloride overnight at 50^{o} C with continuous shaking. The reaction mixture was then evaporated almost to dryness in a rotary evaporator and the acetylcarnitine precipitated by successive additions of acetone. After standing overnight at 4^{o} C the crystals were collected, washed with acetone and recrystallized from n-butanol. The product thus obtained had the following characteristics: (a) it gave a positive Hestrin reaction, indicating the appearance of an ester bond, (b) it gave a single spot on paper chromatography using as solvent methyl ethyl ketone- isopropyl alcohol-0.1N HCl (3:5:2) with an Rf of 0.7 for acetylcarnitine and 0.6 for carnitine, (c) by infrared analysis (Figures 4 and 5) a peak at 1750 cm^{-1} characteristic of the ester linkage was observed and the peak at 3300 cm^{-1} characteristic of the -OH group of carnitine was no longer present. The

Figure 4 - Infrared spectra of D,L-carnitine chloride, made in Nujol phase. Peak A at 3300 cm^{-1} represents the -OH group. Peak C at 1730 cm^{-1} represents the -COOH group.


TRANSMITTANCE (%)
CM⁻¹
WAVELENGTH (MICRONS)

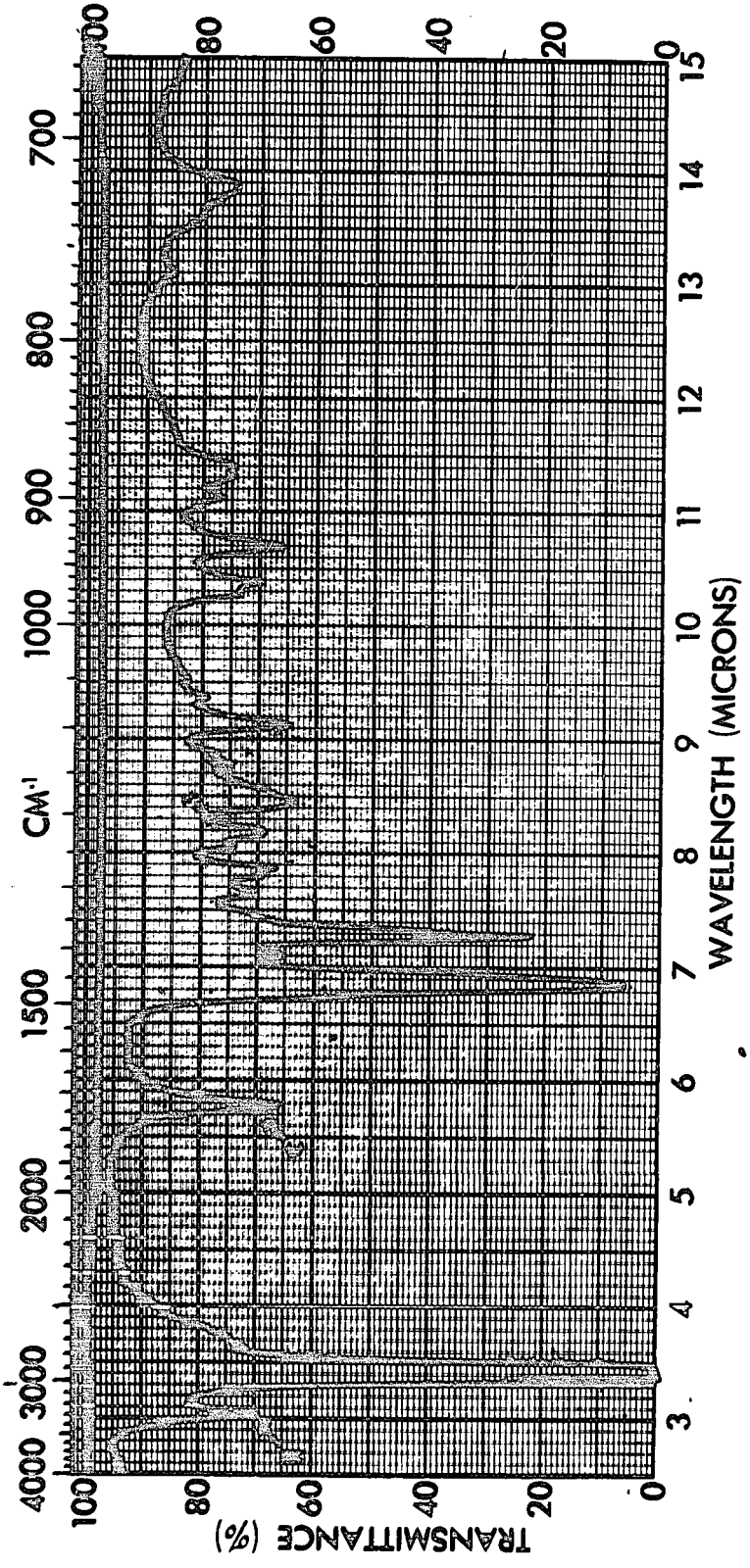
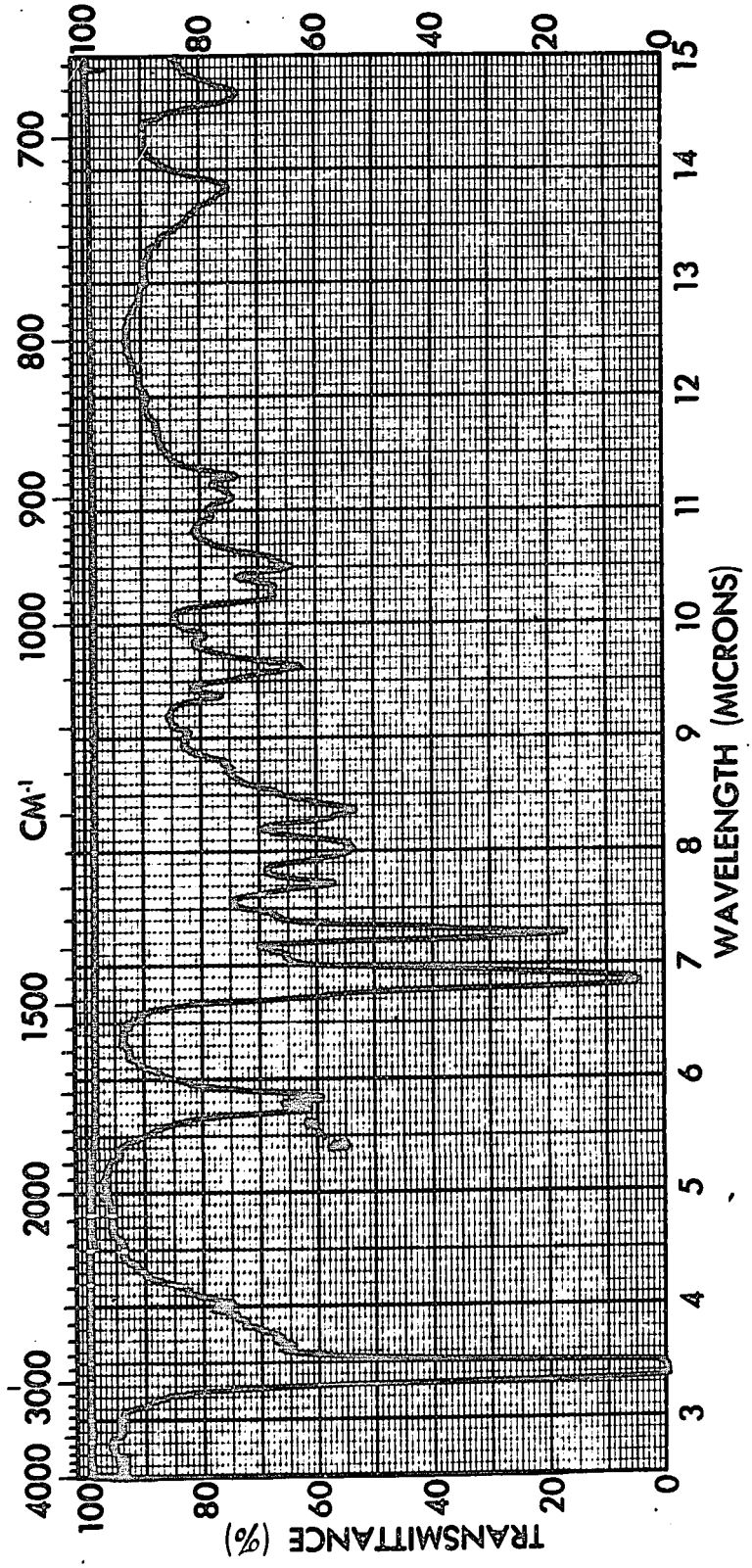
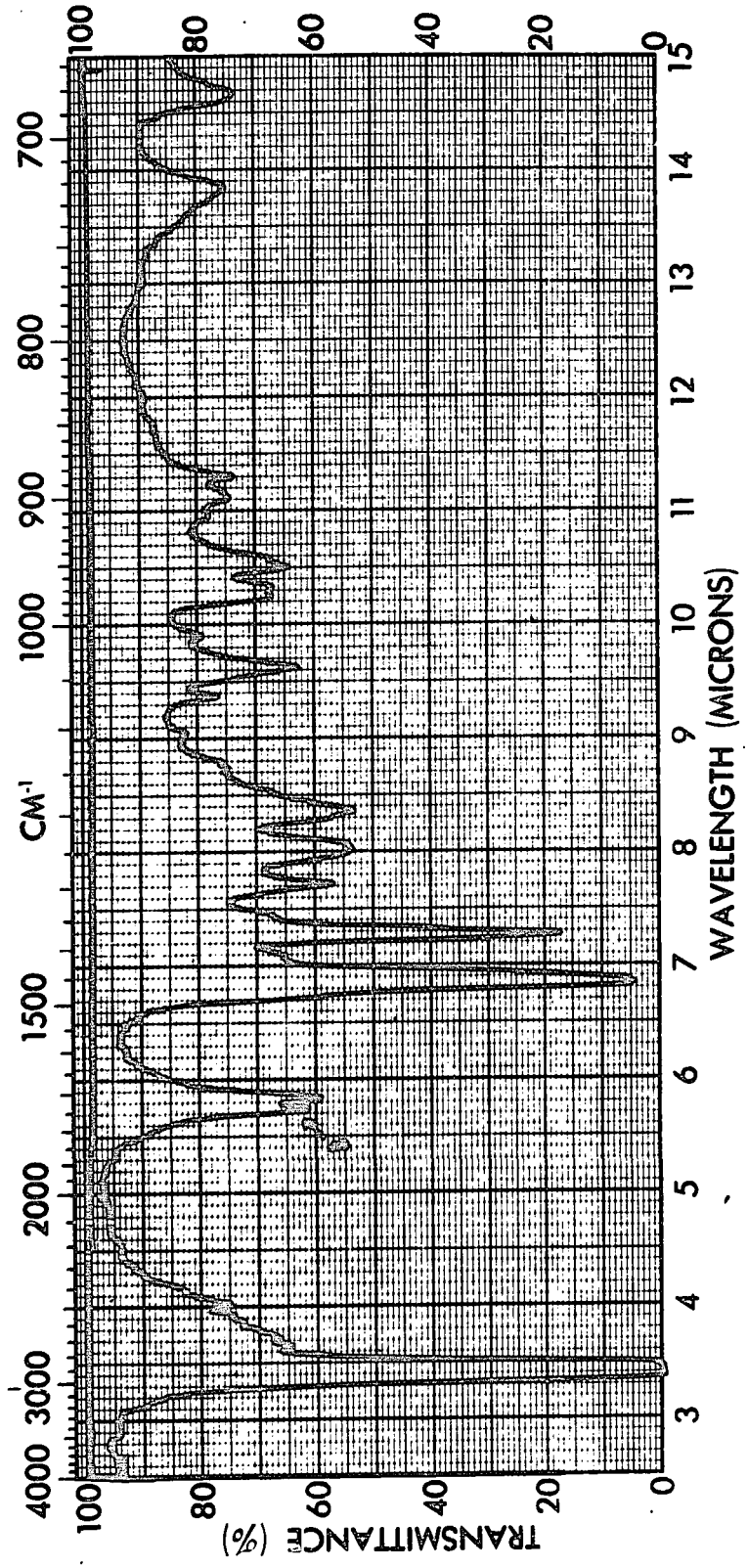

TRANSMITTANCE (%)
CM⁻¹
WAVELENGTH (MICRONS)
4000
3000
2000
1500
1000
900
800
700
100
80
60
40
20
0
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 5 - Infrared spectra of acetyl-D,L-carnitine. HCl, made in Nujol phase. Peak B at 1750 cm^{-1} represents the -CO-O- linkage.


TRANSMITTANCE (%)
100
80
60
40
20
0
4000
3000
2000
1500
1000
900
800
700
CM⁻¹
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)


TRANSMITTANCE (%)
CM-1
WAVELENGTH (MICRONS)
4000
3000
2000
1500
1000
900
800
700
100
80
60
40
20
0
3
4
5
6
7
8
9
10
11
12
13
14
15

purity of the product was 99% when analyzed according to Hestrin (177).

3) sn-^{14}C-Glycerol-3-phosphate (Li^+)

sn-^{14}C-Glycerol-3-phosphate (Li^+) was synthesized by the method of Bublitz and Kennedy (178) as modified by Possmayer (179). In the method glycerol-1-^{14}C is phosphorylated by ATP in the presence of the enzyme, glycerokinase. Glycerol-1-^{14}C was incubated in the presence of enzyme and a slight excess of Mg^{++} and ATP in a glycine buffer medium at pH 9.8. After two hours incubation at 37° C the reaction was stopped by adding an equal volume of 10% TCA containing 200 mg charcoal per ml. After centrifugation the supernatant was collected and the precipitate washed once with TCA. The pooled supernatants were washed with ether. The sn-^{14}C-glycerol-3-phosphoric acid present in the aqueous fraction was purified by the method of Chang and Kennedy (180) which involves elution from a Dowex-1-8-X (formate), 200-400 mesh, column with a formic acid gradient. After evaporation to dryness of the fractions containing the labelled glycerophosphate, the product obtained was diluted in a small volume of water and neutralized to pH 7 with LiOH.

D. Analytical Methods

1) Protein determination

Protein was determined according to the method of Lowry et al. (181) Bovine serum albumin, previously standardized against ammonium sulfate, was used as standard.

2) Nitrogen determination

For the determination of nitrogen in tissues the method of Koch and McMeekin (182) was used as follows: an aliquot of tissue homogenate (equivalent to approximately 10 mg wet weight tissue was digested

with 1 ml 50% H_2SO_4. 3 to 4 drops of 30% H_2O_2 were added at successive intervals until the digestion was complete (usually after 1 hour). The clear digestion was transferred to a 10 ml volumetric flask and made up to volume with distilled water. A sample of 1 ml was taken for nitrogen determination using Nessler's reagent.

3) Non-collagen nitrogen

Collagen was precipitated using dilute alkaline solution according to the method of Lilienthal, Jr. et al (183) in the following way: 1 ml 10% muscle homogenate was mixed with 9 volumes 0.05N NaOH. After standing overnight the mixture was centrifuged for 30 minutes at 90,000 x g. Then 1-2 ml of the supernatant were used for the nitrogen determination as outlined above (182).

4) Cholesterol

Cholesterol was determined by the method of Schoenheimer and Sperry (184) as follows: to the sample of cholesterol (in its digitonide form) dissolved in 1 ml glacial acetic acid were added 4 ml of a mixture of acetic anhydride and sulphuric acid (20 : 1 v/v). After standing for 30 minutes the intensity of the blue colour was measured at 750 nm. A standard curve was done using a standard solution of cholesterol (Sigma).

5) Ribose and Ribose-5-Phosphate

The method of Mejbaum (185) for pentose determination was used to estimate ribose either in its free form or as ribose-5-phosphate. The sample, in a volume of 3 ml, is heated in a boiling water bath for 30 minutes together with 3 mls of a solution of 1% orcinol and 0.1% $FeCl_3$ in concentrated HCl. After cooling, the intensity of the green

colour formed is read at 660 nm. Solutions of ribose were used as standard.

6) Phosphorus

Phosphorus was determined by the method of Ernster et al. (186) which is based on the reaction between ammonium molybdate and inorganic phosphorus in an acid medium to form phosphomolybdic acid. The latter is extracted into isobutanol-benzene and reduced by stannous chloride to give a blue colour which is measured at 730 nm. All measurements were made relative to a water blank.

Samples of phospholipids were converted to inorganic phosphate by wet ashing using 1 ml 60% perchloric acid and boiling for about 30 minutes. Prior to ashing, care was taken to remove all organic solvents from the aliquot of phospholipids.

7) Free fatty acids

Free fatty acids were determined either by titration against a dilute solution of NaOH or by a colorimetric method. These methods were carried out as follows:

(a) Titrimetric method: To the fatty acid sample, dissolved in 2 mls of methanol, were added 2 ml of 0.001% cresol red indicator in 90% methanol. Then, the mixture was titrated with 0.05 N NaOH (in 90% methanol) previously standardized against palmitic acid.

(b) Colorimetric method: The colorimetric method of Duncombe (187) was used. This method is dependent on the reaction of fatty acids with copper and subsequent extraction of the copper-fatty acid salt followed by determination of the copper using diethyl-dithiocarbamate as reagent.

8) Carnitine

The method used is based on that described by Friedman (188), and depends on the complexing of carnitine with bromophenol blue in alkaline solution and subsequent extraction of the coloured complex into a mixture of ethylene dichloride and isoamyl alcohol. Since other quaternary ammonium compounds such as choline also complex, a preliminary separation of carnitine from choline is carried out using a cation-exchange resin (IRC-50) at pH 7. The method was applied to muscle as follows: hind leg muscle of two mice (normal or dystrophic) are pooled and homogenized in 5 volumes 95% ethanol. After filtration the precipitate is washed with 1 volume 95% ethanol. Proteins are then precipitated from the combined ethanol extracts using $Ba(OH)_2$ and $ZnSO_4.7H_2O$ according to Somogyi (189). The mixture is made up to 10 ml and centrifuged. Then 1 ml of the supernatant is mixed with 1 ml 0.1 M Na-phosphate buffer, pH 7. This mixture is applied to a column of IRC-50 resin (about 100 mesh) and eluted with 15 ml of water. The eluate is collected and dried on a water bath at 80° C. The residue is redissolved in 10 ml absolute ethanol and the resulting solution is acidified with 2 drops of concentrated HCl. After shaking slowly for 1 hour in a bath at 80° C, the acidified alcohol is evaporated with an air jet. The residue is mixed with 1 ml 60% K_2HPO_4 and 300 mgs anhydrous Na_2CO_3. After shaking for a few minutes to allow solution to take place 0.5 ml bromophenol blue (75 mg per 100 ml 30% K_2PO_4H) is added. The mixture is shaken for 10 minutes and then 5 ml ethylene dichloride (containing 4% isoamyl alcohol) are added. After further shaking for 10 minutes the upper layer is decanted and the absorption measured in a

Beckman spectrophotometer at 602 nm. Solutions of D,L-carnitine.HCl in 95% alcohol were used as standard. The recovery of carnitine obtained was 80%.

9) Gas-Liquid Chromatography of Fatty Acids

Gas liquid chromatography of the fatty acids from the free fatty acid pool triglycerides and phospholipids of muscle was carried out using the corresponding methyl esters. The methyl esters were obtained by transmethylation following essentially the method developed in this laboratory by Dr. Sinclair (190). Samples of fatty acids dissolved in 5 mls 5% H_2SO_4 in methanol were placed in sealed tubes under nitrogen and heated for 3 hours at 70° C. After cooling the tubes, 3 ml of water were added and the methyl esters were extracted with 5 ml petroleum ether. The latter extract was washed several times with water to remove all traces of H_2SO_4. After evaporating to dryness in a stream of nitrogen the esters were redissolved in 0.1 ml acetone. An aliquot of 10 µl was used for gas-liquid chromatography separation. All the solvents used contained the antioxidant butylated hydroxy toluene (BHT) in a concentration of 10 mg/liter.

The determinations were carried out in a gas chromatograph, Model 1609, (F & M Scientific Co., Avondale, Penn.). The columns used (6 ft. x 1/4 in.) contained 20% ethyleneglycol succinate polyester on acid-washed Diatoport W, 80-100 mesh. The columns were operated at 195° C. A hydrogen flame ionization detector was used; the temperature of the injection port was 230° C; that of the detector block was 250° C and the carrier gas (Helium) flow was 150 ml./min. Peaks on the tracing were identified by comparison of their retention time with those observed

for known fatty acid methyl esters. Peak areas were estimated according to Carroll (191) using the product retention time x peak height.

10) Coenzyme A

The intramitochondrial pool of acid-soluble CoA was determined by the method of Allred and Guy (192) as modified by Lee and Fritz (193). Because of the small amount of muscle available from mice each determination was carried out on the pooled mitochondria from the hind leg muscle of three mice. Mitochondria, isolated as described later, in a total volume of about 0.4 ml were treated with 0.15 ml 15% perchloric acid followed by neutralization with KOH. The clear supernatant was then made up to 1 ml and aliquots of it were taken for CoA determination. The incubation mixture contained in a volume of 1 ml: 150 µmoles Tris-HCl buffer pH 7.2; 13 µmoles Na-malate; 0.45 µmole NAD; 12 µmoles acetylphosphate (Li^+); 15 µmoles KCl; 3 mg dithioerythritol; 4 units malic dehydrogenase and 2 units citrate synthetase. The reaction was started after addition of 30 units phosphotransacetylase. The reaction was carried out at room temperature and CoA was determined as a function of the change in optical density at 340 nm. With this method a linear response was observed for amounts of CoA per cuvette from 0.3 nmole to 4 nmole. The results are expressed in nmoles CoA per unit succinate cytochrome c reductase.

E. Enzymatic Determinations

1) In muscle homogenates

(a) Short-chain triglyceride lipase (Tributyrinase): Tributyrinase activity has been determined by a slight modification of the method of

Martin and Peers (194) which is based on the measurement of CO_2 released from a bicarbonate buffer system by the butyric acid liberated from the hydrolysis of tributyrin. The determinations were done at 37° C using a Warburg apparatus. A 5% homogenate of muscle was prepared in distilled water. The reaction flask contained: 1.5 ml 0.025 M $NaHCO_3$ previously gassed with N_2-CO_2 (95:5) and 1 ml muscle homogenate in the main chamber, and 0.5 ml of 0.06M tributyrin in 0.0148 M $NaHCO_3$ solution in the side arm. Before addition to the side arm the tributyrin was emulsified by sonication for 30 seconds in a Branson Sonic (Model No. 5125) tuned to half the maximal output, in the presence of one drop of 10% Nonidet. After gassing the system with the N_2-CO_2 mixture for 3 minutes, the flasks were placed in the water bath. After equilibration of the temperature for 10 minutes the substrate was tipped into the main chamber and readings were taken every 10 minutes for 1 hour after the first 5 minutes. The results are expressed in micromoles of tributyrin hydrolysed (a) per g wet muscle, (b) per mg N_2, and (c) per mg non-collagen nitrogen (NCN).

(b) Long-chain triglyceride lipase (Tripalmitinase): The isotopic method of Kaplan (195) was used for the determination of Tripalmitinase activity in muscle. A 20% muscle homogenate was prepared in water. After treatment with Cutscum (1% final concentration) for 10 minutes at 0° C, the homogenate was centrifuged at 14,000 x g for 20 minutes. Lipase activity in the supernatant was then determined as follows: Incubation tubes were prepared containing a final volume of 1 ml: 60 μmoles Na-phosphate buffer, pH 6.5; 0.3 ml of supernatant fraction, and 3 μmoles of glycerol-tri-(Palmitate-1-^{14}C) with radioactivity corresponding

to 120,000 d.p.m. After incubation at 37° C for 30 minutes the reaction was stopped by the addition of 5 ml isopropanol/heptane/1 N H_2SO_4 (40: 10: 1). The extraction of the free palmitic acid and counting of radioactivity was carried out as indicated by Kaplan (195).

(c)Monoaminoxidase : Monoaminoxidase was determined by the isotopic method of Wurtman-Axelrod (196), using ^{14}C-tryptamine-bisuccinate as substrate. The incubation was carried out at 37° C for 15 minutes. The incubation mixture contained in a final volume of 0.5 ml: K-phosphate buffer, pH 7.4, 150 μmoles; ^{14}C-tryptamine-bisuccinate, 8 nmoles (containing 150,000 dpm); 5% muscle homogenate in distilled water, 0.1 ml. The incubation was stopped by addition of 0.2 ml 2N HCl and then 6 ml of toluene were added. The mixture was shaken and centrifuged. 3 ml aliquots of the organic layer containing the deaminated matabolites of ^{14}C-tryptamine were transferred to a vial containing 10 ml of counting solution No. 1 described later. The results are expressed in mμmoles of tryptamine oxidized per minute per mg NCN.

(d) Cytochrome oxidase: The activity of cytochrome oxidase in muscle homogenates was determined by the manometric method of Potter (197) as described by Humoller et al. (198). In this method oxygen uptake is determined in a system where muscle homogenate is utilizing ascorbic acid as substrate. The determinations were carried out at two different concentrations of muscle and the results were corrected for autooxidation of ascorbic acid. The incubation mixture contained in a final volume of 3 ml in the main chamber of the Warburg flask: 200 μmoles Na-phosphate buffer pH 7.4; 0.24 μmoles cytochrome c; 120 μmoles of $AlCl_3$; 0.1 ml of 5-10% muscle homogenate in distilled water. The side arm contained 34.2 μmoles Na-ascorbate. The center well of the flask contained 0.2 ml

20% KOH. After preincubation at 37° C for 10 minutes, the Na-ascorbate was tipped into the main chamber and the oxygen uptake was determined at 10, 20 and 30 minutes. The results are expressed in μmoles O_2 uptake per minute per gm w.w., or, per mg NCN, or, per mg N.

(e) Succinate dehydrogenase: The activity of succinate dehydrogenase in muscle homogenates was determined by coupling the oxidation of succinate through phenazine methosulfate to the reduction of 2, 6-dichlorophenolindophenol, and following the reaction at 600 nm. in a Cary 15 spectrophotometer, as described by Alvarado and Blanchaer (199). 5% muscle homogenate in 0.25 M sucrose pH 7.4 was treated with one-tenth vol. of 1% Na-deoxycholate for 20 minutes at 4° C. An aliquot (o.1 ml) of the treated homogenate was then added to a 3 ml cuvette containing 1.5 mM cyanide, 20 mM Na-succinate; 0.05 mM 2,6-dichlorophenolindophenol; 0.1% bovine serum albumin, and one of four concentrations of phenazine methosulfate 1.80, 1.35, 0.90 and 0.45 mM. The curve obtained on plotting the reciprocal of the phenazine methosulfate concentrations against the reciprocal of the velocities is extrapolated to infinite phenazine methosulfate concentration to give the value for succinate dehydrogenase.

2) In subcellular fractions of muscle

(a) Preparation of subcellular fractions: Mice were killed by decapitation, the hind leg muscles were removed and placed in a beaker containing 0.15 M KCl precooled in an ice bucket. All of the steps concerned in the isolation of subcellular fractions were carried out at 0-4° C. After removing connective tissue, the muscle was minced and a 5% homogenate was prepared in 0.25 M sucrose pH 7.4, with 0.001 M Tris-

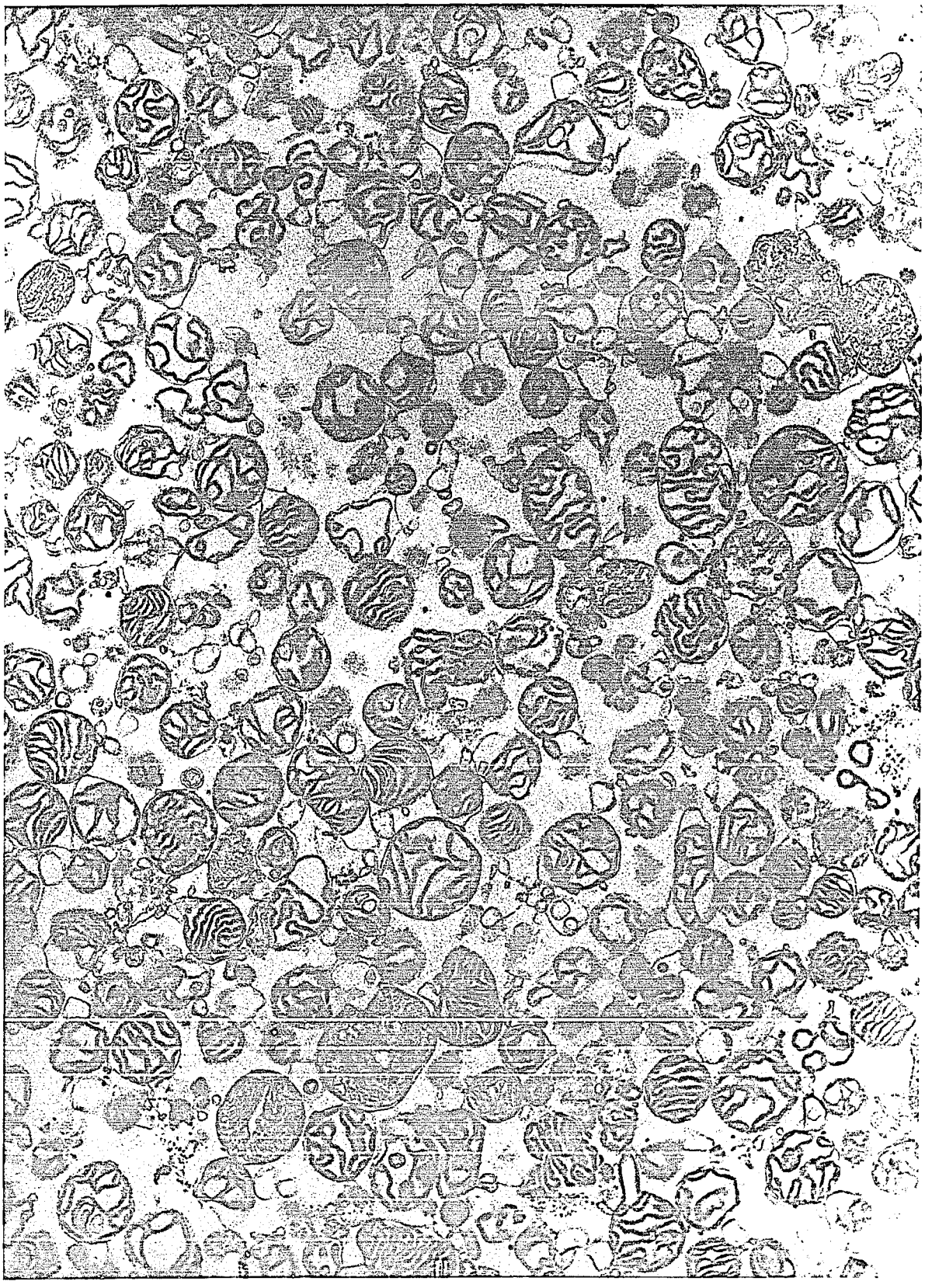
buffer and 0.005 M Na-EDTA. The homogenate was centrifuged at 350 x g for 10 minutes. The supernatant was removed and centrifuged at 9,000 x g for 10 minutes. The precipitate was resuspended in the homogenization medium and centrifuged again at 11,000 x g for 10 minutes to yield a mitochondrial or sarcosomal pellet.

The supernatant obtained after the 11,000 x g centrifugation was then centrifuged at 90,000 x g for 100 minutes in a Spinco Model L ultracentrifuge (No. 40 Head) to yield both a microsomal pellet and a high-speed supernatant fraction.

Mitochondrial and microsomal fractions were tested for NADH-cytochrome c reductase and rotenone insensitive NADH-cytochrome c reductase as described later. As measured, the latter enzyme represents 76.9% of the total cytochrome c reductase in the microsomal pellet and only 8.6% of that found in the mitochondrial pellet. This distribution is consistent with that expected for pellets which are predominantly mitochondrial or microsomal (200). These fractions on examination by electron microscopy yielded the pictures shown in Figures 6 and 7. Experiments carried out in this laboratory gave a respiratory control ratio for the mitochondrial fraction of 6.5, using pyruvate as substrate (201).

(b) Determination of the enzymes of the Krebs cycle: For determination of the activities of the enzymes of the Krebs cycle mitochondria were extracted by treatment with ice-cold 0.1 M K_2HPO_4, pH 8 containing 0.001 M Na-EDTA and 0.1% Na-deoxycholate (6 ml per g muscle). Extraction was allowed to take place for 30 minutes at 0-4° C. After centrifugation

Figure 6 - Mitochondrial fraction obtained from muscle homogenates of mice as described in the Methods. The pellet was fixed with glutaraldehyde and osmic acid and stained with uranyl acetate and lead citrate. Magnification x 20,335.



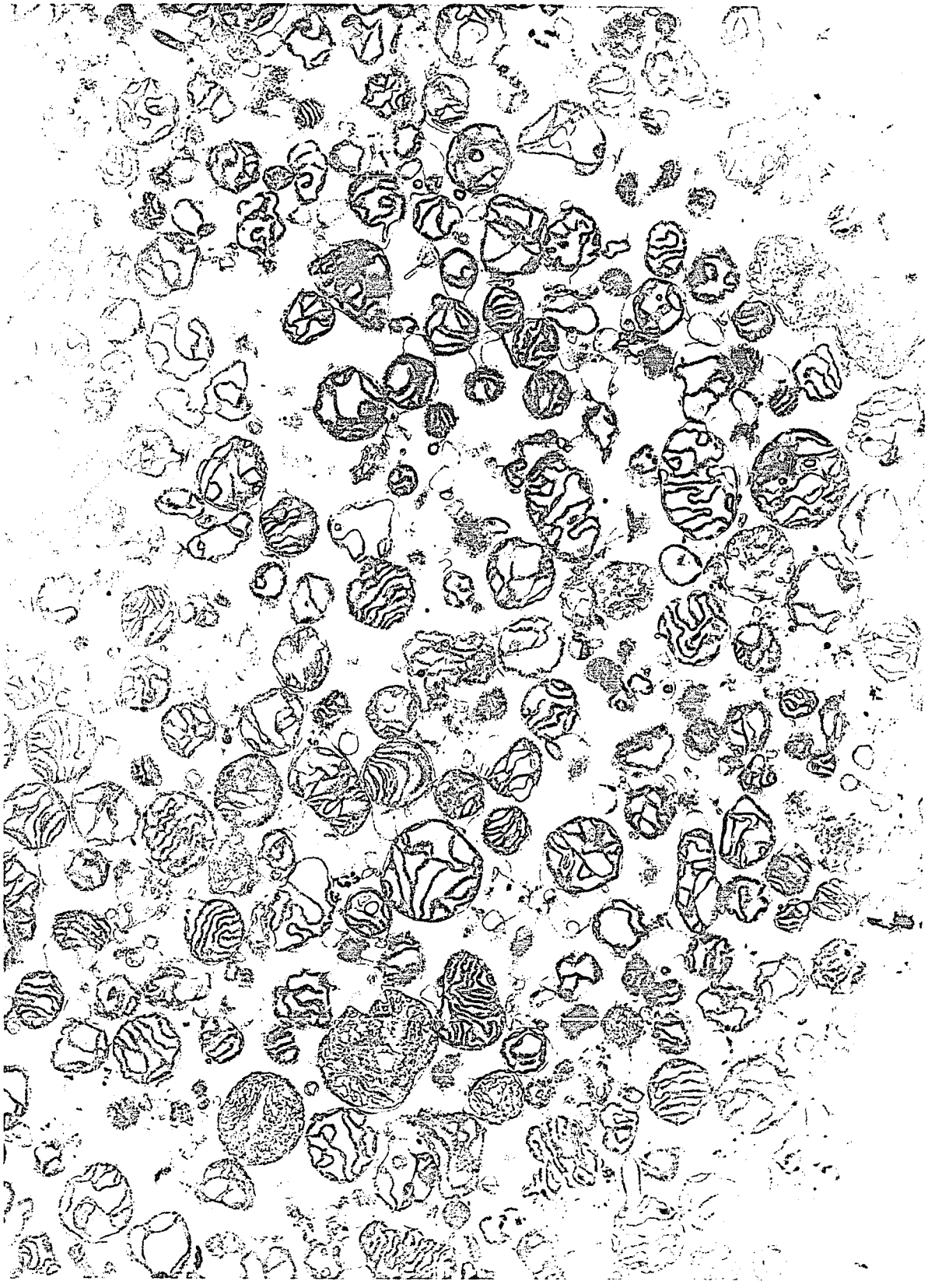
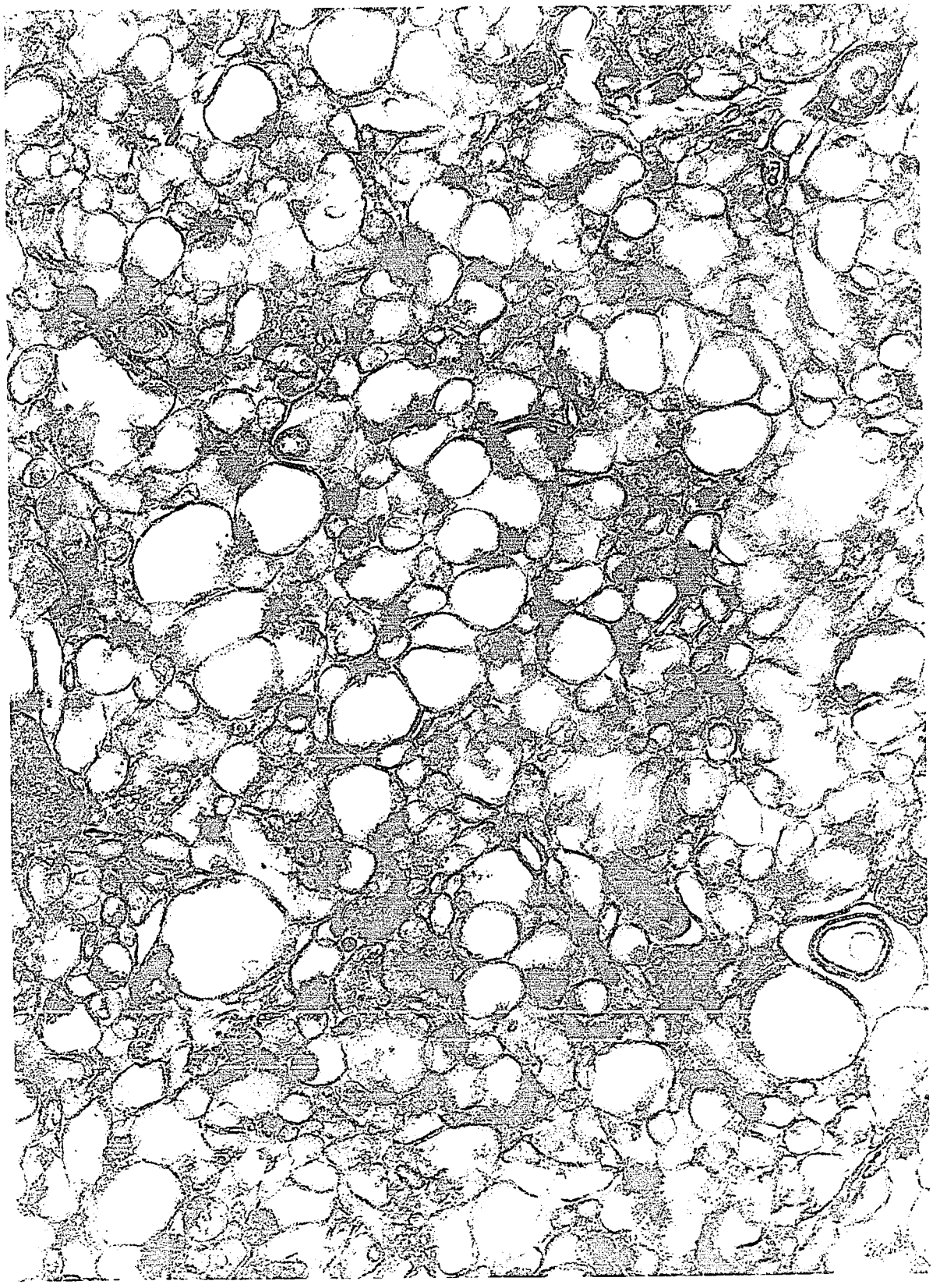


Figure 7 - Microsomal fraction obtained from muscle homogenates of mice as described in the Methods. Fixation and staining of the pellet was carried out as described in Figure 6. Magnification x 51,300.



at 13,000 x g for 30 minutes, aliquots of the supernatant were analyzed for enzymatic activity. Succinate dehydrogenase activity was determined on the deoxycholate-treated mitochondria without further centrifugation.

With the exception of fumarase, aconitase and succinate dehydrogenase, all enzyme activities were measured following the change in optical density at 340 nm due either to the oxidation or reduction of NAD in different systems. Measurements for aconitase were made at 300 nm, for fumarase at 240 nm and in succinate dehydrogenase at 600 nm. The changes in optical density at 300 nm and 240 nm are determined by the synthesis of aconitate and fumarate, respectively. All the determinations were done in a Cary-15 spectrophotometer using a total volume of 3 ml (with the exception of citrate synthetase where 1 ml was used) contained in 1 cm (light-path) cuvettes thermostated at 25° C. The different methods used were taken from the respective references indicated except for slight variations introduced to permit their application to muscle mitochondria. The assays mixtures were as follows:

α) Citrate synthetase (202): 50 μmoles Tris-HCl buffer pH 7.6; 60 μmoles Na-L-malate; 5 μmoles NAD; 0.25 μmoles acetyl CoA; 2.5 μmoles EDTA; 0.1 units malic dehydrogenase, and mitochondrial protein equivalent to 30-60 μg before extraction (b.e.).

β) Aconitase (203): 205 μmoles K-phosphate buffer pH 7.4; 90 μmoles K-citrate; 200-300 mg mitochondrial protein (b.e.).

γ) NADP-Isocitrate dehydrogenase (204): 150 μmoles Tris-buffer pH 7.6; 0.45 μmoles NADP; 2 μmoles $MnSO_4$; 4 μmoles D,L-isocitrate (K^+salt); 40-100 μg mitochondrial protein (b.e.).

δ) NAD-Isocitrate dehydrogenase (205): 150 μmoles Tris-buffer pH 7.6; 1 μmole ADP; 32 μmoles D,L-isocitrate (K^+ salt); 1 μmole NAD; 2 μmoles $MnSO_4$; 40-100 μg mitochondrial protein (b.e.).

ε) α-Ketoglutarate dehydrogenase (206): 150 μmoles Tris-buffer pH 7.6; 0.15 μmoles CoA; 1.5 μmoles NAD; 9 μmoles cysteine; 30 μmoles α-ketoglutarate (K^+salt); 40-100 μg mitochondrial protein (b.e.).

ζ) Succinate dehydrogenase: It was determined as described for muscle homogenates. The amount of mitochondrial protein used in each determination was about 50 μg.

η) Fumarase (207): 250 μmoles K-phosphate buffer pH 7.4; 30 μmoles Na-fumarate; 300-400 μg mitochondrial protein (b.e.).

ϑ) Malate dehydrogenase (208): 225 μmoles Tris-HCl buffer pH 7.6; 0.45 μmole NADH; 0.80 μmole Na-oxaloacetate; 20-25 μg mitochondrial protein (b.e.).

The activity of each enzyme is expressed in units per mg mitochondrial protein. One unit is considered to be the amount of enzyme which causes a change in optical density of 0.001 per minute under the specific conditions used in each individual assay.

(c) Activities of the enzyme complexes of the electron transport chain: Non-extracted mitochondria were used for the determination of the activities. These enzyme complexes were determined measuring the change in optical density at 550 nm (at 22° C) essentially as described by Sottocassa et al. (200). The total volume of the assay mixture was 3 ml contained in 1 cm (light-path) cuvettes. The substrate and KCN (when used) were added after 15 minute preincubation. The different assay mixtures contained:

α) Succinic-cytochrome c reductase: 180 μmoles K-phosphate buffer, pH 7.5; cytochrome c, 1 mg; 3 μmoles KCN; 48 μmoles Na-succinate; mitochondrial protein, about 50 μg.

β) NADH-cytochrome c reductase: 150 μmoles K-phosphate buffer, pH 7.5; 0.3 μmole cytochrome c; 0.3 μmole NADH; 0.9 μmoles KCN; mitochondrial protein, about 50 μg.

γ) Rotenone insensitive NADH cytochrome c reductase: The assay mixture is the same as in the assay for NADH-cytochrome c reductase with the exception that Rotenone was added in a final concentration of 1.3 μmoles.

δ) Cytochrome oxidase: 150 μmoles K-phosphate buffer pH 7.5; reduced cytochrome c, 1.85 mg; mitochondrial protein, about 50 μg.

The activity of each enzyme is expressed as units per mg mitochondrial protein. One unit is the amount of cytochrome c (m μmoles) reduced (or oxidized) per minute. This was calculated from an extinction coefficient for cytochrome c of $18.5 \times 10^{6}\ M^{-1}\ cm^{-1}$.

(d) Monoaminoxidase: It was estimated by the same method used for muscle homogenate. The amount of mitochondrial pellet used was about 100 μg.

(e) Carnitine acetyltransferase (CAT): Mitochondria were extracted as for the Krebs cycle enzyme determinations. CAT activity in the extract was studied by the method of Fritz (209). The NADH formed as a result of the coupling of the reactions catalyzed by malic dehydrogenase and citrate synthetase with that catalyzed by carnitine acetyltransferase. The reaction mixture contained in a final volume of 1 ml the following components: 0.2 μmole acetyl CoA; 10 μmoles Na-malate; 30 μmoles acetyl-

D,L-carnitine; 1 μmole KCN; 0.1 unit citrate synthetase; 0.1 unit malate dehydrogenase; 2.5 μmoles NAD; 100 μmoles Tris-HCl buffer, pH 7.8; and mitochondrial protein. 1 ml cuvettes were preincubated at 37° C for 10 minutes with all of the components of the reaction present except for acetylcarnitine which was added last to start the reaction. The measurement of NADH at 37° C was carried out in a Cary 15 spectrophotometer, at 340 nm.

F. Incubations

1) Incorporation of Palmitate-1-^{14}C and sn-^{14}C-Glycerol-3-phosphate into Triglycerides and Phospholipids of Muscle from Normal and Dystrophic Mice

The incorporation of palmitate-1-^{14}C into triglycerides and phospholipids was studied in a system containing: 15 μmoles glucose; 0.3 μmole NAD; 18 μmoles $MgCl_2$; 15 μmoles ATP, 0.15 μmole CoA; 0.6 μmole of palmitate-1-^{14}C (containing about 215,380 dpm) and 1 ml muscle homogenate (10% homogenate in Krebs-Ringer II medium) in a final volume of 3 ml. The incubation was carried out at 37° C for 20 minutes. When sn-^{14}C-glycerol-3-phosphate was used the incubation medium was: 6 μmoles L-α-glycerophosphate (containing about 500,000 dpm); 30 μmoles ATP; 35 μmoles $MgCl_2$; 60 μmoles phosphate buffer pH 7.5; 75 nmoles CoA and 1 ml muscle homogenate (prepared as before). Incubation was carried out at 37° C for 5 minutes. In both cases the reaction was stopped by the addition of 20 ml chloroform-methanol (2 : 1) and 4 ml methanol. The mixture was shaken, centrifuged and then the supernatant transferred to new tubes. The residue was washed twice with 2 ml of the chloroform-methanol mixture plus 0.4 ml methanol. The pooled supernatants were washed twice with 5 and 3 ml of water respectively. The chloroform

phase was taken to dryness in a rotary evaporator and the residue obtained was redissolved in a small amount of chloroform.

The lipids obtained in this way were passed through small columns of 500 mg activated acid-treated Florisil (210). These columns were prepared in the following way: Pasteur pipettes were sealed at the tip and a small amount of glass wool was placed at the bottom of the thicker portion of the pipette. The latter was filled with chloroform followed by Florisil. After application of the sample the tip of the pipette was broken off and the neutral lipid fraction was eluted with 12 ml chloroform. Then the phospholipid fraction was eluted by the addition of 12 ml methanol. The resolution of the column to separate these two groups was then checked by thin layer chromatography. Only small traces of phospholipid were present in the neutral lipid or chloroform fraction, and the phospholipid fraction was free of any contamination by neutral lipids. The recovery of phospholipid was about 95% and that of triglyceride about 100%. For subsequent analyses both fractions were evaporated to dryness.

When palmitate-1-^{14}C was used the neutral lipid fraction was freed of free fatty acids using a slight modification of the method of Eaton and Steinberg (211). The above residue was dissolved in 10 ml isopropanol-isooctane (40 : 10, v/v). Then 15 ml ethanol-water-1 N NaOH (50 : 44 : 5, v/v/v) were added and the mixture was shaken. After centrifugation the iso-octane phase (upper) was removed and the lower phase was washed twice with 5 ml iso-octane. The combined upper phases obtained in this way were washed again with 10 ml alkaline ethanol. After centrifugation the supernatant was collected, the lower phase was washed twice with 3 ml iso-octane and all of the supernatants were

pooled and made up to 10 ml. 2 ml were taken for radioactivity measurement and the remainder was evaporated to dryness and redissolved in 2 ml petroleum ether.

The lipids obtained from the step above and the phospholipids obtained from the Florisil column were dissolved in 2 ml petroleum ether. Then 0.25 ml was spotted on a silica gel G thin layer plate and developed in the solvent system, petroleum ether-ethyl ether- formic acid-methanol (80 : 20 : 1.5 : 3 v/v/v/v). Standards of free fatty acids and triglycerides were also applied to the plate. After developing, the lipid areas were located using iodine vapour and they were removed by scraping. The lipids in each area were extracted twice with 4.5 ml chloroform-methanol-acetic acid-water (65 : 25 : 4 : 1, v/v/v/v), once with 3 ml methanol, and once with 3 ml methanol-acetic acid:water (94 : 1 : 5, v/v/v). The extracts were pooled into a glass vial used for counting and evaporated to dryness. After the addition of 10 ml of counting solution no. 1 the radioactivity was then determined.

2) Formation of Pentoses and $^{14}CO_2$ from ^{14}C-U-Glucose by high-speed homogenates of muscle

High-speed homogenates of muscle were obtained as already indicated. Incubation was carried out in Warburg flasks at 37° C for 30 minutes using the following system contained in a final volume of 2 ml: potassium phosphate buffer, pH 7.4, 375 moles; hexokinase, 1 mg; ATP, 4 moles; NADP, 0.4 mole; Mg^{++} 8 moles; phenazine methosulfate, 0.4 moles; glucose-U-^{14}C, 10 moles (containing 868,000 d.p.m.) and high-speed supernatant of muscle, about 1 mg protein.

The $^{14}CO_2$ released was trapped in a Hyamine-impregnated filter paper placed in the center well of the Warburg flask. After 30 minutes incubation, the reaction was stopped by the addition of 0.25 ml 50% trichloroacetic acid (TCA) contained in the side arm. After allowing

10 minutes for the release of the CO_2 and its absorption, the filter paper was removed and placed in counting vials together with 0.5 ml methanol used to wash the center well. Radioactivity was determined after adding 10 ml counting solution no. 1.

The above incubation mixture was centrifuged and the protein-free supernatant was washed with ether in order to remove the TCA. The phosphoric esters were precipitated with barium acetate as described by LePage (212). Pentoses were determined as described earlier. The recovery of ribose-5-phosphate was determined to be about 85%.

3) Utilization of Ribose-5-phosphate by high-speed homogenates of muscle

This was studied using the same high-speed supernatant fraction described in the previous section. Before incubation this fraction was dialyzed against 0.01 M K-phosphate buffer, pH 7.4. Incubation was carried out for 0,15 and 30 minutes at 37° C in Warburg flasks containing in a final volume of 2 ml: 375 μmoles potassium phosphate buffer, pH 7.4; 8 μmoles ribose-5-phosphate and high-speed supernatant of muscle (about 1 mg) protein.

After 0,15 and 30 minutes, the amount of pentose in the medium was determined as described before (185). The high-speed supernatant of muscle was also tested for ribose-5-phosphatase activity. Ribose-5-phosphate (final concentration 2 mM) was incubated with Tris-HCL buffer pH 7.5 (0.133 M) and muscle high-speed supernatant (about 1 mg protein) for 60 minutes at 37° C. The incubation was stopped with 0.2 ml 50% TCA. After centrifugation inorganic phosphate was determined. No phosphatase activity utilizing ribose-5-phosphate under the above conditions was found.

G. In vivo experiments

1) Incorporation of Glucose-U-^{14}C into the lipids of tissues of dystrophic mice

Dystrophic mice and their littermate controls were kept in individual cages and fed a high-carbohydrate diet (Table III). After the mice became adjusted to the new diet (3-4 days), food-intake was measured for seven days. Then, the mice were injected intraperitoneally with 0.2 μc per g body weight glucose-U-^{14}C, and after 1 hour they were killed by decapitation. The tissues were removed, weighed and homogenized in 20 volumes chloroform-methanol (2 : 1). After centrifugation the extract obtained was washed once with 4 volumes 0.9% NaCl and twice with 4 volumes "upper phase" solution as described by Folch et al. (213). This procedure removes practically all of the water-soluble substances. After filtration and evaporation to dryness the lipids were redissolved in 5 ml heptane. Then 1 ml of this volume was taken for measurement of radioactivity using counting solution no. 1.

The remainder of the extract was evaporated to dryness. The lipids from liver and muscle were redissolved in chloroform and run through a column of acid-treated Florisil (5 gm), according to the method of Carroll (210) in order to separate the phospholipids from the other lipid fractions. Neutral lipids were eluted with 100 ml chloroform, and the phospholipids with 250 ml methanol. The recovery of radioactivity was about 100%. Phosphorus was determined on the phospholipid fraction.

From each fraction (phospholipids and neutral lipids) an aliquot was taken for radioactivity measurement. The remainder of the fraction was taken to dryness, and saponified at 50° C for 1 hour following

the addition of 0.3 ml 50% KOH in methanol. Cholesterol was extracted with petroleum ether, and after acidification with 1 N HCl, the fatty acids were removed with 3 successive extractions of 5 ml petroleum ether. A portion of the fatty acid extract was used to determine radioactivity and a second portion to determine the fatty acid content by titration. Cholesterol was determined as described before.

The aqueous phase was washed two times with 2.5 ml ether and an aliquot was taken for radioactivity measurement using counting solution no. 2.

In kidney and brain no fractionation of lipids on Florisil was carried out. Except for this difference the procedure was identical to that for liver and muscle.

2) Incorporation of ^{14}C-Palmitic acid into the lipids of tissues of dystrophic mice and loss of ^{14}C in the expired CO_2

Dystrophic and normal mice were fed ad libidum on a Purina laboratory chow diet for about one week from the date of arrival. Prior to the experiment they were fasted for 18 hours and then albumin-solubilized palmitate-1-^{14}C was injected intraperitoneally, at a dose of 0.6 μc per g body weight. One hour after the administration, the mice were sacrificed by decapitation and the tissues (brain, liver, kidney and hind leg muscle) were removed as quickly as possible, dipped into ice-cold normal saline, blotted and weighed. Then, 10% homogenates were prepared at 0° C with water for each tissue. Lipids were extracted at room temperature by the addition of 20 volumes of chloroform-methanol (2 : 1 v/v). After the addition of 4 ml methanol the precipitate was centrifuged. The precipitate was extracted two more times with 2.0 ml chloroform-methanol (2 : 1). The combined extracts were mixed with 7.0

ml of 1.5% NaCl and centrifuged. The lower phase was washed twice with 6 ml of the "upper phase" derived from the mixture chloroform-methanol-0.5 M sodium acetate (8 : 4 : 3, v/v/v). The washed extract was evaporated to dryness under reduced pressure. The lipid residue was dissolved in 3 ml acetone-ethanol-ether mixture (4:4:1, v/v/v). Saponification and extraction of fatty acid and cholesterol was carried out as indicated before. Cholesterol digitonide was formed by addition of 5 ml 0.5% digitonin solution to the cholesterol extracted, followed by heating at 70° C for 5 minutes and standing at room temperature overnight. After collection of the digitonide by centrifugation, it was washed twice with 10 ml of acetone-ether (1:2 v/v) mixture and once with 10 ml of ether before drying under an air stream. The amount of cholesterol and fatty acid and the radioactivity present were determined as already described.

In some experiments mice were injected intravenously through the dorsal tail vein with albumin-palmitate-1-^{14}C (0.4 μc/g body weight). In these experiments the specific activity of the palmitate was 23.7 μc per ml. Mice were killed after 30 minutes and the tissues were removed, extracted and analyzed as described above.

Radioactivity in plasma was determined as follows: after 1 hour injection of palmitate-1-^{14}C the mice were anaesthetized with ether and blood was removed from the heart, mixed with heparin and centrifuged. Then 0.1 ml of plasma was added to a vial containing 1 ml hydroxide of hyamine and heated in the oven at 60° C for 2 hours. Radioactivity was determined after adding 15 ml counting solution no. 2.

For the determination of $^{14}CO_2$ expired after the injection of palmitate-1-^{14}C, the animals were placed into a small metabolic chamber (capacity 250 ml) and the CO_2 expired was trapped in 2 N NaOH. The CO_2 was released from the NaOH solution by acidification and trapped in Hyamine using Conway vessels. A portion of the Hyamine solution was used for radioactivity measurement after adding 10 ml of counting solution no. 1.

H. Determination of radioactivity

Samples for counting were prepared in duplicate using one of the solutions described in the next paragraph. All samples were counted in a Model 6725 Nuclear Chicago Liquid Scintillation Counting System. The counting efficiency was about 70%. All counts were corrected for background to give the net counting rate. Correction for quenching was made by the channels ratio method.

The following solutions were used for scintillation counting: No. 1: 5 g PPO and 0.3 g POPOP per liter of scintillation grade toluene.

No. 2: 100 g naphthalene, 10 g PPO and 0.25 g POPOP per liter of dioxane.

No. 3: 45 g naphthalene, 9 PPO and 0.45 g POPOP dissolved in a mixture of 750 ml dioxane and 150 ml ethylene glycol.

I. Evaluation of results by statistical methods

The statistical methods used in this study have been taken from Croxton (214) and are outlined in the Appendix.

CHAPTER IV

A. In Vivo Incorporation of 1-^{14}C-Palmitic Acid into Lipid Fractions of Tissues of Normal and Dystrophic Mice

1) Introduction

The increased fat content in the muscle from dystrophic mice has been related to an increased lipogenesis (215) based on the findings of an enhancement in the incorporation of acetate-2-^{14}C into cholesterol and fatty acid of several tissues from dystrophic mice. However, what could prove to be of a more quantitative importance in the explanation of fat accumulation is the finding that muscle mitochondria from dystrophic mice shows a reduction in its ability to oxidize palmitate-1-^{14}C. This possibility has resulted in the present study on the *in vivo* metabolism of palmitate-1-^{14}C. Its incorporation into cholesterol and fatty acid of several tissues (brain, liver, kidney and muscle) and into the expired CO_2 by normal and dystrophic mice has been determined. The results obtained are described in this chapter.

2) Results

The fatty acid and cholesterol contents of tissues from dystrophic and normal mice expressed in terms of μmoles/g and mg/g wet tissues, respectively, are shown in Table IV. No differences were observed in brain, kidney and liver, but a large increase in both cholesterol and fatty acid was observed in dystrophic muscle.

In Table V are expressed the total radioactivity (d.p.m. per g wet tissue) and specific activity (d.p.m. per μmole fatty acid) found

TABLE IV

FATTY ACID AND CHOLESTEROL CONTENT OF TISSUES FROM DYSTROPHIC MICE (D) AND THEIR LITTERMATE CONTROL (C)

Fatty acid and cholesterol were isolated and determined as described in Methods

TISSUE	MOUSE	No.	FATTY ACID (μmoles/g wet weight ± SEM)	CHOLESTEROL (mg/g wet weight ± SEM)
BRAIN	D	13	114.0 ± 3.8	12.46 ± 0.61
	C	13	112.6 ± 3.5	12.54 ± 0.67
LIVER	D	13	228.0 ± 31.1	3.17 ± 0.18
	C	13	199.7 ± 19.1	2.83 ± 0.19
KIDNEY	D	13	99.4 ± 7.2	3.39 ± 0.14
	C	13	96.7 ± 5.7	3.50 ± 0.08
MUSCLE	D	13	181.9 ± 15.9	1.05 ± 0.035
	C	13	92.8 ± 11.5	0.58 ± 0.026

TABLE V

TOTAL RADIOACTIVITY AND SPECIFIC ACTIVITY IN FATTY ACIDS FROM TISSUES OF DYSTROPHIC MICE (D) AND THEIR CONTROLS (C) AFTER ADMINISTRATION OF PALMITATE-1-^{14}C

Determinations were carried out as described in Methods
P is the probability that there is no effect caused by dystrophy

TISSUE	MOUSE	No.	TOTAL RADIOACTIVITY (d.p.m. x 10^3 per g wet tissue ± SEM)	P	SPECIFIC ACTIVITY (d.p.m. per μmole fatty acid ± SEM)	P
BRAIN	D	13	23.0 ± 3.50	< 1	208 ± 22	< 1
	C	13	23.5 ± 2.80		209 ± 14	
LIVER	D	13	3,361 ± 320	< 1	17,250 ± 2,300	< 1
	C	13	3,850 ± 199		20,710 ± 1,550	
KIDNEY	D	13	347 ± 48	< 1	4,101 ± 668	< 1
	C	13	445 ± 33		4,756 ± 452	
MUSCLE	D	13	131 ± 15	< 0.005	792 ± 118	< 0.02
	C	13	98 ± 10		1,336 ± 266	

for the fatty acid of different tissues. The greatest radioactivity was found to be in liver, followed by kidney, muscle and brain. No differences were found for brain, liver and kidney but a greater incorporation was observed in dystrophic muscle ($P<0.005$) where, however, there is a decrease in the specific activity ($P<0.01$). When the total lipids from muscle or liver were fractionated into its different fractions by column chromatography on Florisil (216), it was found that nearly all the radioactivity was in the triglyceride fraction and only a small amount could be recovered as free fatty acid. In Figure 8 is shown the general pattern of distribution of radioactivity, obtained in this case, for the lipids of normal muscle.

The total radioactivity and specific activity (d.p.m. per mg cholesterol) for cholesterol of different tissues are listed in Table VI. A significant reduction in both parameters were found for liver, kidney and muscle of dystrophic mice. No differences were observed in brain.

Trial experiments were carried out to determine if the pattern of incorporation was the same when the isotope was injected intravenously. The results seemed to confirm the similarity in the incorporation, however because of the difficulties involved these experiments were only performed in three pair of mice.

The $^{14}CO_2$ expired by normal and dystrophic mice after injection of palmitate-1-^{14}C is shown in Figures 9 and 10. The determinations were made at 10, 30 and 60 minutes after injection. The average recovery of radioactivity as $^{14}CO_2$ in 60 min. was about 20% of the injected dose. The results correspond to the average values of 5 experiments. When the output of $^{14}CO_2$ is expressed in terms of d.p.m. per g body

TABLE VI

TOTAL RADIOACTIVITY AND SPECIFIC ACTIVITY IN CHOLESTEROL FROM TISSUES OF DYSTROPHIC MICE (D) AND THEIR CONTROLS (C) AFTER ADMINISTRATION OF PALMITATE-1-^{14}C

Determinations were carried out as described in Methods
P is the probability that there is no effect caused by dystrophy

TISSUE	MOUSE	No.	TOTAL RADIOACTIVITY ($d.p.m.\ 10^3$ per g wet tissue ± SEM)	P	SPECIFIC ACTIVITY (d.p.m. per mg cholesterol ± SEM)	P
BRAIN	D	13	372 ± 42	< 1	30.8 ± 3.4	< 1
	C	13	397 ± 37		30.7 ± 3.5	
LIVER	D	13	971 ± 222	< 0.001	329 ± 89	< 0.001
	C	13	6,228 ± 1,584		2,332 ± 612	
KIDNEY	D	13	239 ± 32	< 0.001	67 ± 9	< 0.001
	C	13	547 ± 58		140 ± 16	
MUSCLE	D	13	35 ± 6	< 0.001	36 ± 6	< 0.001
	C	13	63 ± 12		105 ± 17	

Figure 8 - Distribution of radioactivity into different lipid fractions from hind leg muscle of normal mice, isolated after 60 minutes injection of palmitate-1-^{14}C. Fractionation was carried out by the method of Carroll (216).

TGC, triglycerides
FFA, free fatty acid

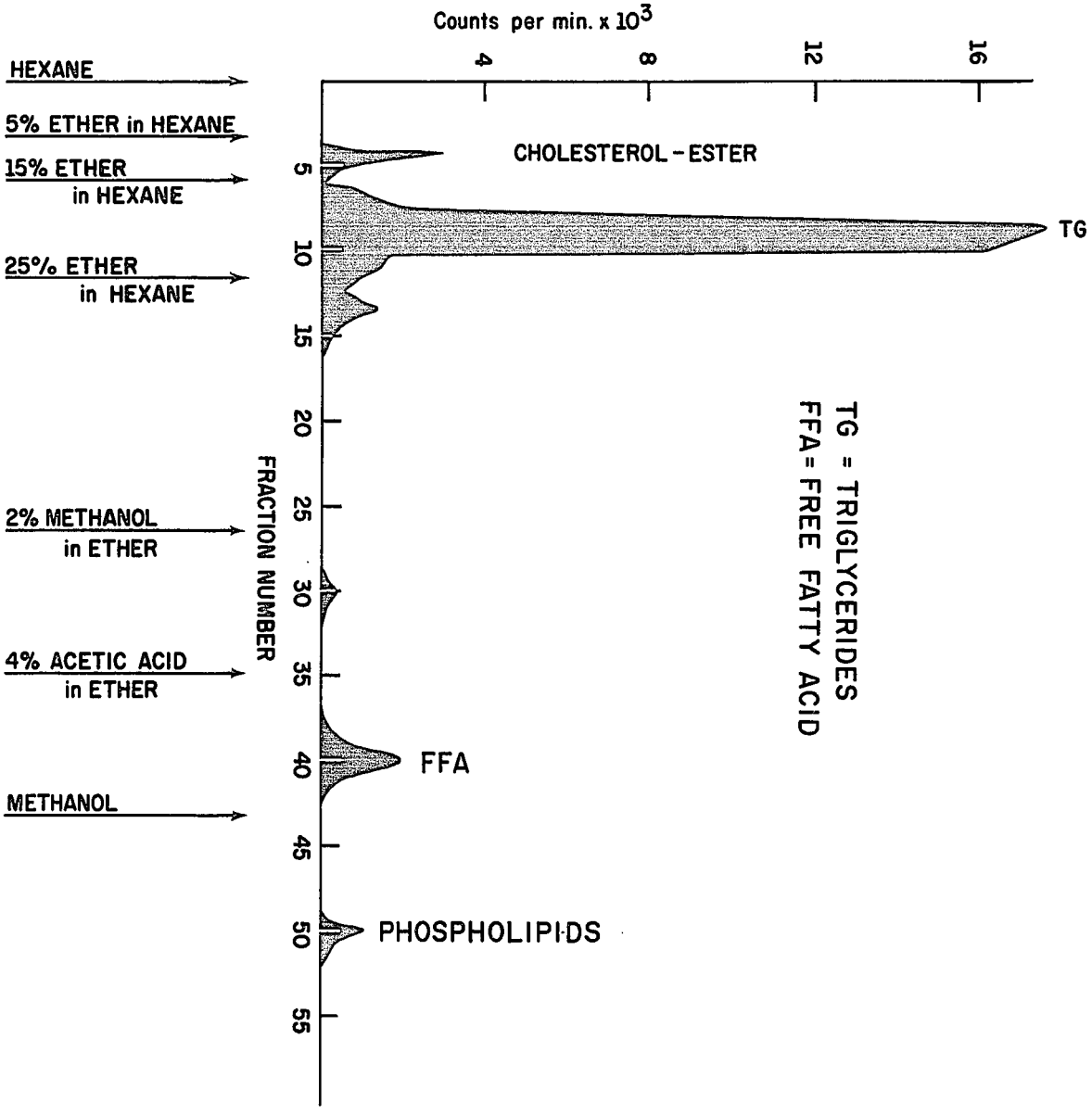

Counts per min. x 10³
4
8
12
16
HEXANE
5% ETHER in HEXANE
15% ETHER in HEXANE
25% ETHER in HEXANE
2% METHANOL in ETHER
4% ACETIC ACID in ETHER
METHANOL
CHOLESTEROL - ESTER
TG
FFA
PHOSPHOLIPIDS
FRACTION NUMBER
5
10
15
20
25
30
35
40
45
50
55
TG = TRIGLYCERIDES
FFA = FREE FATTY ACID

Figure 9 - Incorporation of radioactivity from palmitate-1-^{14}C into the expired CO_2 by normal (●) and dystrophic (o) mice. The results are expressed in terms of radioactivity (d.p.m.) per g body weight. The lines represent the average values of 5 experiments. Determination of radioactivity in CO_2 was done as described in Methods.

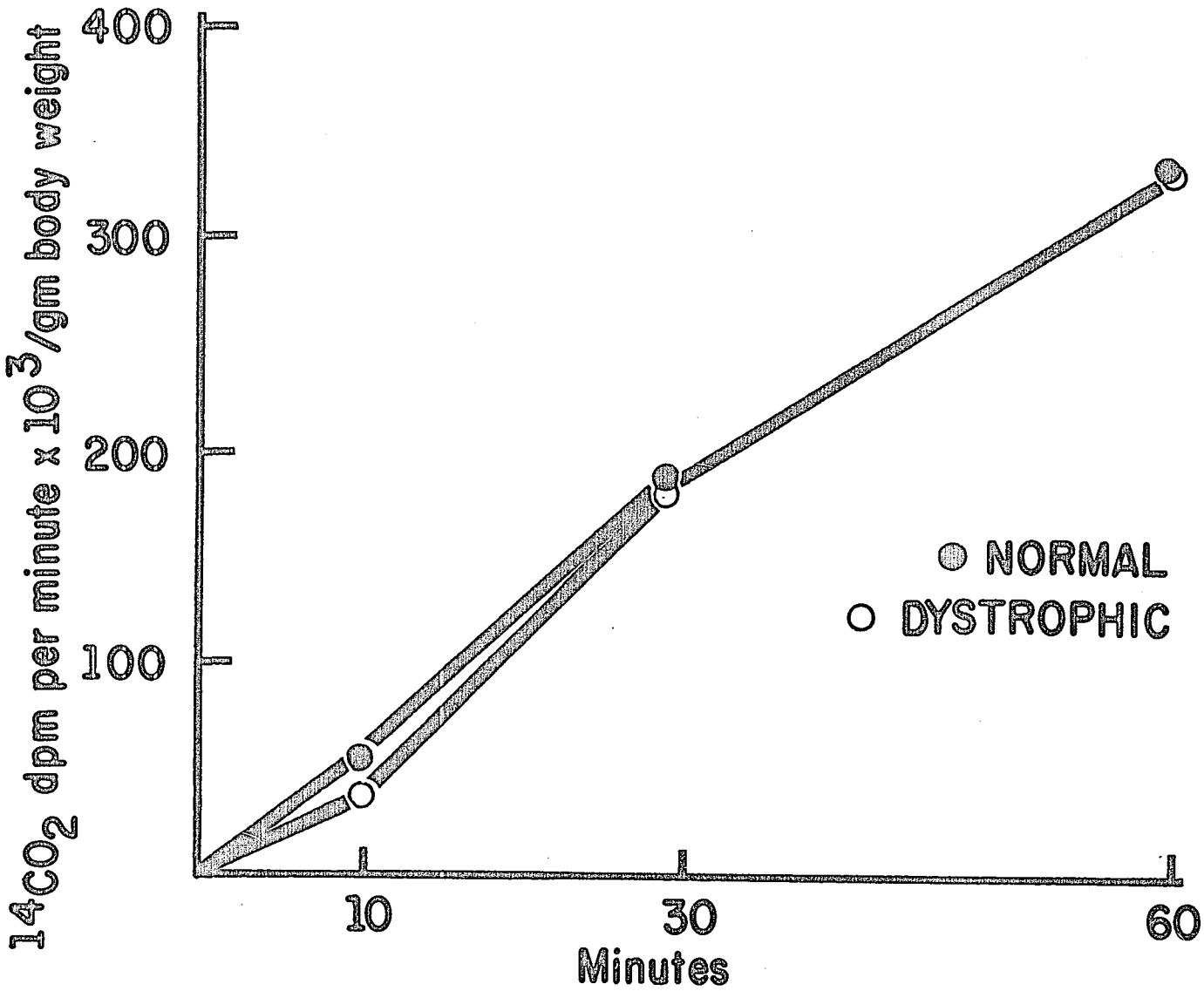
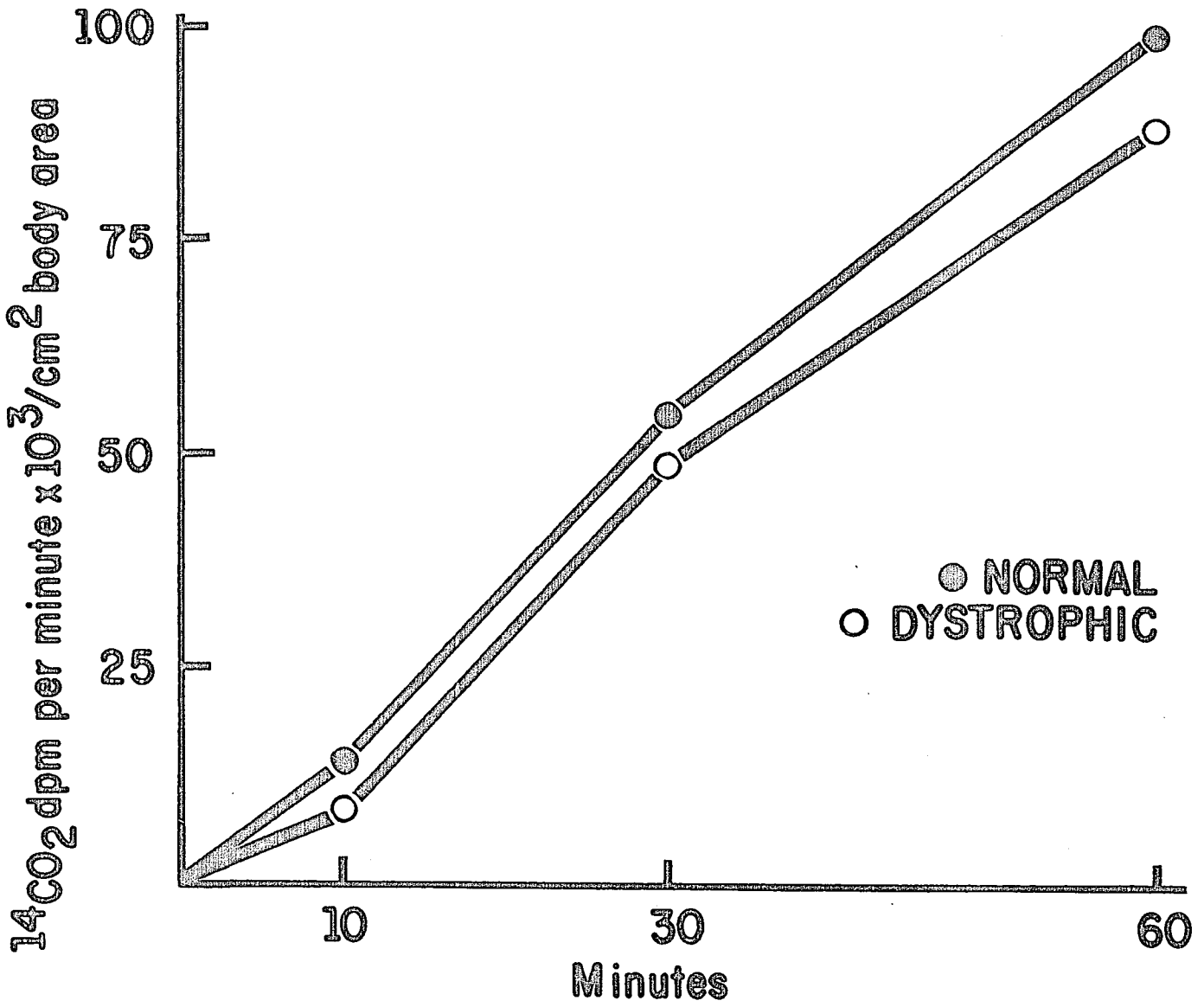

400
300
200
100
$^{14}CO_2$ dpm per minute x 10^3/gm body weight
NORMAL
DYSTROPHIC
10
30
60
Minutes

Figure 10 - Incorporation of radioactivity from palmitate-1-^{14}C into the expired CO_2 by normal (●) and dystrophic (o) mice. The results are expressed in terms of radioactivity (d.p.m.) per cm^2 surface area. The lines represent the average values of 5 experiments. Determination of radioactivity in CO_2 and surface area were done as described in Methods.


100
75
50
25
$^{14}CO_2$ dpm per minute x 10^3/cm^2 body area
10
30
60
Minutes
NORMAL
DYSTROPHIC

weight no differences were observed between normal and dystrophic mice (Figure 9). However, a significant reduction ($P<0.01$) in $^{14}CO_2$ output was observed when expressed as d.p.m. per cm^2 body area (Figure 10). The latter has been calculated by Meeh's formula: $S = k\sqrt[3]{g^2}$, where S = surface area in dm^2, g is the weight in kg and k is a specific constant for each animal species which corresponds to 7.82 for mice from 6-26 g body weight (217).

In Table VII are expressed the radioactivities found in the plasma 60 min. after the intraperitoneal injection of the isotope. The results are expressed in d.p.m./0.1 ml plasma. No differences appear to exist between normal and dystrophic mice.

The percentage of radioactivity in fatty acid compared with the total radioactivity found in the different tissues have been determined. These percentages have been found to be 97, 78, 70 and 35% respectively for liver, kidney, muscle and brain.

3) Discussion

The increased amounts of fatty acid and cholesterol that have been observed in the muscle of dystrophic mice are in agreement with previous reports (132, 133, 218). Considering that in patients affected with different types of muscular dystrophy sometimes alterations occur in other tissues together with the main manifestations in muscle, the question arises as to whether one is dealing with a pure muscular disease or with a more generalized disease even though its main signs appear in muscle. For this reason study has been extended to tissues other than muscle. In the case of liver, kidney and brain, no differences were observed in fatty acid and cholesterol contents.

TABLE VII

RADIOACTIVITY IN PLASMA FROM MICE 60 MINUTES AFTER INTRAPERITONEAL INJECTION OF 1-^{14}C-PALMITIC ACID

Blood was withdrawn by heart puncture 1 hour after intraperitoneal injection of 1-^{14}C-Palmitic Acid (0.6 µc/gm body weight). Plasma was separated by centrifugation. 0.1 ml of plasma was mixed with 1 ml Hyamine, heated at 60° C for 2 hours and the radioactivity was counted after mixing with 15 ml counting solution no. 2.

Pair no.	Radioactivity in 0.1 ml plasma from: (d.p.m.)	
	Normal	Dystrophic
1	30,988	32,411
2	27,925	26,771
3	25,042	28,371
4	28,037	27,321

The first factor to be considered in this study was that of whether an equivalent pool of palmitate-1-^{14}C is available in tissues of both normal and dystrophic mice. The results obtained showed that an equivalent amount of radioactivity in plasma was found in both types of animals and also that in some experiments a similar pattern of incorporation was observed following administration by either the intraperitoneal or intravenous routes. This indicates that the differences in incorporation observed cannot be explained simply by differences in the absorption into the blood of the injected isotope.

Two main factors can help to explain the observation that the uptake of radioactivity in the form of fatty acid was found to be increased in muscle. The first relates to the observation that muscle mitochondria isolated from dystrophic mice show a great reduction in their ability to oxidize fatty acid (135). Secondly, since the pattern of incorporation is similar in both normal and dystrophic muscle and free fatty acid is mainly incorporated into the triglycerides, it is possible that an increased esterification of the free fatty acid has occurred. The latter is supported by the observation (125) that in dystrophic muscle there is a reduction in the activity of several glycolytic enzymes which could cause an accumulation of triose-phosphates. Such an increase could lead to an increased availability of α-glycerophosphate, the most important acceptor of fatty acyl groups.

The decreased incorporation of radioactivity into cholesterol that has been observed in liver, kidney and muscle may be explained on the basis of a decreased degradation of fatty acid to acetyl-CoA, or to a decreased cholesterogenesis from acetyl-CoA. Evidence has been ob-

tained (218) that, under a variety of conditions there is no increase in cholesterogenesis from acetate in dystrophic animals. The increased cholesterol content in dystrophic muscle therefore appears to arise as a consequence of a reduced elimination or degradation rather than an increased synthesis.

The $^{14}CO_2$ expired by the animal has been expressed both on a body weight and on a body area basis; the latter has been shown to be more closely related to the basal metabolic rate of animals. When expressed in the latter way a significant reduction was observed in the dystrophic mice. This reduction, together with the increased incorporation of palmitate-1-^{14}C into dystrophic muscle and the decreased incorporation of radioactivity into cholesterol are compatible with the findings of Lin et al (135) and point to a defect existing in the degradation of fatty acid in dystrophic muscle. This combination of effects could result in the fat accumulation observed.

In brain no differences in incorporation were observed. It is interesting to notice here that in this tissue the percentage of radioactivity recovered as fatty acid represents only 35% of the total radioactivity in the tissue. This observation is difficult to explain but may reflect a very active degradation of palmitic acid by brain tissue or a difficulty in the transport of fatty acids at the level of the blood-brain barrier in which case the uptake of radioactivity might originate from metabolites derived from the breakdown of palmitic acid in other tissues. However, the latter appears to be ruled out since a direct uptake of oleic-1-^{14}C and palmitic-1-^{14}C acid by brain tissue of rat has been demonstrated to occur (219, 220).

B. In Vivo Incorporation of Glucose-U-^{14}C into Lipid Fractions of Tissues of Normal and Dystrophic Mice

1) Introduction

Previous in vitro (215) and in vivo (221) studies with labelled acetate have suggested that in a number of tissues of dystrophic mice (strain 129) there is enhanced lipogenesis. Experiments reported in the previous chapter show that, in vivo, there is no alteration in the incorporation of palmitate-1-^{14}C into the fatty acids of brain, kidney and liver but an increase was found for muscle. Incorporation into cholesterol was found very reduced. These results suggests that a general defect in lipid metabolism exists in tissues of dystrophic mice.

Since carbohydrates are precursors of both the glycerol and fatty acid portions of lipids, and in view of the above implications, we have carried out in vivo experiments in which the incorporation of glucose-U-^{14}C into the lipids of various tissues has been studied. In this study a high carbohydrate diet was fed to induce a condition of high lipogenic activity.

2) Results

In Table VIII is shown the food intake for dystrophic mice and their littermate controls when fed a high carbohydrate diet (see Methods). The results have been expressed as calories of food intake per g body weight per day. A significant increase ($P < 0.001$) has been observed in the caloric intake of dystrophic mice.

In Table IX is expressed the incorporation of glucose-U-^{14}C into the lipids of brain and kidney of dystrophic and normal mice. A significant reduction in incorporation was observed for the total lipids

TABLE VIII

DAILY FOOD INTAKE BY DYSTROPHIC MICE (STRAIN 129) AND THEIR LITTERMATE CONTROLS

Mice were kept in individual cages and fed a high-carbohydrate diet (see Table III) with a caloric content of 3.78 cal. per g. After 3 days adjustment to the new diet food-intake was determined daily. The results indicate the average value for 7 days.

Animal	Number	Weight (g ± SEM)	Food intake (Cal. per day per g body weight ± SEM)
Control	11	20.55 ± 0.87	0.65 ± 0.02
Dystrophic	11	13.15 ± 0.63	0.82 ± 0.02

TABLE IX

INCORPORATION OF GLUCOSE-U-^{14}C INTO LIPIDS OF BRAIN AND KIDNEY OF DYSTROPHIC MICE (D) AND THEIR LITTERMATE CONTROLS (C)

Determinations were carried out as described in the Methods. *Significantly different on basis of "pair comparison test".

Tissue and animal	No. of Pairs	Total lipids (d.p.m./g.wet wt.)	Incorporation of Glucose-U-^{14}C into: Fatty Acid (d.p.m./g.wet wt.)	Cholesterol (d.p.m./g.wet wt.)	Glycerol (d.p.m./g.wet wt.)
Brain					
C	6	12,947 ± 1,007	5,386 ± 614	189 ± 22	7,084 ± 505
D	6	9,215 ± 715*	3,635 ± 280	172 ± 14	5,142 ± 412
Kidney					
C	6	8,086 ± 312	3,102 ± 132	140 ± 11	4,728 ± 360
D	6	8,427 ± 528	2,865 ± 204	141 ± 16	5,204 ± 415

of brain but this reduction could not be attributed to one specific fraction. No differences were found in kidney. Due to the fact of the great variability in the observed results, these were statistically analyzed using paired data comparison. The same analysis has been applied to the results in Tables X and XI.

In liver and muscle no differences between normal and dystrophic were observed in the radioactivity incorporated into the phospholipid fraction. Similarly, the total content of phospholipid, expressed as mg P per g wet weight tissue does not show any difference (Table X). In Table XI are shown the results obtained in the study of the neutral lipid fraction from muscle and liver. No differences were found for liver in the radioactivity incorporated in all the fractions studied. The latter includes total lipids, cholesterol, fatty acids and glycerol. In muscle, increases in fatty acid and cholesterol contents and incorporation of radioactivity into fatty acids and glycerol were observed. No appreciable amount of radioactivity was found in cholesterol in both normal and dystrophic mice.

3) Discussion

In vivo lipogenesis from acetate-2-^{14}C by dystrophic mice has been studied in this laboratory (218). The results did not provide clear evidence of an enhanced incorporation of acetate into cholesterol and fatty acids in dystrophic mice. Considering that the incorporation of acetate into lipids is, in part, dependent on the acetic-thiokinase activity of the tissues under study and that glucose rather than acetate is the predominant substrate for lipogenesis, it was considered important to extend the investigation by using glucose-U-^{14}C instead of acetate-2-^{14}C.

TABLE X

INCORPORATION OF GLUCOSE-U-C^{14} INTO PHOSPHOLIPIDS
OF MUSCLE AND LIVER OF DYSTROPHIC MICE (D)
AND THEIR LITTERMATE CONTROLS (C)

Determinations were carried out as described in Methods

Tissue and animal	No. of pairs	Total fraction (d.p.m/g.wet wt.)	mg.P/g.wet wt.	Specific activity (d.p.m/mg.P)	Activity (d.p.m/g. wet wt.) in Fatty acids	Non-fatty acids
Liver						
C	6	10,384 ± 866	1.17 ± .02	8,860 ± 815	1,367 ± 309	10,842 ± 732
D	6	11,384 ± 924	1.16 ± .05	9,831 ± 746	2,980 ± 688	10,266 ± 814
Muscle						
C	6	1,017 ± 201	0.45 ± .02	2,222 ± 148	311 ± 42	791 ± 102
D	6	1,461 ± 232	0.45 ± .01	3,161 ± 167	436 ± 38	989 ± 228

TABLE XI

INCORPORATION OF GLUCOSE-U-C^{14} INTO NEUTRAL LIPIDS
OF MUSCLE AND LIVER OF DYSTROPHIC MICE (D)
AND THEIR LITTERMATE CONTROLS (C)

Determinations were carried out as described in Methods.

Tissue and animal	No. of pairs	Total fraction (d.p.m/g.wet wt.)	Fatty Acid (μmoles/g.wet wt.)	Fatty Acid d.p.m/g.wet wt.	Cholesterol (mg./g.wet wt.)	Cholesterol d.p.m/g.wet wt.	Glycerol (d.p.m/g.wet wt.)
Liver							
C	6	17,483 ± 2,023	172 ± 16	1,237 ± 182	2.79 ± 2	422 ± 32	15,439 ± 1,704
D	6	24,381 ± 3,161	189 ± 18	1,422 ± 101	2.84 ± 2	541 ± 68	21,036 ± 2,896
Muscle							
C	6	3,140 ± 414	57. ± 7.	904 ± 182	0.52 ± .03	-	1,810 ± 284
D	6	8,177 ± 892*	146. ± 11.*	3,428 ± 684*	0.99 ± .04*	-	4,226 ± 622*

The lipogenic capacity of tissues is inversely related to the amount of fat contained in the diet (222). In order to obtain a high degree of lipogenesis in our experiments a high-carbohydrate diet was used containing only 1.5% corn oil. When the mice were changed to this diet they were found to require a period of about 3 days for adaptation (determined by the changes in weight of the mice). Food-intake, measured for 7 days after the period of adaptation, is greater in dystrophic animals then normal when expressed as calories per g body weight per day. This may reflect a higher basal metabolic rate in dystrophic mice.

The greatest incorporation of glucose-U-^{14}C occurs in liver followed by brain, kidney and muscle. Most of the radioactivity incorporated into the lipids from liver and muscle is into the glycerol moiety followed by the fatty acid portion. Incorporation into cholesterol was very low in liver, brain and kidney and negligible in muscle. This pattern of labelling suggests that glucose-U-^{14}C gives rise to the glycerol moiety at a more rapid rate than the fatty acid portion. It is likely that this occurs by way of α-glycerophosphate (223). It is also interesting to note that all tissues studied were capable of appreciable conversion of radioactivity into fatty acids with brain showing the largest followed by kidney, muscle and liver.

Of all the tissues studied only muscle has shown an increased capacity to incorporate glucose-U-^{14}C into the neutral lipids (both into the fatty acid and into the glycerol moiety). It has been reported (224) that no differences in the production of $^{14}CO_2$ from glucose-U-^{14}C could be observed between dystrophic mice and their controls.

It is possible that even if a defect in the oxidation of glucose is present in dystrophic muscle the whole animal can maintain a normal production of $^{14}CO_2$ by increasing its production from non-muscular tissues or by an increased activity of the pentose-shunt. This last possibility could also explain the pentosuria we have mentioned before.

In vitro experiments (215, 225) on acetate incorporation into lipids indicate that there is a general enhancement of lipogenesis in tissues of dystrophic mice which might, at least in part, account for the accumulation of lipid in dystrophic muscle. This enhanced lipogenesis from acetate has been also demonstrated using high-speed supernatants of muscle (71,000 x g) as the enzyme preparation (135).

Coleman (125) has reported deficiencies in several enzymes of the glycolytic pathway which result in the accumulation of triose-phosphates. The latter could produce an increased amount of glycerol-3-phosphate for triglyceride synthesis. Since one of the primary controlling mechanisms in fatty acid synthesis is the feed-back inhibition of acetylCoA carboxylase by long-chain acyl CoA (226), it is possible that any factor affecting the level of long chain acyl CoA might indirectly affect fatty acid synthesis. The accumulation of glycerophosphate could depress the inhibition of acetyl CoA carboxylase by acylation of long chain acyl CoA and thereby result in an increased formation of fatty acid. Considering that a partial blockade does exist in the glycolytic pathway in the muscle from dystrophic muscle, it is easy to explain the increased incorporation of radioactivity from glucose-U-^{14}C into the glycerol moiety of neutral lipids. However, the increased incorporation into fatty acids is not so easily explained.

It is possible that a decreased utilization of acetyl CoA by muscle mitochondria occurs and that the acetyl groups accumulating are diverted into fatty acid synthesis. This could lead to an increased incorporation of radioactivity into fatty acid. Further increase could also result from the enhanced fatty acid synthetase activity that has been reported (135).

No differences were observed in the total phospholipid content (expressed as mg P per g w.w. tissue) or incorporation of radioactivity into this fraction either in muscle or liver. However, it still is possible that alterations in incorporation into any one or several of the phospholipids may have occurred in dystrophic muscle (or other tissues). It was not possible to fractionate the different classes of phospholipids because of the small amount of muscle available from each animal.

CHAPTER V

Formation of Pentose-5-Phosphate from Glucose and Utilization of Ribose-5-Phosphate by High-Speed-Supernatants of Muscle Homogenates of Mice

1) Introduction

Pentosuria has been reported to exist in patients with Duchenne MD (227, 228). This alteration in the urine composition could be originated by a primary defect in the production or utilization of pentoses by dystrophic patients or be secondary to their release from muscle cells undergoing breakdown.

Increases in the activities of the dehydrogenases of the hexose-monophosphate (HMP) shunt in the metabolism of the glucose have been reported to exist in muscle from dystrophic mice (122) and in man with Duchenne MD (229). These observations have been confirmed by histochemical methods (230). Such increases could be of significance since they might lead to an enhancement in the production of pentoses, and therefore to pentosuria. The present chapter describes experiments carried out to study the hexose-monophosphate pathway. The first group of experiments was done to assess the production of pentose-5-phosphate using glucose as substrate, and the second group was designed to assess the utilization of these pentoses by determining the disappearance of ribose-5-phosphate from the incubation medium.

2) Results

(a) Pentose and $^{14}CO_2$ production from glucose-U-^{14}C by high-speed supernatant of muscle homogenates

The cofactor requirements for the release of $^{14}CO_2$ from glucose-U-^{14}C by high-speed supernatant of mouse skeletal muscle have

been determined (Table XII). It can be seen that the release of $^{14}CO_2$ does not require NAD or Mn^{++} but is very dependent on NADP. Phenazine methosulfate has been added to regenerate NADP through oxidation of the NADPH formed in the reaction. Its presence stimulates the production of $^{14}CO_2$ from glucose-U-^{14}C about 10 times. Hexokinase and ATP were added to the system in order to phosphorylate glucose. In some experiments where phenazine methosulfate was not used, hexokinase did not increase the rate of formation of $^{14}CO_2$. These observations seem to indicate that the potential activity of the dehydrogenases of the HMP shunt are greater than the activity of the endogenous hexokinase contained in the high-speed supernatant of muscle.

In the system used there existed the possibility that the Embden-Meyerhoff pathway of glucose metabolism was operative and that the pyruvate formed could be decarboxylated giving rise to $^{14}CO_2$. With arsenite, an inhibitor of pyruvate oxidation, added, it has been observed (231) on incubation of strips of muscle with glucose-1-^{14}C or glucose-6-^{14}C that there is an increase in the ratio of $^{14}CO_2$ derived from C1 to $^{14}CO_2$ derived from C6 (i.e. the C1/C6 ratio). We have tested the effect of arsenite (final concentration 2 mM) in our preparation and, as is indicated in Table XIII, no change in the production of $^{14}CO_2$ was observed. This indicated that little or no decarboxylation of pyruvate occurs during the above incubation.

The optimal concentrations for ATP, phenazine methosulfate and NADP were those established in Figures 11, 12 and 13. Optimal concentrations were: 2 mM for ATP, 0.01 mM for phenazine methosulfate and 0.2 mM for NADP. The optimal pH for the reaction was observed to

TABLE XII

COFACTOR REQUIREMENTS FOR THE METABOLISM OF GLUCOSE BY HIGH-SPEED SUPERNATANT OF MUSCLE HOMOGENATES OF MICE (Strain 129)

The complete system contained: potassium phosphate buffer pH 7.4, 375 μmoles; hexokinase, 1 mg; ATP, 4 μmoles; NADP, 0.4 μmole; Mg^{++} 8 μmoles; phenazine methosulfate, 0.4 μmole; glucose-U-^{14}C, 10 μmoles (containing 868,000 dpm); high-speed supernatant, about 1 mg protein, in a final volume of 2 ml. The incubation was carried out in Warburg flasks at 37° C for 30 minutes. $^{14}CO_2$ was determined as indicated in the Methods.

Incubation Mixture	$^{14}CO_2$ released (d.p.m/mg protein)
Complete system	29,559
Less phenazine methosulfate	3,842
Less ATP	1,447
Less NADP	570
Less hexokinase	9,809
Plus NAD	29,825
Plus Mn^{++}	28,035

TABLE XIII

ARSENITE EFFECT ON THE METABOLISM OF GLUCOSE BY HIGH-SPEED SUPERNATANT OF MUSCLE HOMOGENATES OF MICE (Strain 129)

Incubation conditions as in Table XII.

Final concentration of arsenite (Na^+) (mM)	$^{14}CO_2$ Production (d.p.m. per mg protein)
0	22,428
2	23,076

Figure 11 - The effect of ATP on the production of $^{14}CO_2$ from glucose-U-^{14}C by high-speed supernatant (90,000 x g for 100 minutes) of mouse skeletal muscle. Incubation conditions were as in Figure 14. Incubation time was 30 minutes.

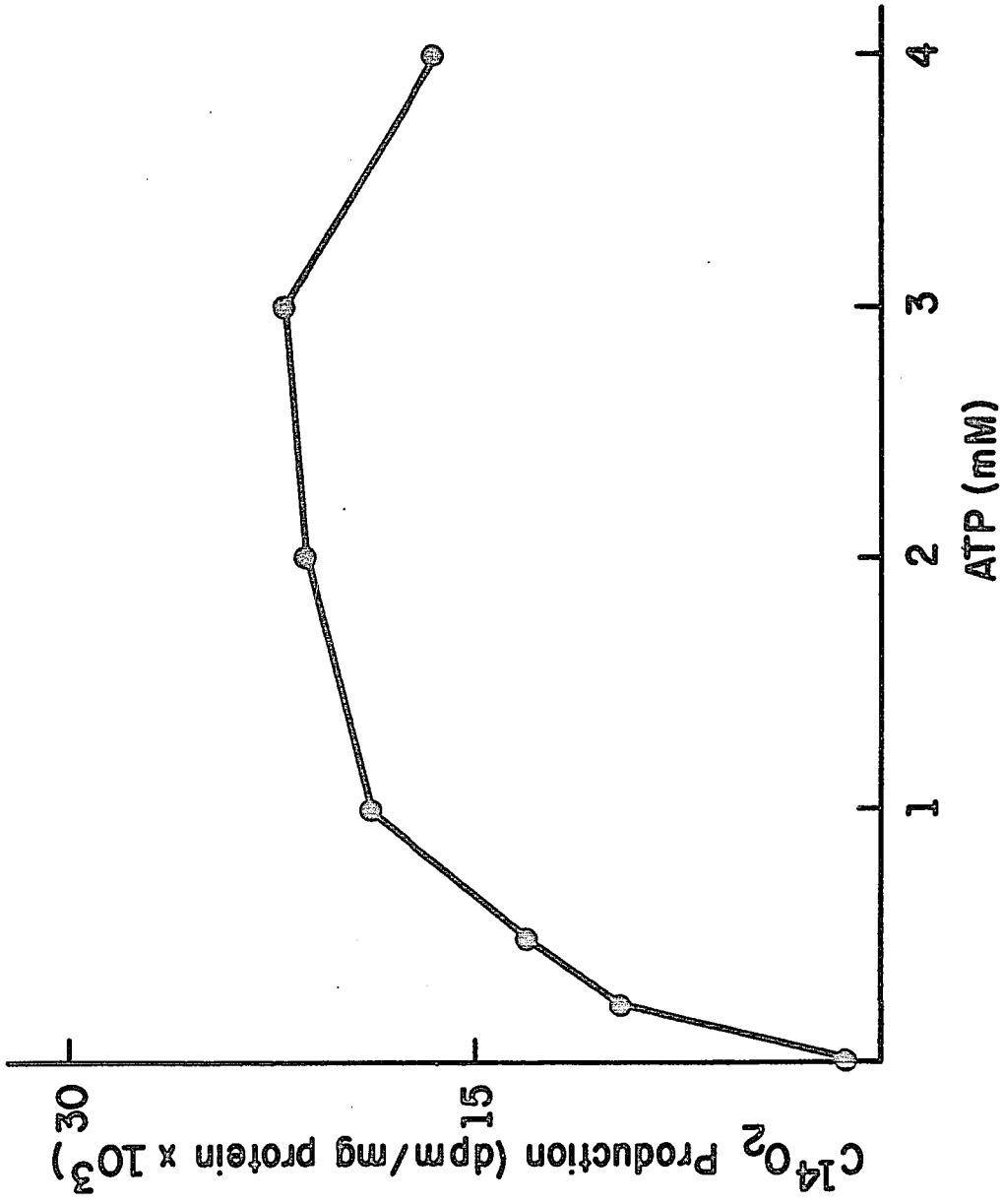

$C^{14}O_2$ Production (dpm/mg protein x 10^3)
30
15
1
2
3
4
ATP (mM)

Figure 12 - The effect of phenazine methosulfate on the production of $^{14}CO_2$ from glucose-U-^{14}C by high-speed supernatant (90,000 x g for 100 minutes) of mouse skeletal muscle. Incubation conditions were as in Figure 14. Incubation time 30 minutes.

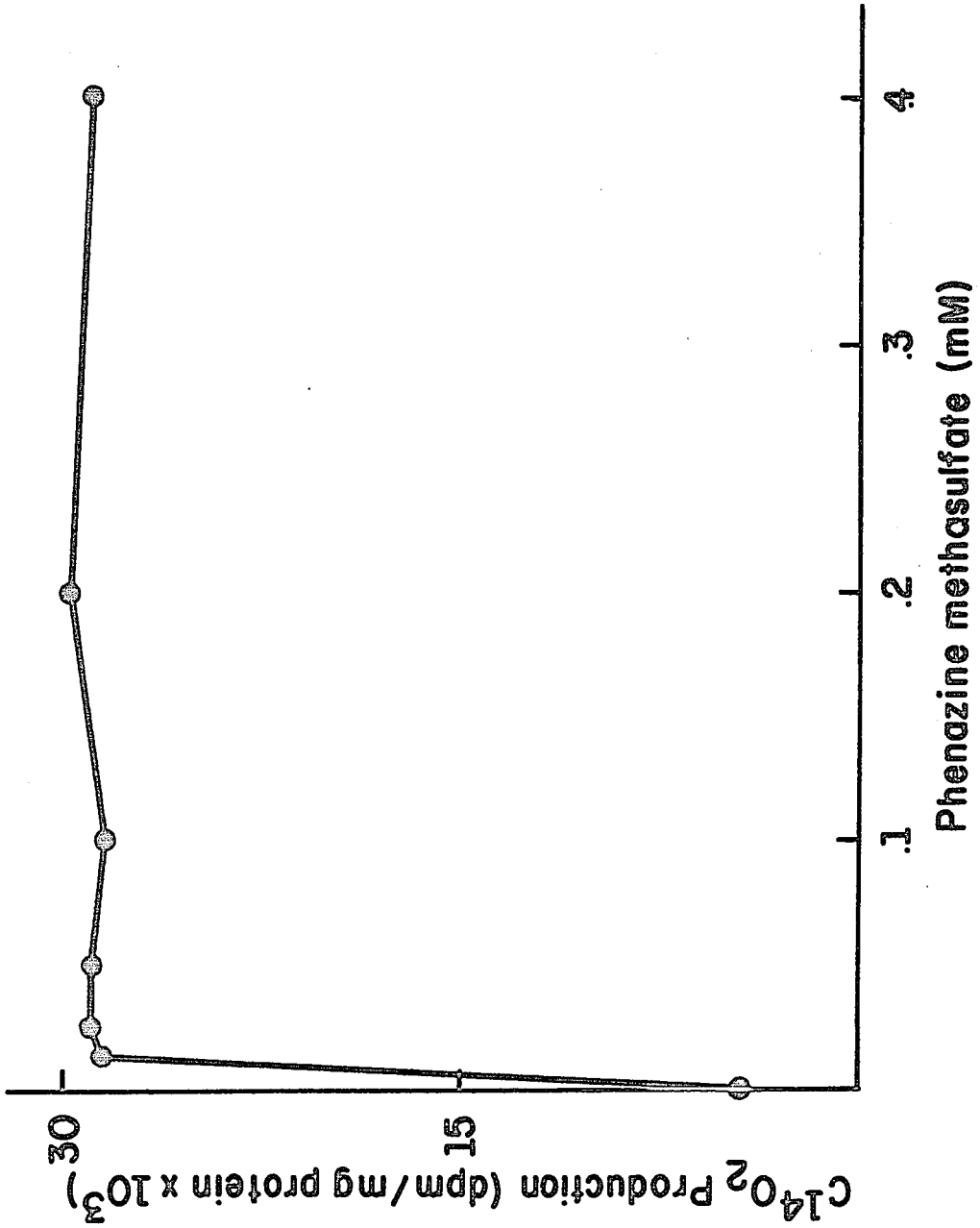
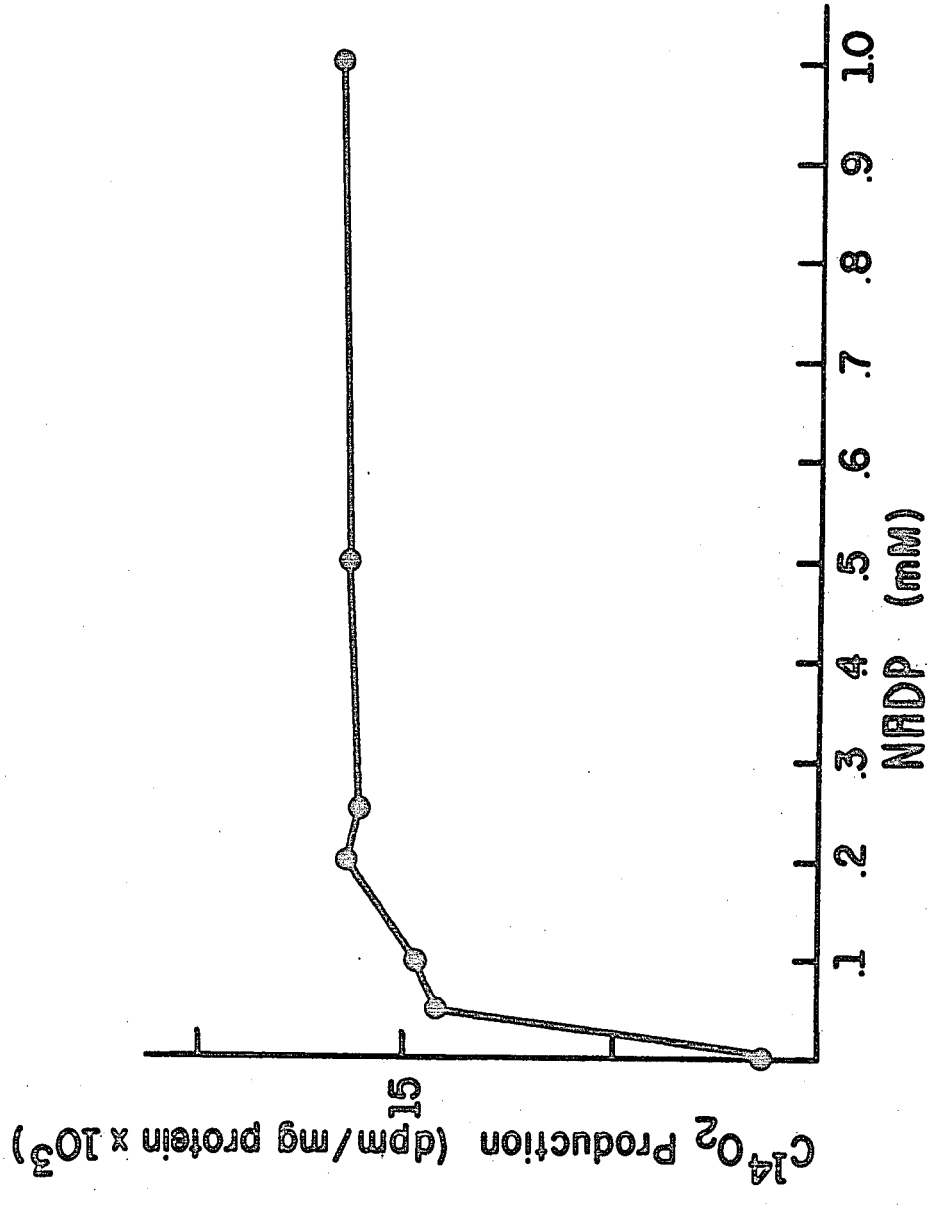

$C^{14}O_2$ Production (dpm/mg protein x 10^3)
30
15
.1
.2
.3
4
Phenazine methasulfate (mM)

Figure 13 - Effect of NADP on the production of $^{14}CO_2$ from glucose-U-^{14}C by high-speed supernatant (90,000 x g for 100 minutes) of mouse skeletal muscle. Incubation conditions as in Figure 14. Incubation time 30 minutes.


$C^{14}O_2$ Production (dpm/mg protein x 10^3)
15
1 2 3 4 5 6 7 8 9 10
NRDP (mM)

be between 7.4 and 8. As shown in Figure 14 the reaction was linear for 30 minutes. This was the incubation time used in our experiments.

Using the optimal system that has been already described, the production of $^{14}CO_2$ from glucose-U-^{14}C and the accumulation of pentoses have been determined in normal and dystrophic mice. The production of $^{14}CO_2$ is expressed in d.p.m. per mg protein and pentose accumulation as μmoles per mg protein in 30 minutes (Table XIV). A great increase was observed in the dystrophic animals for both $^{14}CO_2$ production and pentose accumulation. The fact that the ratio Dystrophic/Normal is not the same for pentose accumulation and $^{14}CO_2$ production is probably the consequence of differences in the subsequent steps in the non-oxidative part of the HMP shunt.

(b) Utilization of Ribose-5-Phosphate by high-speed supernatants of muscle

The conditions for these experiments are as indicated in the Methods. Because of the non-linear disappearance of ribose-5-phosphate from the medium observed with time, the determinations have been made at two different times, 15 and 30 minutes. The time course for the disappearance of ribose-5-phosphate by high-speed supernatants of muscle homogenates is seen in Figure 15.

The comparative results between dystrophic and normal mice shows that dystrophic muscle utilizes more ribose-5-phosphate than does the normal muscle. This can be seen in Table XV.

3) Discussion

The amount of glucose that is being metabolized in skeletal muscle through the HMP shunt pathway has been calculated to be no more than 2% (232). Therefore a quantitative study of this pathway offers

Figure 14 - The effect of time on the production of $^{14}CO_2$ grom glucose-U-^{14}C by high-speed supernatant (90,000 x g for 100 minutes) of mouse skeletal muscle. Incubation conditions and determination of $^{14}CO_2$ as described in the Methods.

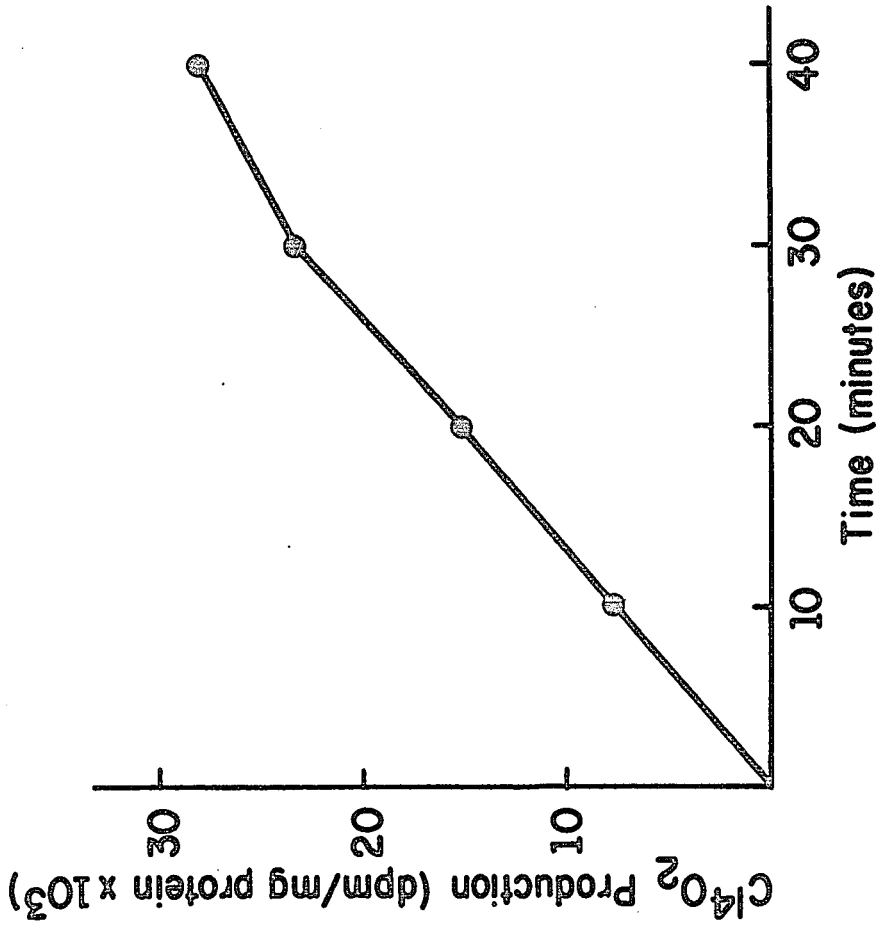

$C^{14}O_2$ Production (dpm/mg protein x 10^3)
30
20
10
10
20
30
40
Time (minutes)

TABLE XIV

$^{14}CO_2$ PRODUCTION AND PENTOSE ACCUMULATION BY HIGH-SPEED SUPERNATANT OF MUSCLE FROM NORMAL AND DYSTROPHIC MICE (Strain 129) INCUBATED WITH GLUCOSE-U-^{14}C

Details of incubation and determination of $^{14}CO_2$ and pentoses are described in the Methods. P indicates the probability that there is no effect caused by dystrophy.

Animal	Number	$^{14}CO_2$ Production (d.p.m. per mg protein ± SEM)	Pentose accumulation (μmoles per mg protein ± SEM)
NORMAL	5	18,820 ± 900	0.27 ± 0.05
DYSTROPHIC	5	40,860 ± 3,030	0.73 ± 0.08
		$P < 0.001$	$P < 0.001$

Figure 15 - The effect of time on the disappearance of ribose-5-Phosphate from the incubation medium brought about by high-speed supernatant from muscle homogenates of mice (Strain 129). The incubation mixture contained in a total volume of 2 ml: 375 μmoles potassium phosphate buffer pH 7.4; 8 μmoles ribose-5-phosphate, and 1 mg high-speed supernatant of muscle. Determination of ribose was carried out as described in the Methods.

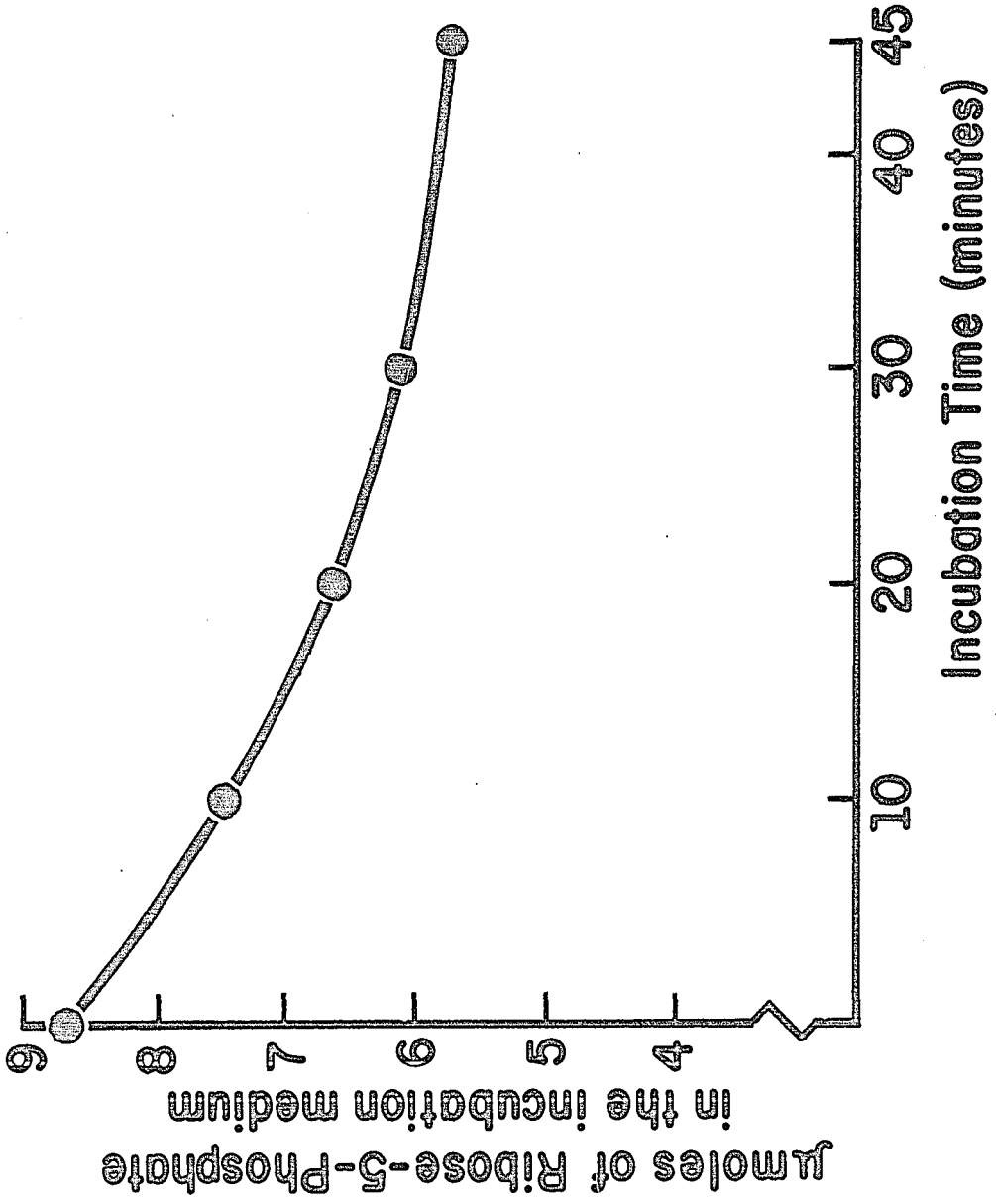

µmoles of Ribose-5-Phosphate
in the incubation medium
3
4
5
6
7
8
9
10
20
30
40
45
Incubation Time (minutes)

TABLE XV

THE DISAPPEARANCE OF RIBOSE-5-PHOSPHATE FROM THE MEDIUM WHEN INCUBATED WITH HIGH-SPEED SUPERNATANT (90,000 x g) OF MUSCLE FROM NORMAL AND DYSTROPHIC MICE (Strain 129)

Incubation procedure and determination of ribose are as indicated in the Methods. P indicates the probability that there is no effect caused by dystrophy.

Incubation time (minutes)	Number	Disappearance of ribose-5-phosphate μmoles per mg protein ± SEM NORMAL	DYSTROPHIC	P
15	5	0.64 ± 0.05	1.00 ± 0.09	<0.001
30	6	1.01 ± 0.06	1.50 ± 0.06	<0.001

some difficulties considering that any small contribution from the Krebs cycle easily masks the activity of the HMP shunt. The most common way to study the contribution of these pathways is by comparison of the production of $^{14}CO_2$ from glucose-1-^{14}C and glucose-6-^{14}C. Values in excess of 1 for the ratio CO_2 (C1)/CO_2(C6) indicates some operation of the HMP pathway.

Because all the enzymes of the HMP pathway are located in the soluble part of the cell, we have studied this pathway using the high-speed supernatant of muscle homogenate obtained after centrifugation at 90,000 x g, as the enzyme preparation. By this procedure, which eliminates mitochondria from the system, the release of $^{14}CO_2$ from glucose can only occur through the HMP pathway.

Several functions are attributed to the HMP pathway. These are: a) to supply the ribose and deoxyribose required for the synthesis of nucleic acids and high energy nucleotides. It is important to mention here that a pentose derivative, 5-phosphoribosyl-1-pyrophosphate is a rate limiting precursor not only of RNA (233) but also of histidine (234); b) to form NADPH which is required for a number of synthetic reactions such as in the reduction of folic acid to tetrahydrofolic acid (a donor of 1-C units), in the biosynthesis of purine and pyrimidinic bases, in the biosynthesis of fatty acids, cholesterol and steroids, in the synthesis of certain amino acids, creatinine, and in the reduction of dithiol groups, thereby being possibly an important factor in the regulation of the ratio S-S/SH. Also NADPH plays an important role in the general mechanism of detoxification.

All the above functions are related to anabolic reactions. Therefore, it is reasonable to expect an increase in the activity of the

HMP pathway to occur in all conditions requiring an increased synthesis. Accordingly, increased activity has been reported in embryonic tissues (235) and in tissues undergoing regeneration (236). In dystrophic muscle both regeneration and degeneration processes are working simultaneously. Studies carried out with cultures of breast muscle of normal and dystrophic chicken embryos have shown that the dystrophic fibers grew and degenerated at a faster rate, so that, in young cultures there were more fibers in dystrophic than in normal, the reverse being true in old cultures (237). It, therefore, seems probable that an increase in the HMP pathway takes place in dystrophic muscle in a response to injury and is not related to the primary mechanism of the disease. However some practical importance could be derived from the study of the HMP pathway, particularly if it can be used as a measure of the regenerating reactions. Comparison of this parameter with some parameter indicating the destructive process (e.g. activity of proteolytic enzymes activity) might give a ratio of some prognostic or diagnostic value.

One of the characteristics of dystrophic muscle is its accumulation of fat. It has been found (135) that a decreased capacity to oxidize fatty acids does exist in the mitochondria from dystrophic muscle of mice together with an increased fatty acid synthetase activity. The increased production of NADPH could potentiate this increased capacity to synthesize fatty acids. Preliminary experiments involving a system for fatty acid synthesis (135) in combination with the system used in these studies but without phenazine methosulfate, failed to yield an overall system capable of fatty acid synthesis. Since this

failure may have resulted from a depletion of ATP (required in both pathways) by the excess of hexokinase added, glucose-6-phosphate was substituted for glucose and hexokinase. However, still no fatty acid synthesis could be demonstrated. Further experiments are required to resolve this problem. We have not tested the reverse situation, that is the influence of the fatty acid synthesis on the activity of the HMP pathway. We have found (see Table XII) that phenazine methosulfate increases the HMP pathway about 10 times. If this occurs by removal of NADPH that could inhibit the HMP pathway, then fatty acid synthesis could increase the HMP shunt through the same mechanism.

Our results confirm those obtained by Canal and Frattola (238) and are in agreement with the study of the dehydrogenases of the HMP shunt in mice and in patients with Duchenne MD (122,229).

CHAPTER VI

In Vitro Metabolism of Triglycerides by Muscle Preparations of Normal and Dystrophic Mice (Strain 129)

1) Introduction

The phenomenon of fat accumulation has been the object of extensive investigation in this laboratory. In this respect it has been shown (135) that muscle mitochondria from dystrophic mice are unable to oxidize long-chain fatty acids to the same extent as normal mitochondria. Also dystrophic muscle shows an increased capacity to synthesize fatty acids from acetate.

Because the main bulk of fat is accumulated in the form of triglycerides (TG) we have studied the extent to which 1-^{14}C-palmitic acid and sn-^{14}C-glycerol-3-phosphate (^{14}C-GP) is incorporated in this lipid fraction. In the same experiments we have studied the incorporation into the phospholipid fraction. It should be noted here that the incubation conditions used have been those determined to be optimal for incorporation into TG. Some experiments have been carried out using unlabelled palmitic acid and glucose-U-^{14}C.

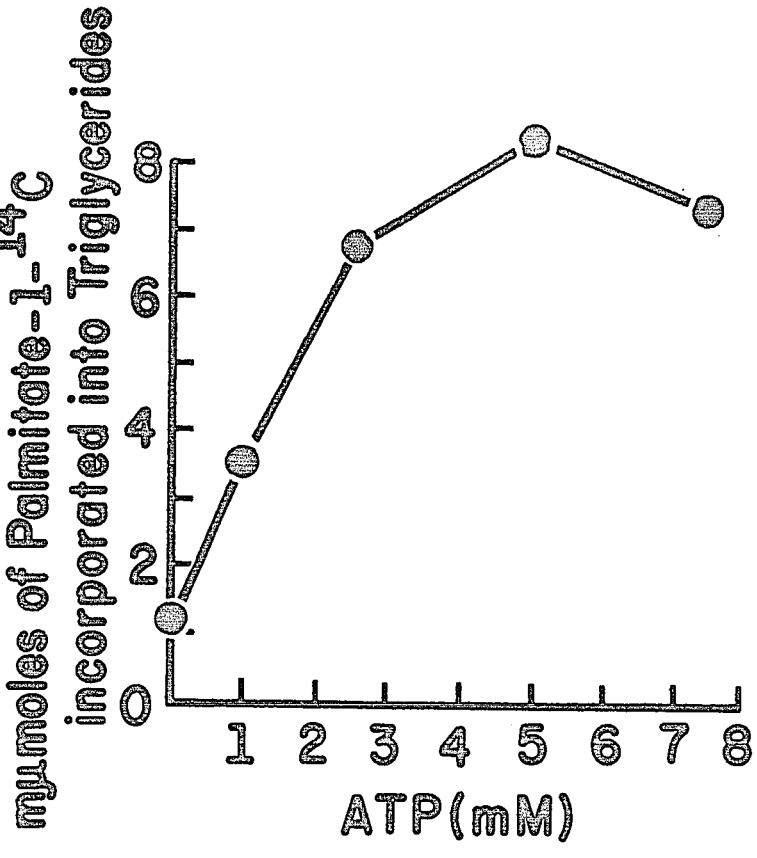
The second part of this chapter deals with the reactions of catabolism of TG. Two different lipases have been studied, one that uses tributyrin as substrate and another using tripalmitin.

2) Results

(a) Incorporation of 1-^{14}C-palmitic acid and ^{14}C-GP into triglycerides by muscle homogenates

In Figures 16, 17, 18 and 19 are shown the dependence of the incorporation of 1-^{14}C-palmitic acid into TG on the concentrations

Figure 16 - The effect of ATP on the incorporation of 1-^{14}C-palmitic acid into triglycerides by muscle homogenates of mice. The incubation mixture contained in a final volume of 3 ml : 0.3 µmole NAD; 18 µmoles $MgCl_2$; 0.15 µmoles CoA; 15 µmoles glucose; 0.6 µmole 1-^{14}C-palmitic acid (containing about 215,380 d.p.m.); and 1 ml muscle homogenate (10% homogenate in Krebs-Ringer II medium). Radioactivity in triglycerides was determined as described in the Methods.


mμmoles of Palmitate-1-14c
incorporated into Triglycerides
0
2
4
6
8
1 2 3 4 5 6 7 8
ATP(mM)

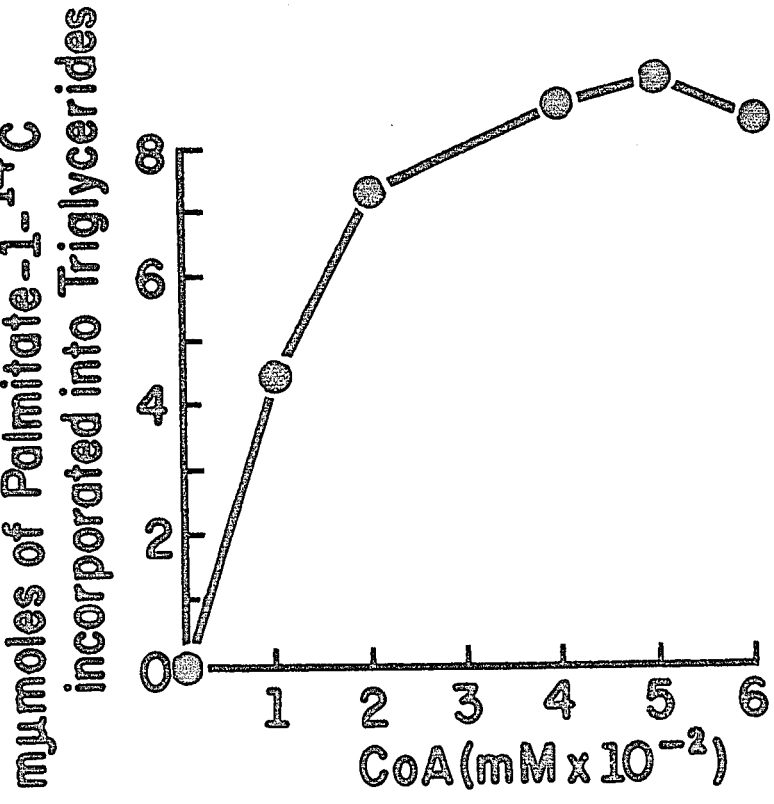


Figure 17 - The effect of CoA on the incorporation of 1-^{14}C-palmitic acid into triglycerides by muscle homogenates of mice. The system contained 15 μmoles ATP (i.e. 5 mM). Other incubation conditions as described in Figure 16.

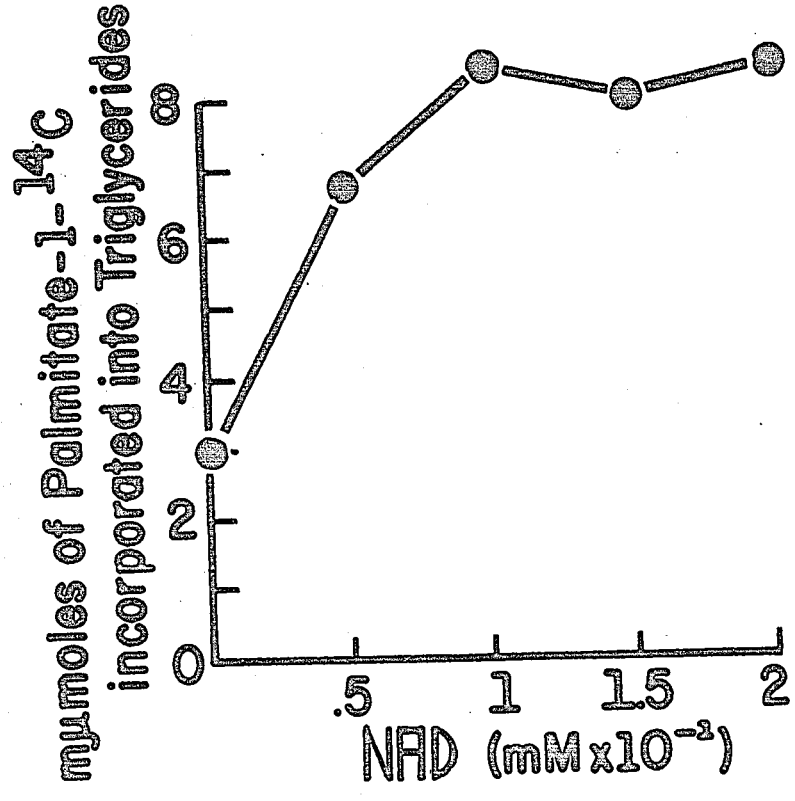


Figure 18 - The effect of NAD on the incorporation of 1-^{14}C-palmitic acid into triglycerides of muscle homogenates of mice. Incubation conditions as in Figure 16 with the exception that NAD was added in the final concentration indicated.

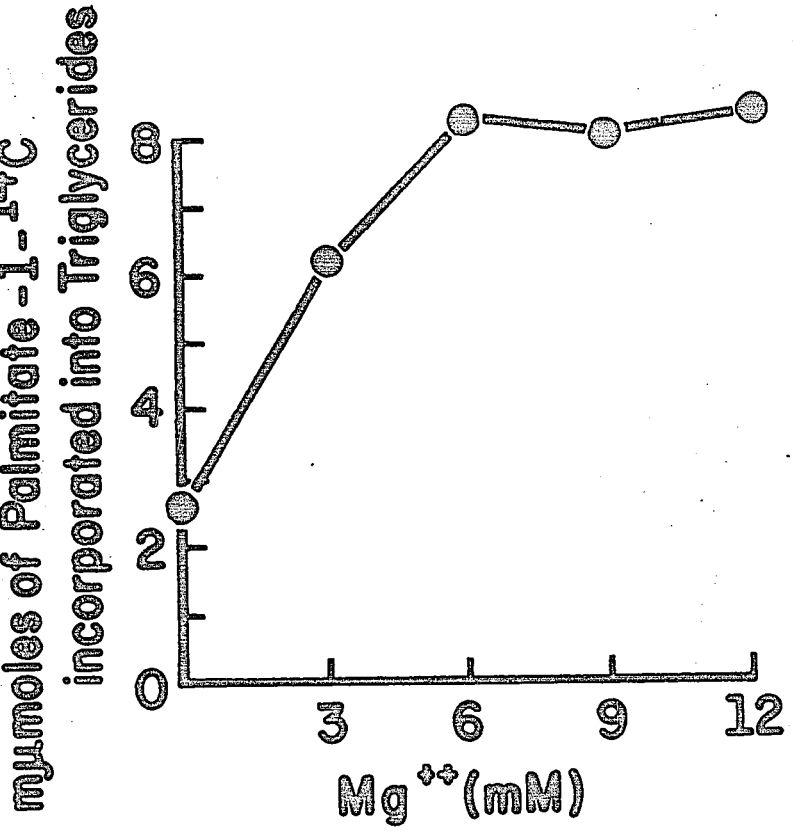


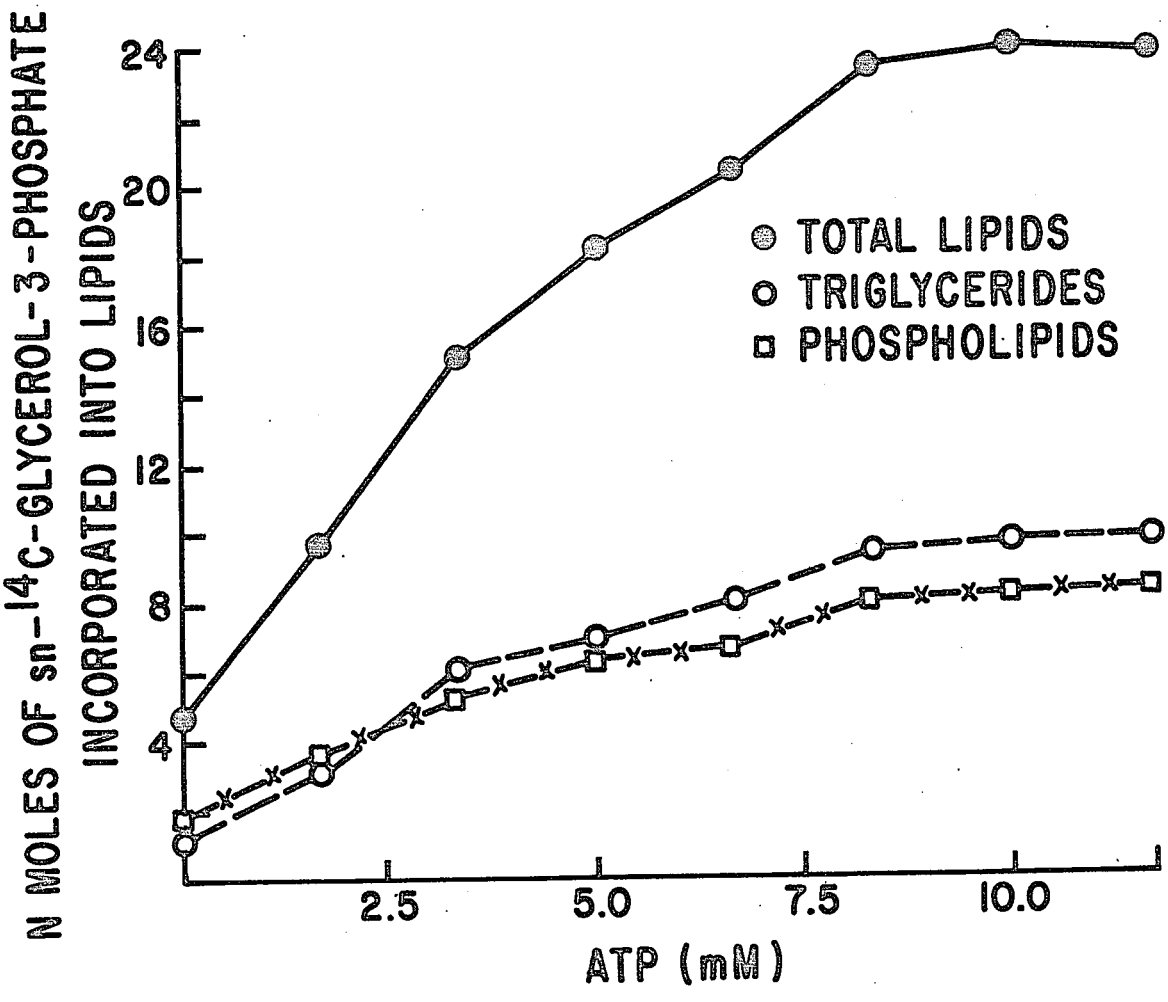
Figure 19 - The effect of Mg^{++} on the incorporation of 1-^{14}C-palmitic acid into triglycerides by muscle homogenates of mice. Incubation conditions as in Figure 16.

of ATP, CoA, NAD and Mg^{++}. The concentrations giving maximal incorporation were used in our experiments. They are 5 mM for ATP, 0.05 mM for CoA, 0.1 mM for NAD and 6 mM for Mg^{++}. Similar studies for ATP and CoA have been carried out using as substrate sn-^{14}C-glycerol-3-phosphate (GP). The results are shown in Figures 20 and 21. The optimal concentrations were found to be 10 mM for ATP and 0.025 mM for CoA.

In Table XVI can be seen the cofactor requirements for the incorporation of palmitate-1-^{14}C and ^{14}C-GP into TG. As shown, both precursors are dependent for its incorporation on ATP, Mg^{++} and CoA. When palmitate was used, it was necessary to add both glucose and NAD. These requirements were not necessary for ^{14}C-GP. It was curious to note in the former case glucose could not be substituted by α-GP. Fluoride, in a concentration of 1.2 mM, strongly inhibits the incorporation of palmitate and it has no effect on the incorporation of ^{14}C-GP.

In order to assess the dilution of labelled palmitate by endogenous fatty acid, concentrations of free fatty acids were determined colorimetrically in both normal and dystrophic muscle. Taking this factor into consideration the influence of different concentrations of free fatty acids on the incorporation of 1-^{14}C-palmitate into TG has been studied. The rate of incorporation does not change until the free fatty acid concentration is above 640×10^{-6} M. In all the incubations carried out with normal muscle the concentration of free fatty acid was below this value whereas in the dystrophic muscle all but pair 5 (see Table XX) were above this value.

Figure 20 - The effect of ATP on the incorporation of sn-^{14}C-glycerol-3-phosphate into total lipids, triglycerides and phospholipids by muscle homogenates of mice. The incubation mixture contained in a final volume of 3 ml: 35 μmoles $MgCl_2$; 60 μmoles phosphate buffer pH 7.5; 75 nmoles CoA; 6 μmoles sn-^{14}C-glycerol-3-phosphate (containing about 500,000 d.p.m.); 1 ml muscle homogenate (10% in Krebs II) and ATP as indicated. Incorporation of substrate was determined as described in the Methods.


N MOLES OF sn-^{14}C-GLYCEROL-3-PHOSPHATE INCORPORATED INTO LIPIDS
24
20
16
12
8
4
2.5
5.0
7.5
10.0
ATP (mM)
TOTAL LIPIDS
TRIGLYCERIDES
PHOSPHOLIPIDS

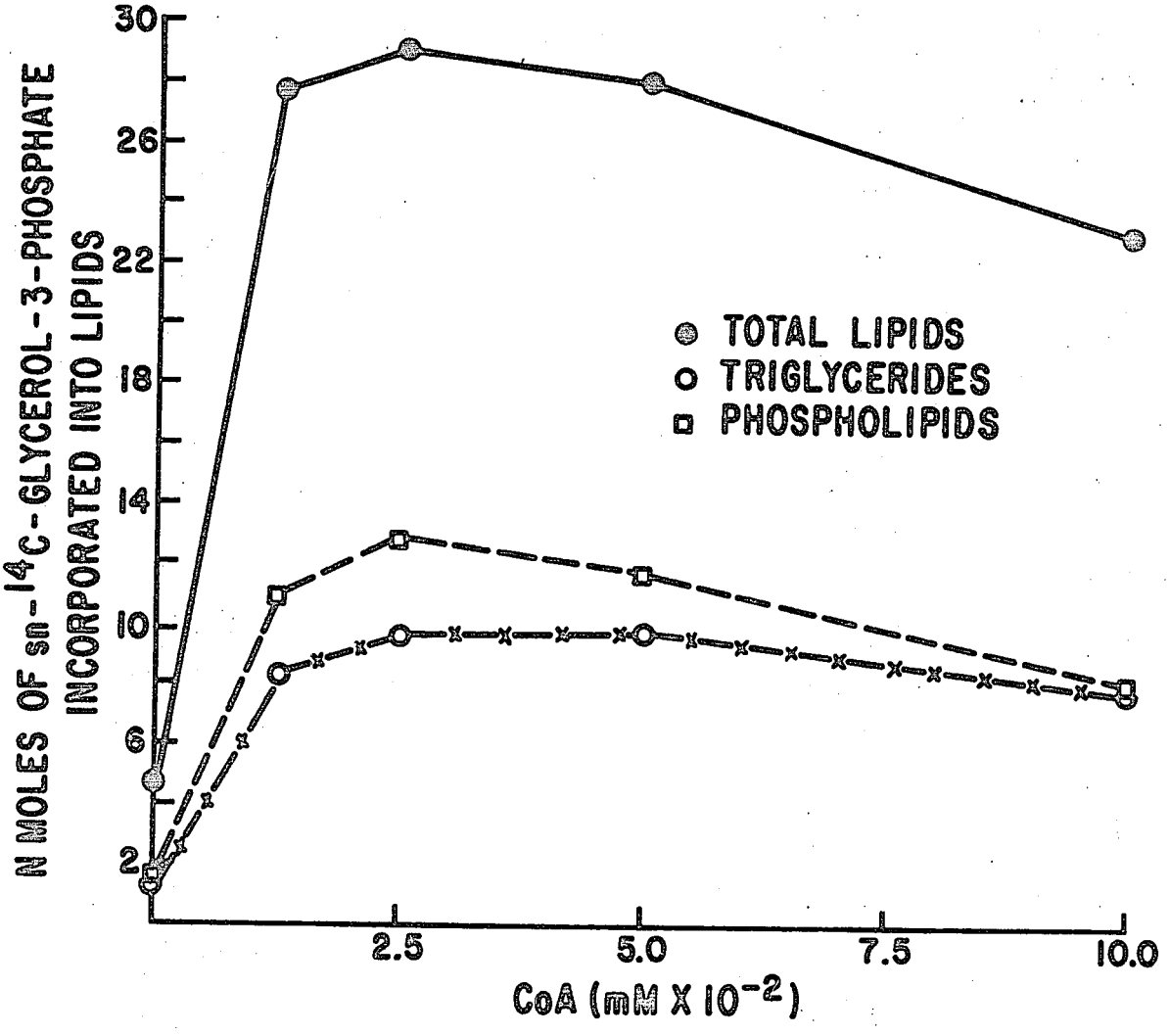


Figure 21 - The effect of CoA on the incorporation of sn-^{14}C-glycerol-3-phosphate into total lipids, triglycerides and phospholipids by muscle homogenates of mice. Incubation conditions were as in Figure 20.

TABLE XVI

COFACTOR REQUIREMENT FOR THE INCORPORATION OF PALMITATE-1-^{14}C OR sn-^{14}C-GLYCEROL-3-PHOSPHATE INTO TRIGLYCERIDES BY MUSCLE HOMOGENATES OF MICE (STRAIN 129)

The complete incubation mixture and the determination of radioactivity in Triglycerides (TG) are described in Methods. Incubation at 37° C was carried out for 20 minutes when incubated with palmitate-1-^{14}C and for 5 minutes in the case of sn-^{14}C-glycerol-3-phosphate.

Incubation mixture	Palmitate-1-^{14}C incorporated into TG (n moles)	sn-glycerol-^{14}C-3-phosphate incorporated into TG (n moles)
Complete system	9.50	9.60
Less glucose	2.65	9.52
Less NAD	2.84	9.68
Less ATP	1.20	1.25
Less CoA	0.23	1.00
Less Mg^{++}	2.60	1.64
Plus sn-glycerol-3-phosphate (α-GP)	9.58	--
Plus α-GP, less glucose	2.88	--
Plus Hexokinase (1 mg)	9.50	--
Plus F^- (1.2×10^{-3} M)	3.16	9.62

Since it is possible that there were differences in the rate of esterification due to the specificity of different fatty acids it was of interest to determine the fatty acid distribution in the free fatty acid, triglyceride and phospholipid fractions from normal and dystrophic muscle. Using gas-liquid chromatography no appreciable differences were found (see Table XVII, XVIII, and XIX). In the free fatty acid fraction the main components were found to be palmitic (about 25%), stearic (a. 20%), oleic (a. 17%), linoleic (a. 16%) and arachidonic (a. 20%) acids. In the TG fraction were found palmitic (a. 19%), oleic (a. 33%) and linoleic (a. 30%) acids. In the phospholipid fractions palmitic (a. 20%), stearic (a. 15%), oleic (a. 13%), linoleic (a. 16%) and arachidonic (a. 25%) were the predominant fatty acids found.

The time course for the incorporation of palmitate-1-^{14}C and ^{14}C-GP into TG by muscle homogenates are shown in Figures 22 and 23. The incorporation is linear for 20 minutes in the case of palmitate. With ^{14}C-GP the response was not linear over this period, and, therefore, the time used in further experiments was set at 5 minutes.

A comparative study of the incorporation of 1-^{14}C-Palmitic acid into the TG and phospholipids of muscle from normal and dystrophic mice is shown in Table XX. The free fatty acid content is markedly increased in dystrophic muscle (17.12 $\pm$ 0.94 μmoles per g wet weight versus 11.03 $\pm$ 0.68) μmoles in normal). No differences were found in the incorporation of palmitic acid into the phospholipids of normal and dystrophic muscle under the experimental conditions described. The incorporation into TG is very much increased in dystrophic muscle in

TABLE XVII

FATTY ACID DISTRIBUTION IN THE FREE FATTY ACID FRACTION FROM NORMAL AND DYSTROPHIC MICE (STRAIN 129)

Fatty acids were obtained and analyzed in their methyl-ester forms by Gas-Liquid Chromatography as described in Methods. Numbers in brackets represent the average value.

Fatty Acid Designation	Fatty Acid content (% of total) in:			
	Normal Muscle		Dystrophic Muscle	
16 : 0	28.3 23.3	(25.8)*	22.4 20.4	(21.4)
16 : 1	1.6 2.0	(1.8)	1.7 2.1	(1.9)
X_1*	0.52 0.56	(0.54)	0.54 0.70	(0.62)
X_2*	0.28 0.34	(0.31)	0.21 0.40	(0.31)
18 : 0	19.7 19.6	(19.65)	20.4 20.5	(20.45)
18 : 1	13.1 17.9	(15.5)	17.6 21.3	(19.5)
18 : 2	14.8 14.9	(14.85)	17.8 18.0	(17.9)
20 : 4	21.7 21.4	(21.55)	19.4 16.8	(18.1)

*X_1 and X_2 are unknown fatty acids

TABLE XVIII

FATTY ACID DISTRIBUTION IN THE TRIGLYCERIDE FRACTION FROM NORMAL AND DYSTROPHIC MICE (STRAIN 129)

Analysis as in Table XVII

Fatty Acid Designation	Fatty Acid content (% of total) in:	
	Normal Muscle	Dystrophic Muscle
16 : 0	18.7 (19.65) 19.6	16.7 (18.5) 20.3
16 : 1	5.5 (7.7) 9.9	3.5 (4.65) 5.8
X_1*	0.8 (0.9) 1.0	0.7 (0.7) 0.7
18 : 0	5.5 (3.95) 2.4	9.5 (8.7) 7.9
18 : 1	30.3 (31.8) 33.3	30.4 (35.2) 40.0
18 : 2	37.2 (32.45) 27.7	37.4 (28.3) 19.2
18 : 3	2.0 (2.25) 2.5	1.8 (2.15) 2.5

*X_1 is unknown fatty acid

TABLE XIX

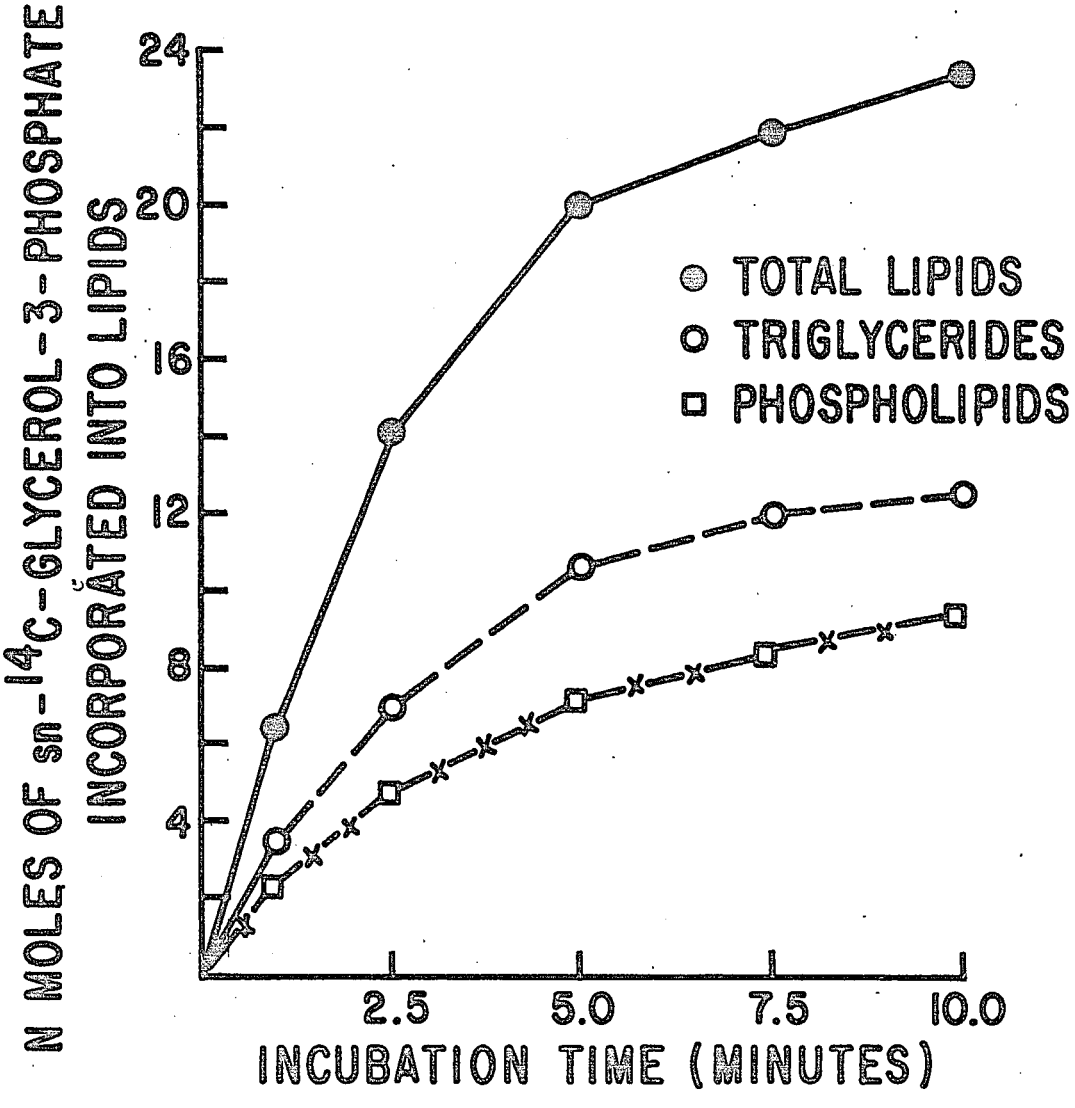
FATTY ACID DISTRIBUTION IN THE PHOSPHOLIPID FRACTION FROM NORMAL AND DYSTROPHIC MICE (STRAIN 129)

Analysis as in Table XVII

Fatty Acid Designation	Fatty Acid content (% of total) in:			
	Normal Muscle		Dystrophic Muscle	
16 : 0	25.2 18.2	(21.7)	17.3 21.9	(19.6)
16 : 1	4.7 9.1	(6.9)	5.9 6.0	(5.95)
X_1*	1.7 1.3	(1.5)	1.3	(1.4)
18 : 0	17.8 16.1	(16.95)	10.9 18.7	(14.8)
18 : 1	10.7 12.9	(11.8)	16.8 13.1	(14.95)
18 : 2	15.7 12.8	(14.25)	22.5 17.1	(19.8)
18 : 3	1.1		2.0	
20 : 4	23.1 30.8	(26.95)	23.3 23.1	(23.2)

*X_1 is unknown fatty acid

Figure 22. The effect of time on the incorporation of sn-^{14}C-glycerol-3-phosphate into triglycerides and phospholipids by muscle homogenates of mouse. Incubation conditions as described in the METHODS.


N MOLES OF sn-14C-GLYCEROL-3-PHOSPHATE INCORPORATED INTO LIPIDS
24
20
16
12
8
4
TOTAL LIPIDS
TRIGLYCERIDES
PHOSPHOLIPIDS
2.5
5.0
7.5
10.0
INCUBATION TIME (MINUTES)

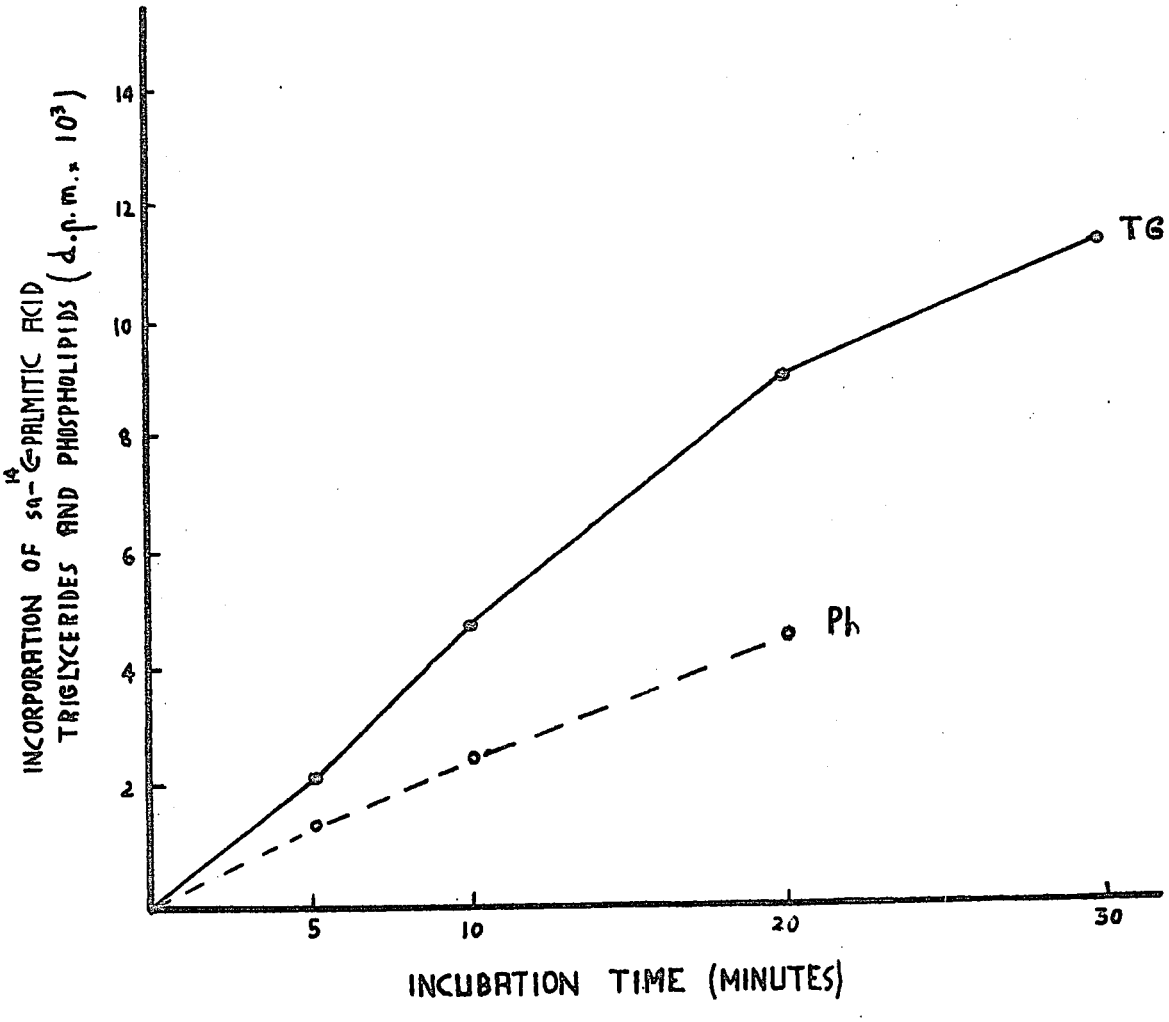


Figure 23. The effect of time on the incorporation of 1-^{14}C-palmitic acid into triglycerides and phospholipids by muscle homogenates of mice. Incubation conditions as in Figure 20.

TABLE XX

INCORPORATION OF PALMITATE-1-^{14}C INTO TRIGLYCERIDES AND PHOSPHOLIPIDS BY MUSCLE HOMOGENATES OF NORMAL AND DYSTROPHIC MICE (Strain 129)

Incubation conditions are indicated in Methods. Statistical analysis was carried out on a pair comparison basis. P is the probability that there is no effect caused by dystrophy.

Pair	Free Fatty Acid concentration (μmoles /g w.w. muscle)		Incorporation of Palmitate into: (nmoles /100 mg w.w. muscle)			
			Triglycerides		Phospholipids	
	N	D	N	D	N	D
1	9.35	18.70	200	510	71	114
2	10.36	18.20	356	297	183	156
3	13.15	16.83	271	398	132	90
4	11.45	17.07	209	319	104	95
5	10.32	13.84	136	199	53	51
6	11.56	18.10	158	322	64	68
	11.03 ± 0.68	17.12 ± 0.94				
	$P < 0.001$		$P < 0.01$		$P < 1$	

5 of the 6 pairs of mice examined and no differences were found in pair no. 2. On pair comparison basis the difference in incorporation between normal and dystrophic tissue was found to be significant, however this is not so when the results were analyzed using the "t" student test.

In some experiments glucose-U-^{14}C and unlabelled palmitic acid were added under the conditions used for palmitate incorporation. In these experiments a reduction was observed in the incorporation of radioactivity into the TG and phospholipids of muscle of dystrophic mice (Table XXI).

In Table XXII are shown the results obtained when ^{14}C-GP was used as substrate. No differences between normal and dystrophic muscle were observed when the results are expressed as nmoles of ^{14}C-GP incorporated per g wet weight muscle per minute.

(b) Hydrolysis of TG by muscle homogenates

The activities of two different lipases in normal and dystrophic muscle have been studied. These are described as "tributyrinase" and "tripalmitinase" because of the nature of the substrates used in this study.

In Table XXIII are shown the results concerning the tributyrinase activity in muscle homogenates from normal and dystrophic mice. The results are expressed in terms of μmoles tributyrin hydrolyzed per hour per mg N, per mg NCN, or per g wet weight muscle. Because of infiltration of dystrophic muscle with non-muscular components the use of NCN was considered the best baseline to express the results (183). In normal muscle NCN accounts for

TABLE XXI

INCORPORATION OF RADIOACTIVITY FROM GLUCOSE-U-^{14}C INTO TRIGLYCERIDES AND PHOSPHOLIPIDS OF MUSCLE FROM NORMAL AND DYSTROPHIC MICE (STRAIN 129)

Incubation conditions are indicated in Methods.

Pair no.	Glucose-U-^{14}C incorporated (dpm/min-incubation) in: Triglycerides		Phospholipids	
	N	D	N	D
1	656	193	---	---
2	916	590	390	201
3	515	302	210	122
4	708	287	340	160

TABLE XXII

INCORPORATION OF sn-^{14}C-GLYCEROL-3-PHOSPHATE INTO THE TRIGLYCERIDES AND PHOSPHOLIPIDS OF NORMAL AND DYSTROPHIC MUSCLE OF MICE (Strain 129)

The incubation contained in a final volume of 3ml:35 µmoles $MgCl_2$; 60 µmoles phosphate buffer pH 7.5; 75 nmoles CoA; 6 µmoles sn-^{14}C-glycerol-3-phosphate (containing about 500,000 dpm); 30 µmoles ATP;1 ml muscle homogenate (10% in Krebs II). Radioactivity incorporated and lipid fractionation on thin layer chromatography were carried out as indicated in Methods.

		sn-^{14}C-glycerol-3-phosphate incorporated in: (nmoles/g wet w. muscle-minute)		
Animal	No.	Total Lipids	Triglyceride	Phospholipid
Normal	6	48.0 ± 3.3	21.3 ± 2.1	20.3 ± 1.8
Dystrophic	6	48.2 ± 3.6	21.4 ± 2.4	20.3 ± 2.2

TABLE XXIII

NITROGEN CONTENT AND TRIBUTYRINASE ACTIVITY OF MUSCLE HOMOGENATES FROM NORMAL AND DYSTROPHIC MICE (STRAIN 129)

The determinations were carried out as indicated in Methods. Values reported are averages ± SEM. P values apply to a comparison between normal and dystrophic.

Mice	Number	Nitrogen content (mg/g wet wt. muscle)		Tributyrinase Activity (μmoles butyric acid released per hour per:)		
		Total	NCN	mgN	mg NCN	g wet wt.
NORMAL	7	26.5 ± .68	23.5 ± .50	3.01 ± .08	3.42 ± .19	80.0 ± 2.9
DYSTROPHIC	7	24.8 ± .79	19.9 ± .91	7.35 ± .21	9.13 ± .51	180.5 ± 9.0

89% of the total N whereas in dystrophic it is about 80%. Tributyrinase activity has been found to be greatly increased in dystrophic muscle when the results are expressed relative to any of the three base-lines used ($P < 0.001$). The value in the dystrophic muscle reaches 265% of that of the normal muscle.

Similar experiments have been carried out on muscle undergoing atrophy after denervation. The results expressed in Figure 24 show that there is a large increase in the tributyrinase activity of the muscle from the denervated leg when compared with the normal. This increase was 18% 1 day after denervation, 60% 3 days later, 172% after 5 days, 169% after 7 days, and reached a maximum of 328% 13 days after denervation.

Tributyrinase activity has also been determined in muscle from patients with different muscle disorders and in normal controls. As seen in Figure 25 no differences from the normal controls with an average of about 2 units per mg NCN (one unit is defined as μl CO_2 released/minute-mg NCN) have been observed in a female carrier of Duchenne MD and in two cases diagnosed to have a myopathy of late onset. However, increased values were found in two cases with limb-girdle MD, two with facioscapulo-humeral MD, two with congenital myopathia, and in 7 (out of 9) cases with Duchenne MD. All the determinations were carried out on gastrocnemius muscle.

Tripalmitinase was determined by the isotopic method of Kaplan as described in the Methods. In Figure 26 is shown the influence of "Cutscum" treatment of muscle on the lipase activity. No lipase activity is evident in non-treated homogenate. The treatment with

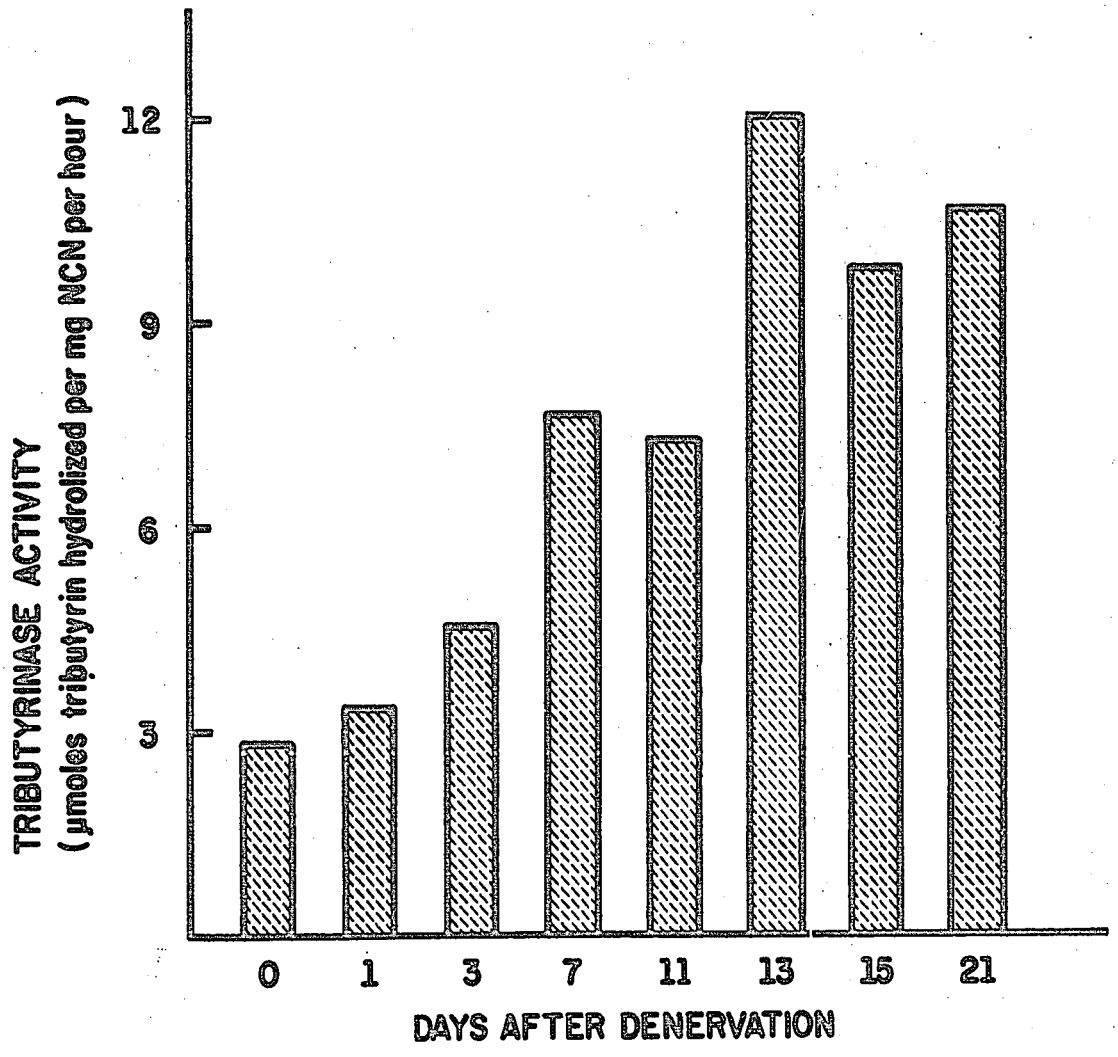


Figure 24 - Short-chain triglyceride lipase (tributyrinase) activity in denervated skeletal muscle of mice. The mice were anaesthesized with ether and the sciatic nerve was cut at the level of the great trochanter. The results were compared with those obtained from the contralateral non-operated leg. Values represent the average of two experiments.

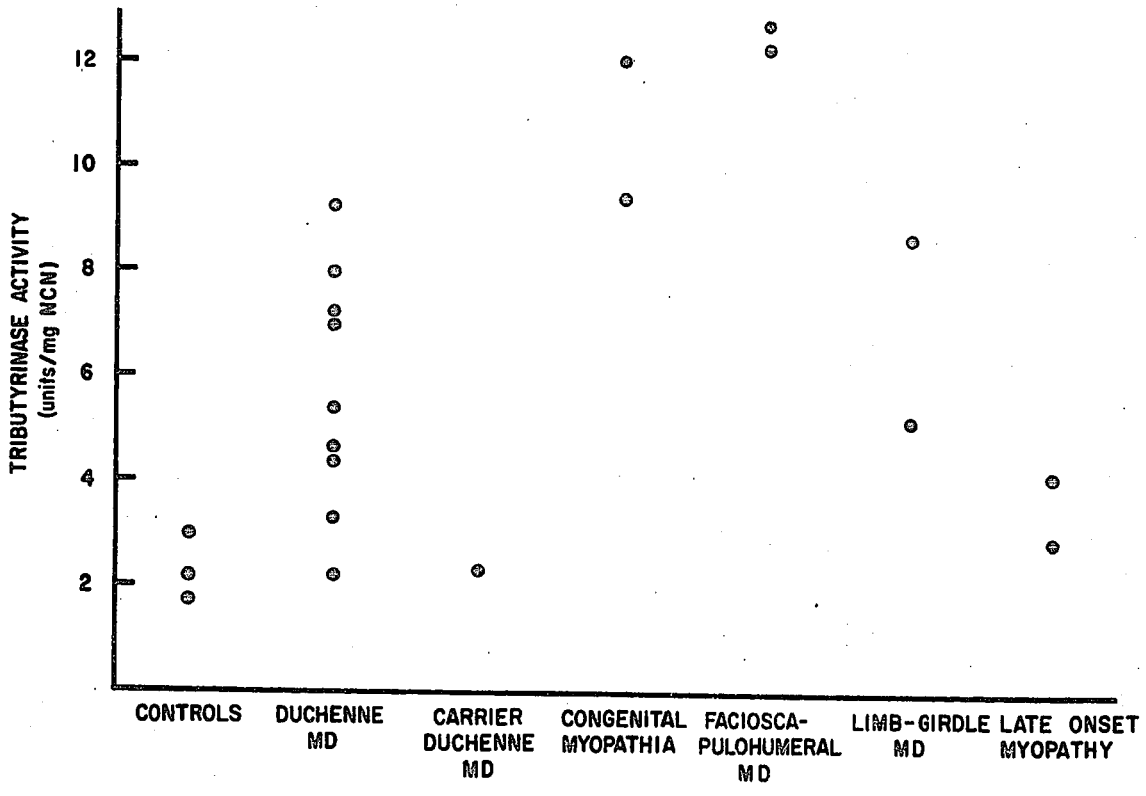


Figure 25 - Short-chain triglyceride lipase (tributyrinase) activities in skeletal muscle (gastrocnemius) from patients affected with various muscular disorders and in normal controls. Determinations were carried out as described in Methods.

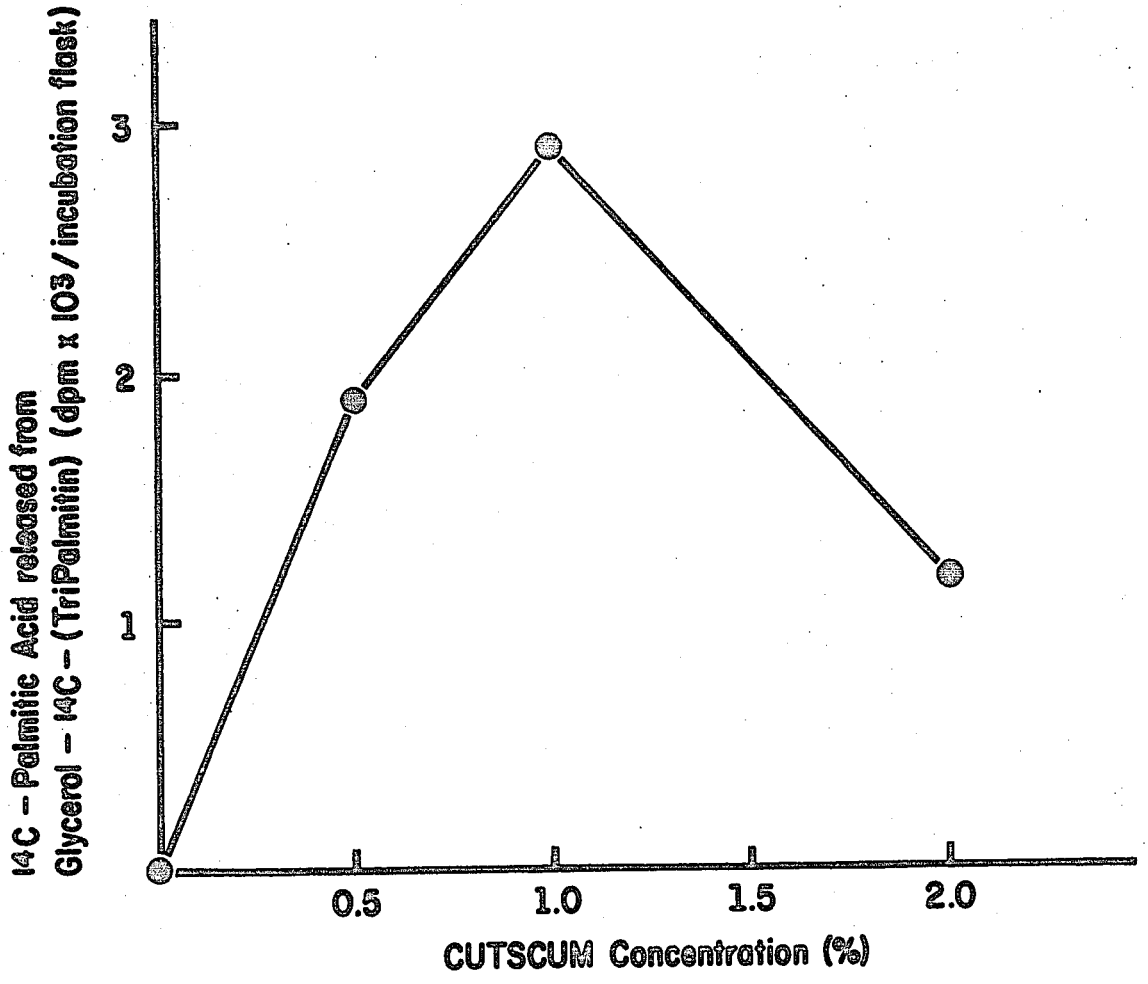


Figure 26 - Influence of "Cutscum" treatment on the tripalmitinase activity in muscle homogenate from mouse skeletal muscle. Each reaction vessel contained in a total volume of 1 ml: 60 μmoles Na-phosphate buffer, pH 6.5; 0.3 ml of 14,000 x g supernatant of muscle homogenate (20% w/v) (treated with "Cutscum" at the final concentration indicated in the figure) and, 3 μmoles Glycerol-^{14}C-(Tripalmitin) containing 120,000 d.p.m. Incubation time was 30 minutes.

"Cutscum" at a final concentration of 1% gave the maximum activity, and an inhibition of activity was observed when the concentration was increased to 2%.

The time course for the hydrolysis of tripalmitin by muscle homogenates is shown in Figure 27. Since the hydrolysis was almost linear over 30 minutes, this time was used in further experiments. In Figure 28 is expressed the relationship between lipase activity and the amount of Cutscum-treated muscle added to the incubation. A linear response was observed up to 0.3 ml. This amount was used in subsequent experiments. The effect of pH on enzyme activity is shown in Figure 29. The optimal pH was found to be 6.5. In the study of dependence on pH the buffer used was Na-phosphate in all determinations except for Na-acetate/acetic acid at pH 5.6 and glycine-NaOH at pH 8.9.

As is shown in Figure 30 the addition of albumin has only a very slight effect on lipase activity. With 10 mg added to the system there is an increase in activity of about 15%. As amounts are increased to 20 or 30 mgs a slight inhibition occurs. Therefore, albumin has been omitted in later experiments.

The inhibition characteristics of tripalmitinase are shown in Table XXIV. It is strongly inhibited by NaF at a final concentration of 0.1 M (86% inhibition), by DTNB (5×10^{-4} M), (39% inhibition), EDTA (10^{-3} M) (58% inhibition), N-ethyl-maleimide (10^{-3} M) (65% inhibition). The addition of NaCl (0.2M), $CaCl_2$ (2×10^{-3}), $MgCl_2$ (10^{-3}), epinephrine (0.6 µg/ml) or Na-taurocholate (2 mg/ml) has no effect on the enzyme. These results may be compared with the inhibition characteristics for the tributyrinase enzyme. With the latter the only inhibitions ob-

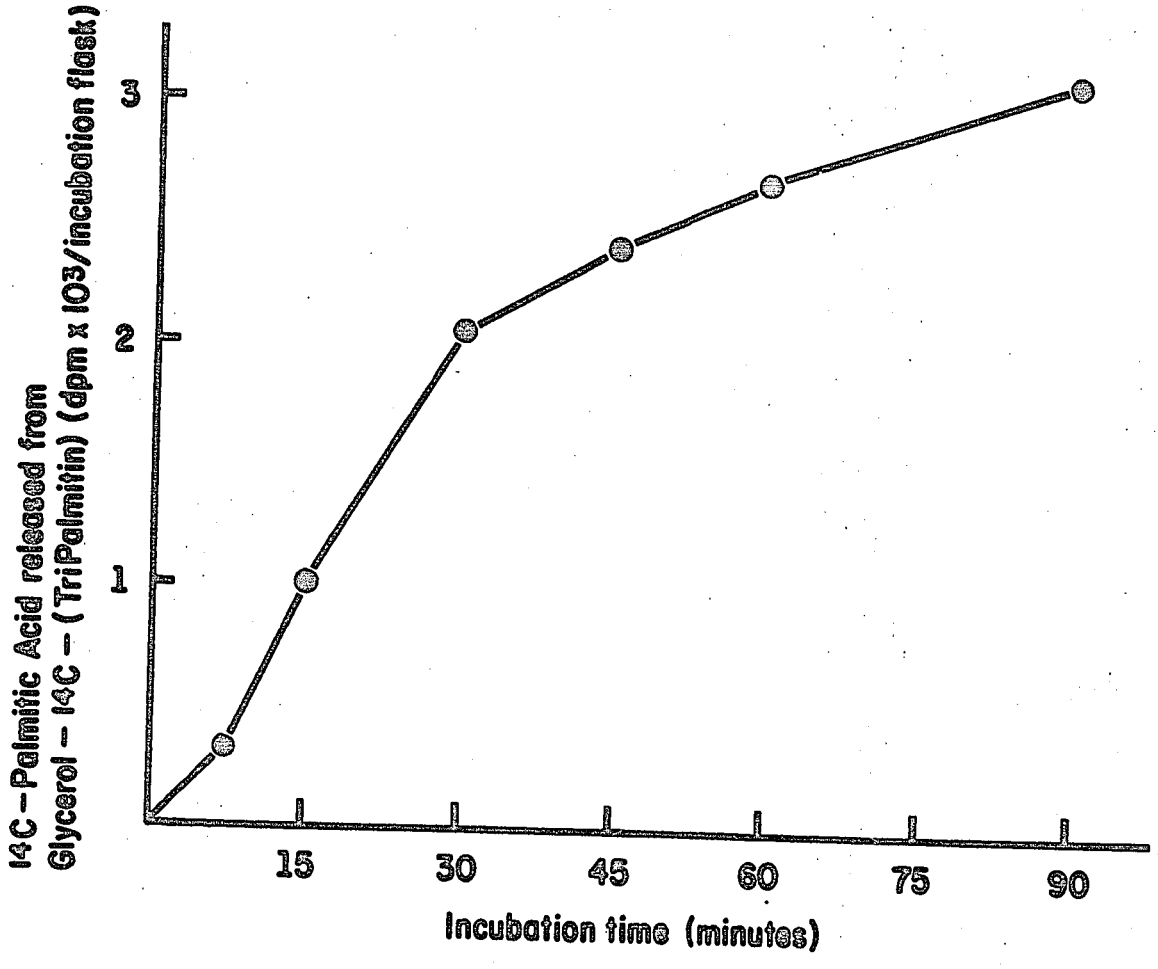


Figure 27 - Time course of the hydrolysis of Glycerol-^{14}C-(Tripalmitin) by skeletal muscle homogenates from mice. Incubation conditions as in Figure 26 except that the reaction was stopped at the times indicated. Muscle homogenates were treated with "Cutscum" at a final concentration of 1%.

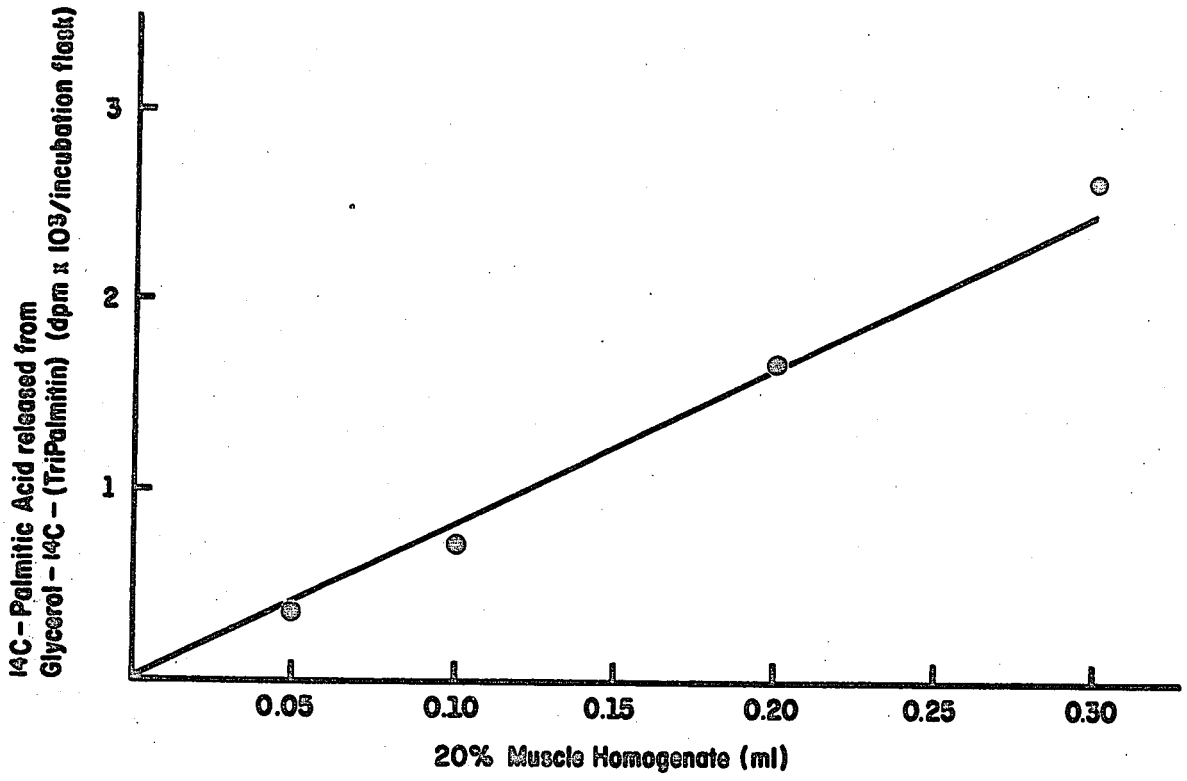


Figure 28 - Tripalmitinase activity. Effect of increasing amounts of "Cutscum"-treated muscle homogenate from mice. Incubation conditions as in Figure 26.

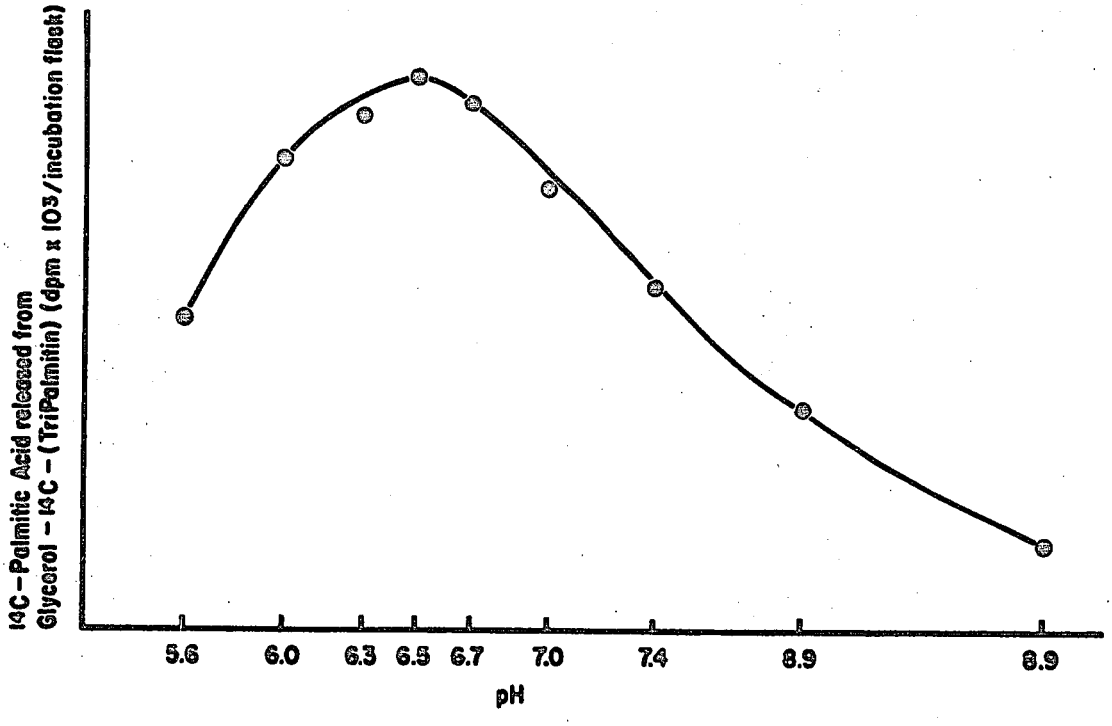


Figure 29 - Effect of pH on the tripalmitinase activity of skeletal muscle from mice. The buffer used was Na-phosphate for pH 6.8, Na-acetate/acetic acid for pH 5.6 and glycine-NaOH for pH 8.9. Other incubation conditions were as in Figure 26.

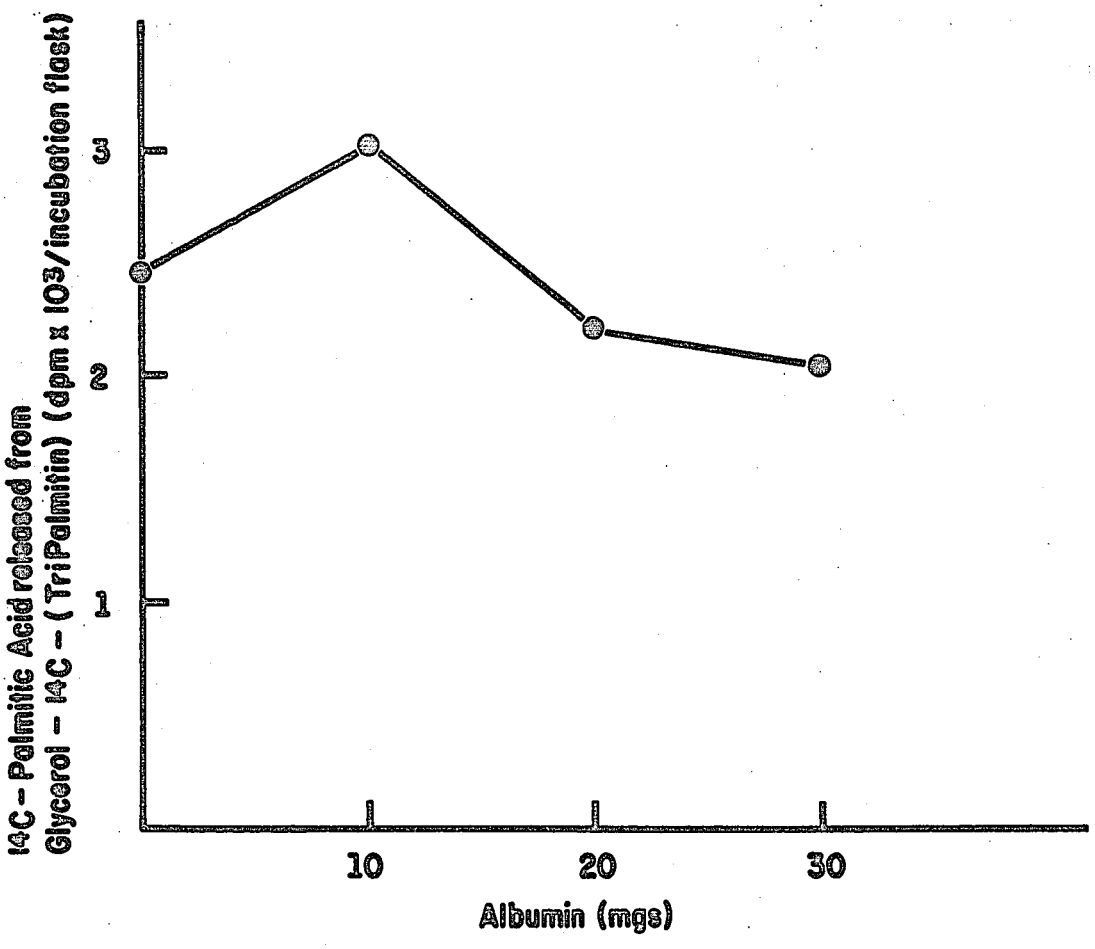


Figure 30 - The effect of increasing amounts of albumin on the tripalmitinase activity of skeletal muscle from mice. Incubation conditions as in Figure 26.

TABLE XXIV

INHIBITION CHARACTERISTICS OF TRIBUTYRIN LIPASE AND TRIPALMITIN LIPASE FROM MOUSE SKELETAL MUSCLE (STRAIN 129)

Determinations were carried out as described in Methods.

Inhibitor	Concentration	"Tributyrinase" % inhibition	"Tripalmitinase" % inhibition
NaF	10^{-3} M	-------	7
NaF	10^{-1} M	52	86
DTNB	2.10^{-4} M	-------	25
DTNB	5.10^{-4} M	-------	39
EDTA	10^{-3} M	0	59
N-ethyl-maleimide	10^{-3} M	0	65
NaCl	2.10^{-1} M	-------	0
$CaCl_2$	2.10^{-3} M	0	0
$MgCl_2$	10^{-3} M	33	4
Epinephrine	0.6 µg/ml	0	0
Na-taurocholate	2 mg/ml	0	0
Eserine	10^{-5} M	0	-------
Nu 683	10^{-8} M	0	-------
Nu 1250	10^{-7} M	0	-------

served were with NaF (10^{-1} M) (52% inhibition) and $MgCl_2$ (10^{-3} M) (33% inhibition). No effect was caused by EDTA (10^{-3} M), N-ethylmaleimide (10^{-3}M), $CaCl_2$, epinephrine, Na-taurocholate, eserine, Nu683 and Nu1250 (the latter three are cholinesterase inhibitors).

A study was undertaken to determine the subcellular distribution of the tripalmitinase. As it is shown in Table XXV the fraction giving the highest specific activity (activity per mg protein) was the pellet obtained after centrifugation at 90,000 x g which is called the "microsomal pellet".

In Table XXVI is shown a comparative study of tripalmitinase activity for normal and dystrophic mice. A significant increase was observed in the dystrophic muscle when the results are expressed in nanomoles (mμmoles) ^{14}C-palmitic acid released per g wet weight muscle. This increase is minimal since no allowance has been made for the presence of endogenous triglyceride which is significantly increased in dystrophic muscle. If it is assumed that this triglyceride simply dilutes the added substrate then the specific activity of the substrate becomes reduced to about one-half that of normal. The net effect of this dilution would be a reduction in the tripalmitinase activity of dystrophic muscle which would take away from rather than add to any observed increase. Thus the increase noted for dystrophic muscle must be regarded as minimal. This situation is also true when activities are expressed per mg NCN.

Discussion

The level of triglycerides in tissues will depend upon the balance between uptake and synthesis and that of catabolism and elim-

TABLE XXV

INTRACELLULAR DISTRIBUTION OF LONG-CHAIN TRIGLYCERIDE LIPASE (TRIPALMITINASE) FROM SKELETAL MUSCLE OF MICE (STRAIN 129)

Muscle fractionation and determination of Tripalmitinase were carried out as described in Methods.

Fraction	Specific Activity (^{14}C-Palmitic Acid released per mg protein)	
	Experiment 1	Experiment 2
500 x g pellet	0.10	0.11
12,000 x g pellet (Mitochondrial)	0.40	0.00
90,000 x g pellet (Microsomal)	32.70	28.31
90,000 x g supernatant	0.32	0.00

TABLE XXVI

LONG-CHAIN TRIGLYCERIDE LIPASE ("TRIPALMITINASE") ACTIVITIES OF MUSCLE HOMOGENATES FROM NORMAL AND DYSTROPHIC MICE (STRAIN 129)

Methods as indicated in the text. P indicates the probability that there is no effect caused by dystrophy.

Mice	Number	"Tripalmitinase" Activity (nanomoles ^{14}C-Palmitic acid released per g w.w. muscle)	P Value
NORMAL	8	723 ± 42	< 0.001
DYSTROPHIC	8	1,100 ± 68	

ination. In this chapter we have considered two of these aspects of triglyceride metabolism, viz., synthesis and degradation.

Two main pathways for the synthesis of triglycerides in animal tissues have been established (223). The first one uses sn-glycerol-3-phosphate (GP) as acceptor of fatty acids and results in triglyceride formation through several intermediate steps involving the formation of phosphatidic acid and diglyceride. In the second pathway monoglycerides are used as the acceptor of the fatty acid.

The study of the synthesis of triglycerides in muscle from normal and dystrophic mice was carried out at the beginning using palmitate-1-^{14}C as substrate and determining the extent of its incorporation into the triglyceride fraction. It was observed that there was no stimulation of acylation upon addition of GP. However, the incorporation was stimulated by the addition of NAD and glucose, but GP could not substitute for glucose (Table XVI). These results are not entirely consistent with the GP pathway for triglyceride synthesis being primarily operative in skeletal muscle of mice. The glucose and NAD introduced in the system could normally supply GP, but since added GP fails to stimulate the acylation reaction, it becomes difficult to say this is the entire role of glucose. It may be that glucose "spares" the palmitate from oxidation so it can be used in acylation reactions.

When glucose-U-^{14}C was used in some experiments, a significant incorporation of radioactivity into triglycerides was observed. It is not known whether all of this incorporation was into the glycerol moiety or a part or all into the fatty acid moiety.

Real proof that the GP pathway is operative in skeletal muscle is best demonstrated by observing the incorporation of ^{14}C-GP into triglycerides. ^{14}C-GP is a rather ideal precursor to use because the negligible amounts of GP in the tissues cause minimal dilution of the isotope. Based on the requirement necessary for the incorporation of GP and palmitate into triglycerides (Table XVI), it can be deduced that two different processes are being measured and that probably the latter does not reflect a de novo synthesis of triglycerides. It would appear that part of the incorporation of palmitate is taking place by an exchange reaction mechanism which involves the other fatty acids of endogenous triglycerides. This explanation is consistent with the tendency observed in dystrophic muscle of an increased incorporation of palmitate which was in parallel with an increased lipase activity (Table XXVI). Further confirmation for this is given by the fact that F^- strongly inhibits both tripalmitinase activity and palmitate incorporation into TG without affecting the incorporation of ^{14}C-GP.

F^- has been used as an inhibitor of ATPase in order to maintain a more constant supply of ATP in the system. It has been reported that it produces an increased incorporation of ^{32}P-GP into the lipids of rat brain homogenates (239). This effect has not been observed in the experiments reported here.

The lack of any differences observed in the distribution of fatty acid in the various lipid fractions confirms the findings of previous workers (130) and supports the contention that this type of muscular dystrophy is not caused by an essential fatty acid deficiency.

The results obtained here do not support the possibility that the increased fat content in dystrophic muscle is due to an alteration in the biosynthetic pathway of TG. This type of conclusion then caused us to examine the lipase activity of these tissues. In the initial investigations a manometric technique was used with tributyrin as substrate. Later it was considered that more useful information could be obtained using the more physiological long chain triglyceride, tripalmitin, as substrate.

The existence of a short-chain triglyceride lipase has been reported in pigeon skeletal muscle (240). Also, with histochemical methods it has been found that skeletal muscle is able to hydrolyze Tween-80 (polyoxyethylene (20) Sorbitan Monooleate) (241). In rat skeletal muscle the existence of four different lipases has been demonstrated (242), but none of these catalyzed the hydrolysis of triglycerides with the fatty acids of chain lengths longer than 9 carbons. However, the existence of such a lipase can be assumed if it is considered that long-chain triglycerides form the main bulk of lipids in muscle (133), that they are consumed by rat diaphragm during incubation in vitro (243), and that depletion of long-chain triglycerides occurs during exercise (244).

It is difficult to evaluate the importance of the increased activity of tributyrinase found in dystrophic muscle, denervated muscle and in the muscle affected with different myopathies, since tributyrin does not appear to be an important physiological substrate in the mouse or man. The possibility that some pseudo-cholinesterases were responsible for the observed activity can be ruled out on the basis that specific

inhibitors (eserine, Nu 683 and Nu 1250) of the latter enzyme did not affect the results.

Long-chain triglyceride lipase in muscle could be detected only after the muscle homogenate was treated with the detergent "Cutscum", the maximal activity being obtained when the final concentration of "Cutscum" was at 1%. This finding strongly suggests that the activity of this enzyme is masked through binding to some structure from which it is released by the action of the detergent. The same phenomenon has been observed with other enzymes of lipid metabolism such as CDP-diglyceride: inositol transferase of rat brain (245).

Long-chain triglyceride lipase from skeletal muscle of mice is very sensitive to the action of DTNB or N-ethyl maleimide which shows that intactness of SH groups is important for optimal activity. Also cations play some role as shown by the inhibition originated by the use of EDTA. In contrast to the observation for certain lipases in adipose tissue (246), epinephrine did not enhance lipase activity in skeletal muscle. The same occurred for Na-taurocholate, an activator of pancreatic lipase (247). The different inhibition characteristics found for tributyrinase and tripalmitinase (Table XXIV) show that there are likely two different enzymes, and not the same one, acting on these different substrates. Experiments have not been carried out to determine the specificity of these enzymes to other substrates.

The study on the cellular distribution of this enzyme shows that it is localized mainly in the pellet obtained after centrifugation at 90,000 x g for 100 minutes. Probably long-chain triglyceride lipase

is localized in the lysosomes, considering that it is a hydrolase and that it is released by the action of "Cutscum".

The increase in activity found for "tripalmitinase" and "tributyrinase"are in agreement with similar increases reported for other lipases from skeletal muscle of dystrophic mice such as monopalmitin lipase, monomyristin lipase and tricaproin lipase (248). This may represent an actual increase in lipase activity in response to the accumulation of fat in the dystrophic muscle, or increased lysosomal activity accompanying the breakdown of muscle.

CHAPTER VII

Enzymatic Studies, Carnitine Content and Intramitochondrial Content of CoA in Skeletal Muscle from Normal and Dystrophic Mice (Strain 129)

1) Introduction

Mitochondrial alterations in dystrophic muscle have been reported by several authors (38, 59), the most common mitochondrial change observed being a separation of the cristae usually in regions of myofibrillar destruction but sometimes also in parts of the muscle with normal myofibrils. These observations and supporting biochemical evidence strongly suggest that there is some impairment of mitochondrial function in muscular dystrophy. This is further substantiated by the findings of an altered fatty acid oxidation by muscle mitochondria from dystrophic muscle (135) and of a decreased respiratory control ratio (249).

Because of these findings we have studied the activities of several mitochondrial enzymes. These include enzymes of the tricarboxylic acid cycle, the complexes of the electron transport chain, acetylcarnitine transferase, and monoaminoxidase. At the same time cytochrome oxidase and monoaminoxidase have been determined in muscle homogenates. Determinations of carnitine in muscle and the intramitochondrial content of CoA from normal and dystrophic mice are also included in the studies reported in this chapter.

2) Results

All the enzymes of the tricarboxylic acid cycle except succinate dehydrogenase, were easily extracted by the Methods previously described (see Chapter III). When comparing enzyme activity in the supernatant obtained after centrifugation of the deoxycholate-treated mitochondria with the activity in the same mitochondria before centrifugation, the recovery was of the order of 100%. The same effect can be obtained using Triton X-100 instead of deoxycholate.

When using deoxycholate-treated mitochondria it was necessary to use CN^- in the assay of any of the enzymes which involved the measurement of changes in optical density at 340 nm in order to inhibit cytochrome oxidase, but this was not necessary when working with the supernatant obtained after centrifugation. The results in Table XXVII illustrate these points for α-ketoglutarate dehydrogenase.

In Table XXVIII are shown the activities of the enzymes involved in the tricarboxylic acid cycle. When the results were analyzed using the student's "t" test no differences were observed between normal and dystrophic muscle even though there appears to be some decrease in the activity of all enzymes tested with the exception of NAD-isocitrate dehydrogenase.

Succinate dehydrogenase has been determined in whole homogenate and in isolated mitochondria. From the data, the mitochondrial content of both normal and dystrophic muscle was determined. As seen in Table XXIX there is a tendency towards an increase in mitochondria in dystrophic muscle on a per g wet weight basis. However, the difference is not statistically significant.

TABLE XXVII

ACTIVITY OF α-KETOGLUTARATE DEHYDROGENASE FROM MUSCLE MITOCHONDRIA

The incubation contained in a final volume of 3 ml: 150 μmoles Tris-buffer, pH 7.6; 0.15 μmole CoA; 1.5 μmoles NAD; 9 μmoles cysteine; 30 moles α-ketoglutarate (K^+ salt); 40-100 μg mitochondrial protein. One unit of activity is the amount of enzyme which causes a change in optical density of 0.001.

Enzymatic preparation	Activity (units/ mg protein)
Deoxycholate-treated mitochondria	
Whole mitochondria	25.5
Whole mitochondria plus KCN*	32.2
After centrifugation	
Supernatant	31.8
Precipitate	00.0

*0.2 mg.

TABLE XXVIII

ACTIVITIES OF ENZYMES OF THE KREBS CYCLE IN MITOCHONDRIA ISOLATED FROM MUSCLE OF NORMAL AND DYSTROPHIC MICE (Strain 129)

Mitochondria have been isolated and enzymatic activity determined as indicated in Methods. Activities are reported as units of activity per mg mitochondrial protein ± SEM. P is the probability that there is no effect caused by dystrophy.

Enzyme	Number	Enzymatic activity (Units per mg protein ± SEM) NORMAL	DYSTROPHIC	P
Citrate synthase	7	469 ± 20	399 ± 21	< 1
Aconitase	6	51.7 ± 2.9	41.7 ± 4.3	
NADP-Isocitric Dehydrogenase	7	25.4 ± 2.1	21.6 ± 2.3	
NAD-Isocitric Dehydrogenase	6	53.3 ± 3.2	56.9 ± 3.8	
α-Ketoglutarate Dehydrogenase	5	22.2 ± 2.2	21.2 ± 1.5	
Fumarase	5	4.43 ± 0.16	4.26 ± 0.19	
Malic Dehydrogenase	6	981 ± 86	785 ± 76	

TABLE XXIX

SUCCINATE DEHYDROGENASE ACTIVITIES IN WHOLE HOMOGENATE AND IN ISOLATED MITOCHONDRIA FROM HIND LEG MUSCLE OF DYSTROPHIC MICE AND NORMAL LITTERMATES (Strain 129)

Determinations were carried out as described in Methods. One unit expresses the μmoles of succinate oxidized per minute. Mitochondrial content is expressed as mg mitochondrial protein per g wet weight muscle.

Mice	Number	Succinate Dehydrogenase Activity a) In whole homogenate (units per g muscle)	Succinate Dehydrogenase Activity b) In isolated mitochondria (units per mg mitochondrial protein)	Mitochondrial content (a/b) = (mg/g muscle)
NORMAL	6	2.10 ± 0.13	0.180 ± 0.011	10.15 ± 1.6
DYSTROPHIC	6	2.97 ± 0.21	0.178 ± 0.015	17.35 ± 2.4

In Table XXX are shown the activities of a number of electron transport enzyme systems. No differences were observed between normal and dystrophic muscle. Mitochondrial yield has been also determined and when expressed as mg mitochondrial protein per gm wet weight muscle it shows a statistically significant decrease in dystrophic muscle.

The distribution of monoaminoxidase in the muscle fiber has been determined and it is expressed in Table XXXI. As can be seen, both mitochondrial and microsomal fractions contain significant portions of the enzymatic activity. A comparative study between normal and dystrophic muscle is also shown. A significant increase was observed in dystrophic muscle. Monoaminoxidase was also determined in muscle homogenates from patients affected with muscular diseases and from normal controls. The results are shown in Figure 31. Similar determinations have been carried out in hind leg muscle from mice after section of the sciatic nerve, and the results obtained are depicted in Figure 32.

In Table XXXII is shown the subcellular distribution for carnitine acetyltransferase (CAT). The highest activity of the enzyme is associated with the 11,800 x g pellet which corresponds to the mitochondrial pellet. Next to this fraction is the 350 x g pellet with an activity of about one-half that of the 11,800 x g pellet. Probably this activity comes from mitochondria precipitated with the myofibrils. In fact, the yield of mitochondria can be increased using proteinase during the homogenization procedure. We did not use proteinase because of the possibility it could damage the transferase enzyme. However later, we tested this possibility and found that proteinase (nagarse) in concentrations as used by Lin et al (135) or Wrogeman et al (149) (that is 1

TABLE XXX

ACTIVITIES OF ENZYME SYSTEMS OF THE ELECTRON TRANSPORT CHAIN IN MITOCHONDRIA ISOLATED FROM NORMAL AND DYSTROPHIC MUSCLE (Strain 129)

Mitochondria have been isolated and enzymatic activities determined as indicated in Methods.

Enzyme	Number	Mitochondrial protein (mg per gm muscle)	Enzyme activity (units/mg protein ± SEM) NORMAL	DYSTROPHIC
Succinate cytochrome c reductase	6		53.0 ± 2.4	58.7 ± 3.4
NADH-cytochrome c reductase	8	N = 2.71 ± 0.12	97.4 ± 14.2	126.9 ± 6.5
Rotenone insensitive NADH-cytochrome c reductase	6	D = 1.76 + 0.18	23.05 ± 2.07	35.1 ± 3.95
Cytochrome oxidase	6		45.4 ± 5.1	37.1 ± 3.1

TABLE XXXI

DISTRIBUTION OF MONOAMINOXIDASE (MAO) IN SUBCELLULAR FRACTIONS OF SKELETAL MUSCLE FROM NORMAL AND DYSTROPHIC MICE (Strain 129)

Subcellular fractions were obtained and enzymatic activity determined as described in Methods. The results are expressed in units per mg NCN for whole homogenate and in units per mg protein for each of the other fractions. One unit is equivalent to the oxidation of 1 nanomole tryptamine per minute. P is the probability that there is no effect caused by dystrophy.

Preparation	Animal	No.	MAO activity	P value
Whole Homogenate	N	6	0.14 ± 0.004	< 0.01
	D	5	0.21 ± 0.013	
11,000 x g pellet	N	9	0.32 ± 0.07	< 0.001
	D	9	0.96 ± 0.10	
90,000 x g pellet	N	5	0.52 ± 0.06	< 0.001
	D	5	1.42 ± 0.12	
90,000 x g supernatant	N	4	0.038 ± 0.004	< 1
	D	4	0.034 ± 0.003	

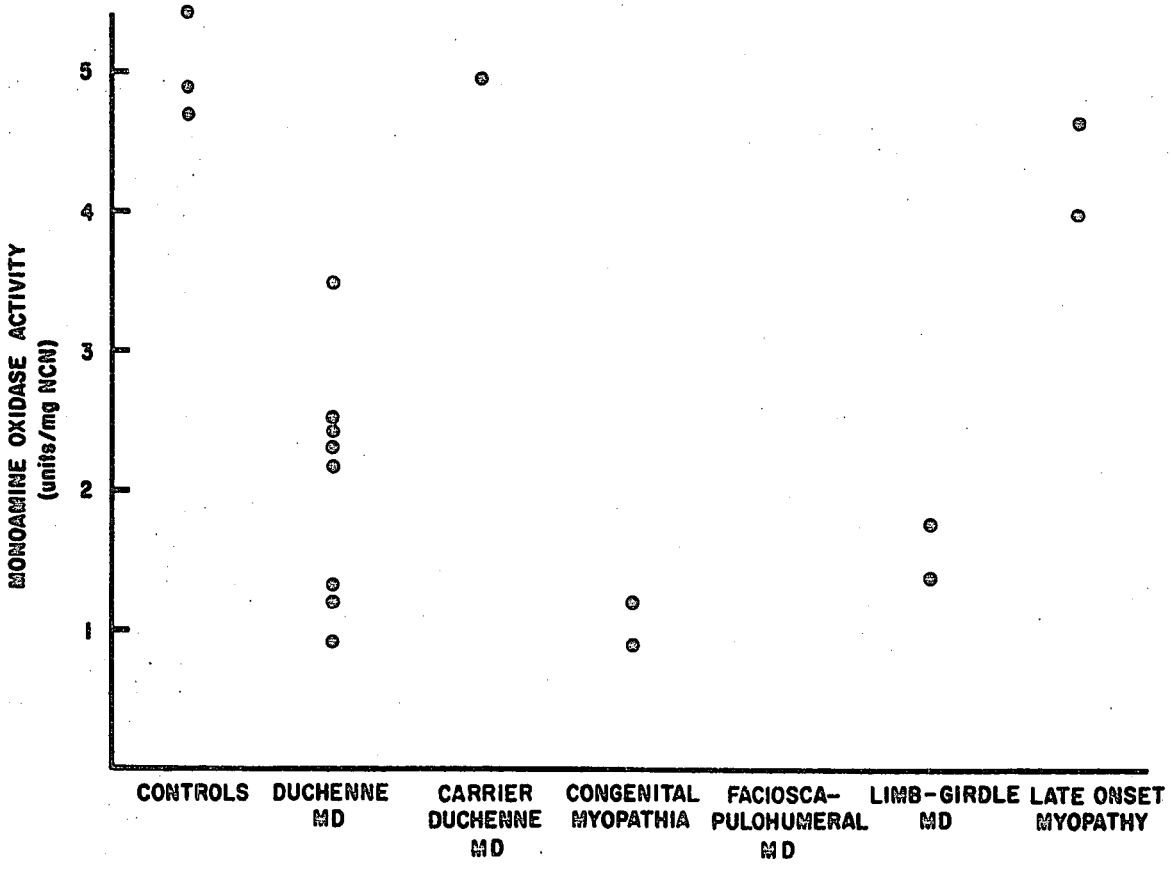


Figure 31 - Monoaminoxidase activity in muscle homogenates from patients affected with muscular diseases and from normal controls. The activity has been determined as indicated in the Methods, and expressed in units per mg non-collagen N. One unit of activity represents 1 nanomole of tryptamine oxidized per minute.

Figure 32 - Monoaminoxidase activity in homogenates of muscle undergoing atrophy after denervation. Determinations were carried out as described in Table XXXI.

MONOAMINE OXIDASE ACTIVITY
(μmoles tryptamine oxidized per mg NCN per minute)
0.3
0.2
0.1
0
1
3
7
11
13
15
21
DAYS AFTER DENERVATION

TABLE XXXII

INTRACELLULAR DISTRIBUTION OF CARNITINE-ACETYLTRANSFERASE IN SKELETAL MUSCLE FROM MOUSE

Fractions were separated by centrifugation and the pellets were extracted and transferase activity was determined as described in the Methods.

Fraction	Activity nmoles DPNH formed/min-g muscle
350 x g pellet	500
11,000 x g pellet (Mitochondrial fraction)	
Deoxycholate-phosphate extractable	1,100
Residue after extraction	0
11,000 x g supernatant	0

and 15 mg/30 ml respectively) did not have any effect on the CAT enzyme, while this treatment increased the yield of mitochondria (Table XXXIII).

All the transferase activity was found in the deoxycholate-phosphate-extractable fraction, and no activity was found in either the residue after extraction or in the 11,900 x g supernatant fraction. These results are shown in Tables XXXIV. It can be seen that most of the succinic-cytochrome c reductase activity (96%) remained in the residue after extraction by deoxycholate-phosphate buffer. This reflects the membrane-bound nature of this enzyme.

The comparative study of CAT enzyme between normal and dystrophic muscle mitochondria is shown in Table XXXV. A decrease of about 35% was noted in dystrophic mitochondria when the results are expressed in units of activity per mg mitochondrial protein.

In Table XXXVI are expressed the concentrations of carnitine found in normal and dystrophic muscle. The amounts reported correspond to total carnitine in the tissue. No significant differences were observed. The small difference observed in the average value is reduced even further if the results are expressed relative to non-collagen N.

Cytochrome oxidase has been determined in homogenates of muscle from patients with muscular diseases and from normal controls. The results are expressed in Figure 33. Although the values are quite scattered, no appreciable differences can be observed between myopathic muscle and normal.

In Table XXXVII is shown the amount of CoA contained in muscle mitochondria from normal and dystrophic mice. The results are expressed in nmoles CoA (free CoA + acetyl CoA) per unit succinate-cytochrome c

TABLE XXXIII

EFFECT OF PROTEINASE ON MITOCHONDRIA YIELD AND CARNITINE ACETYLTRANSFERASE SPECIFIC ACTIVITY FROM SKELETAL MUSCLE OF MOUSE

Mitochondria were isolated and transferase activity was measured as described in the Methods.

Proteinase concentration in homogenization media (mg/ml)	Mitochondrial yield (mg mitochondrial protein/g muscle)	Transferase activity (units/mg mit. protein)
0	2.51	246
1 : 30	3.76	262
15 : 30	3.99	240

TABLE XXXIV

CARNITINE ACETYLTRANSFERASE AND SUCCINIC CYTOCHROME C REDUCTASE DISTRIBUTION IN SKELETAL MUSCLE MITOCHONDRIA FROM MICE (Strain 129)

Mitochondria were isolated and enzyme activities were determined as described in the Methods.

Preparation	CAT (units)	Succinate cytochrome c reductase (units)
Extractable deoxycholate-phosphate fraction	250	1.8
Residue after deoxycholate-phosphate extraction	0	42.8

TABLE XXXV

CARNITINE ACETYLTRANSFERASE AND SUCCINATE-CYTOCHROME C REDUCTASE ACTIVITIES IN SKELETAL MUSCLE MITOCHONDRIA FROM DYSTROPHIC MICE AND NORMAL LITTERMATES (Strain 129)

Mitochondria were isolated and protein and enzyme activities were determined as described in the Methods.

P represents the probability that there is no effect caused by dystrophy.

Enzyme	Animal	No.	Mitochondrial protein yield mg/g muscle ± SEM	Enzyme Activity ± SEM units /mg mitochondrial protein	P.*
Carnitine-acetyltransferase	N	6	3.416 ± .352	259 ± 12	< .001
	D	6	2.341 ± .260	170 ± 5	
Succinic cytochrome c reductase	N	6	3.382 ± .280	53.0 ± 2.4	< 1.
	D	6	2.416 ± .216	58.7 ± 3.4	

TABLE XXXVI

CARNITINE CONTENT OF MUSCLE FROM DYSTROPHIC AND NORMAL MICE (Strain 129)

Carnitine content was determined as indicated in the Methods. The values are expressed as μg carnitine per gm fresh tissue ± SEM.

Mice	No	Carnitine content μg/ g fresh tissue ± SEM
NORMAL	6	127.1 ± 6.3
DYSTROPHIC	6	105.9 ± 11.1

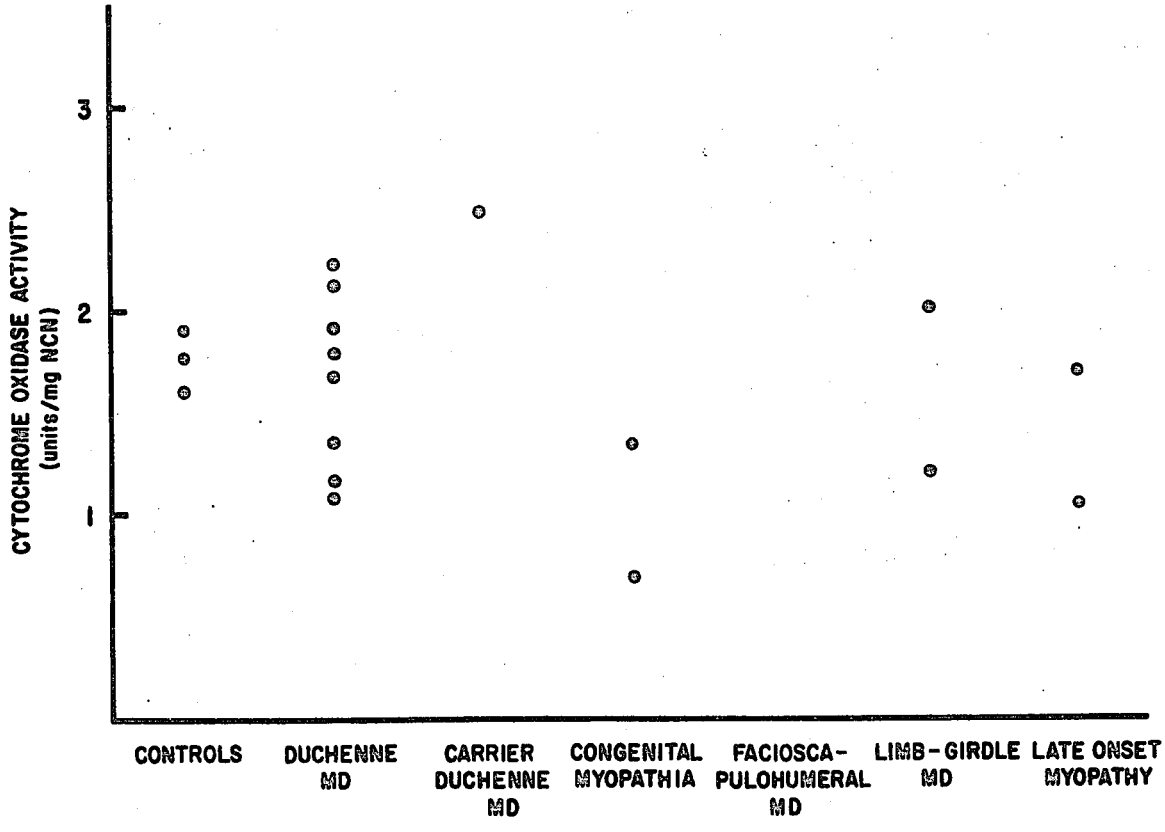


Figure 33 - Cytochrome oxidase activity in muscle homogenates from patients affected with muscular diseases and from normal controls. Activities were determined as described in the Methods. Values are expressed in units per mg non-collagen N. One unit equals 1 μmole O_2 uptake per minute.

TABLE XXXVII

COENZYME A CONTENT IN MITOCHONDRIA ISOLATED FROM THE HIND LEG MUSCLE OF DYSTROPHIC MICE (D) AND THEIR LITTERMATE CONTROLS (N) (Strain 129)

Mitochondria were isolated and CoA determined as described in the Methods. P indicates the probability that there is no effect caused by dystrophy.

Animal	No.	CoA content (nmoles CoA/unit succinate - cyt. c reductase ± SEM)	P
N	5	0.0209 ± 0.0022	
			< 0.01
D	5	0.0083 ± 0.0010	

reductase. A highly significant ($P < 0.01$) decrease (55%) in the amount of CoA was observed in the mitochondria isolated from dystrophic muscle.

3) Discussion

The main demonstrable biochemical manifestation of a genetic disease is that of an abnormality in the amount or configuration of one or several proteins or enzymes. Only when this defect, which is immediately secondary to a mutation in the DNA molecule, is clearly demonstrated, will a clear understanding of the disease be obtained. It is not only the vast number of enzymes and proteins to be tested that makes this search a difficult task but also other factors such as the mechanism of induction or repression which affects the formation of these proteins which may to some extent mask the primary defect. It is therefore possible to find several enzymes with significant differences in activity, but no single difference is able to account for the pathogenesis of the disease. In theory there are several criteria that may be used to help determine the primary defect of a metabolic disease. For example, study may be made, (a) of the order of appearance of different changes with time, (b) of common defects evident in all patients or animals with the same disease, or (c) the administration of a specific inhibitor of an enzyme suspected of being responsible for the disease should reproduce its clinical characteristics. However, in practice these criteria very seldom can be used, since at birth there may already exist a wide spectra of metabolic abnormalities, or alternatively the clinical manifestations seen may arise in response to a number of different primary defects, or thirdly, because of the lack of a specific inhibitor for the particular enzyme in question.

Another point to consider is regarding the relation between the enzymatic activities found and the disease itself. It must be considered that the laboratory tests used to determine enzyme activities are carried out under optimal conditions giving a 100% efficiency that probably never exists in situ. This point may be illustrated by reference to other genetic diseases. Thus, in galactosemia, heterozygous carriers with values as 25% of the normal controls have been reported (250), and in phenylketonuria values of phenylalanine hydroxylase as low as 10% of the normal have been found, without any evident clinical symptoms of the disease (251).

The third problem in this type of study is that of finding a satisfactory basis for expression of the results. Several baselines have been used. For example, wet or dry weight of the tissues is often used, but this has the disadvantage of not accounting for any infiltration of the diseased organ or tissue by other types of cells. DNA as a baseline reference is usually quite reliable but it does not take into account any nuclear proliferation in tissues that are undergoing a degenerative-regenerative process such as occurs in muscle. In our determinations we have used as baseline the mg of non-collagen N (183) for the determinations of activities in muscle homogenates, and mg of particulate protein for the determinations of activities in mitochondrial and microsomal fractions.

In the particulate preparations it is important that the fractions be as pure as possible. Several criteria have been used to evaluate this situation, mainly in the case of mitochondrial fractions. We can offer the following data in favor of reasonable purity of the isolated mitochondria used in our studies:

1) Electron microscopy (Figure 6) shows the bulk of the preparation to be mitochondrial in nature with very few loose membranes that could be attributed to broken outer membranes or to sarcoplasmic reticulum membranes or lysosomes. This figure may be compared with Figure 7 which shows an electron micrograph of a microsomal preparation where no mitochondria can be seen.

2) Rotenone-insensitive NADH cytochrome c reductase activity has been postulated to exist in both the outer mitochondrial membrane and microsomal fractions, but predominantly in the latter (200). The mitochondrial preparation used in these experiments contained 20% of the total activity of this reductase while the microsomal fraction contained 77% of the total activity.

3) Tripalmitin lipase activity has been found to be located in the pellet obtained after centrifugation at 90,000 x g for 100 minutes of the supernatant obtained following removal of the mitochondrial fraction. Considering that this enzyme is a hydrolase and that its activity is released after treatment with the detergent Cutscum, the possibility exists that this enzyme originates from the lysosomes. If so lysosomal contamination in mitochondria seems unlikely since in two experiments the specific activity in the mitochondrial fraction has been found to be zero in one experiment and only 1.2% of that obtained in the 90,000 x g fraction in a second (see Table XXV).

Even though it was not possible to use glucose-6-phosphatase (negligible amount in skeletal muscle) or NADPH-cytochrome c reductase as marker enzymes to assess microsomal contamination of mitochondria, the above data strongly favour only slight contamination and suggest that

this fraction can be considered quite pure.

Another characteristic that was studied in the mitochondrial fraction was that of changes in mitochondrial fragility. In this respect NADH-cytochrome c reductase has been determined in isolated mitochondria before and after being subjected to osmotic shock. The results indicate that the activity in undamaged mitochondria is only 18% of the value obtained after treatment. This value was similar for mitochondria from both normal and dystrophic muscle.

The functional state of the isolated mitochondria have been tested with the oxygen electrode as reported in the Methods. Values for the respiratory control ratio of the order of 6.5 show the presence of intact tightly coupled mitochondria in preparations obtained from normal muscle.

The total content of mitochondria in muscle was determined using succinate dehydrogenase contents in whole homogenate and in isolated mitochondria (252). The data obtained gave a value of 10.15 ± 1.6 mg mitochondrial protein per gm of normal muscle and 17.35 ± 2.4 mg in the case of dystrophic muscle. Because the normal yield of mitochondria obtained from muscle is lower in the case of dystrophic muscle, these data suggest the possibilities: (a) that a lower yield results from increased losses of mitochondria in dystrophic muscle because less muscle was available to start the fractionation, (b) that several physicochemical characteristics (e.g., increased concentration in fat, changes in viscosity of myosin, etc.) of the dystrophic muscle are responsible for a different centrifugation behaviour for both normal and dystrophic tissue, and (c) even if the final preparations obtained have similar characteristics,

the different yield could reflect a selection of one type of mitochondria. For example, in dystrophic muscle a larger population of more fragile mitochondria may exist which do not survive the homogenization and centrifugation. It is entirely possible that the leakage of enzymes into the plasma can express an alteration in membranes that could extend to these of the mitochondria.

A number of enzyme activities have been determined on the mitochondrial preparation just discussed. The study initially focussed on the enzymatic steps of the tricarboxylic acid cycle, the electron transport systems and acetylcarnitine transferase (CAT) because each could in some way be related to the defects in the oxidation of palmitic acid, pyruvic acid (135) and acetylcarnitine (253) observed in mitochondria isolated from skeletal muscle of dystrophic mice.

Carnitine acetyltransferase (CAT) has been reported to be localized mainly in the 1,000 x g residual fraction (254) obtained from rat heart muscle. This fraction was found to consist mainly of nuclei and unbroken cells with some heavy mitochondria trapped in the pellet. Next to this fraction significant activity was present in the mitochondrial pellet and a lesser amount occurred in the soluble cytoplasmic fraction.

In our results (Table XXXII) most of the CAT activity was found in the mitochondrial pellet, followed by the 350 x g residual pellet. Activity in the latter fraction probably derives from mitochondria trapped in the pellet. This conclusion is supported by the observation that proteinase added during the isolation procedure increased the yield of mitochondria and the enzymatic activity, probably as a result of the disruption of the myofibrils (Table XXXIII). No activity was found in

the supernatant fraction after 11,000 x g. These differences between the results of Marquis and Fritz (254) and those reported here may be related to differences in tissue used (heart muscle versus skeletal muscle) and/or to differences in techniques used. Our results are in agreement with those of Beenakkers and Klingenberg (255) who concluded that CAT occurs exclusively in the mitochondria. In addition, Lowenstein (256) has reported that CAT is absent from the high-speed supernatant of rat liver, while Norum (257) has reported that 20% of the CAT activity is found in this fraction.

All the CAT activity was extracted from the mitochondria with phosphate buffer, pH 8, containing Na-EDTA and Na-deoxycholate. The residue after extraction was completely free of transferase activity while it retained most of the succinic cytochrome c reductase (Table XXXIII). This observation confirms the different mitochondrial distribution for these enzymes. Succinic-cytochrome c reductase has been found to be localized in the inner membrane (258). The exact localization of CAT remains uncertain. Subfractionation of mitochondria from rat liver and pig kidney using digitonin treatment and sonication suggests that at least 25% of CAT is located in the outer mitochondrial compartment and 75% in the inner compartment (259). All the evidence, including that reported here, favors the view that the enzyme is readily soluble and that it is not firmly bound to mitochondrial membranes.

A reduction of about 35% of the CAT activity has been found in the skeletal muscle mitochondria from dystrophic mice. This could be related to the decrease in the oxidation of acetylcarnitine (253) and pyruvate (135) by isolated mitochondria of dystrophic mice and also to

the decrease in palmitic acid oxidation (135) if we take into account the proposal by Fritz (260) that the CAT enzyme may be required in the transport of acetyl CoA between two pools within the mitochondria. In this way CAT would act as a bridge between the pool of acetyl CoA formed from β-oxidation and that used in the Krebs cycle. However, since this enzyme seems to behave in a typical Michaelis-Menten (261) fashion, it seems unlikely that the reduction in activity of 35% could entirely account for a decrease of 80% in the oxidation of acetylcarnitine (253).

No significant differences were found in the activities of the tricarboxylic acid cycle enzymes or the electron transport systems studied. Even though there is no major difference in any of the activities of these enzymes, this study does not rule out the possibility of a deficiency in the overall function of the cycle. Such a deficiency could result from alterations in regulatory mechanisms or in the spatial relationship between the different enzymes. It is important to remember here that morphological alterations have been observed in these mitochondria (38, 59).

Because CAT is only partially localized in the outer membrane and this study shows a deficiency in this enzyme in the mitochondria from dystrophic muscle, determinations of monoaminoxidase(MAO) were carried out since this enzyme was considered to be a good marker enzyme for outer membrane of liver mitochondria (262). However in skeletal muscle it was found that this enzyme localization is not restricted to mitochondria but is present also in the microsomal fraction (Table XXXI). MAO activity has been reported to be increased in muscle homogenates from dystrophic mice (263). This was confirmed in our experiments. This same

enzyme has been determined in muscle undergoing atrophy after denervation. As seen in Figure 32 an increased MAO activity did not appear until 13 days after denervation. When MAO was studied in the muscle of patients suffering from several myopathies a reverse type of phenomenon was observed; in all the cases of genetically determined myopathies, MAO values were below those of the normal controls. Normal values were found in the muscle of an MD carrier and in two myopathies of late onset.

One important cofactor involved in the utilization of fatty acid by mitochondria is carnitine. Because the reaction catalyzed by long-chain acyl CoA-carnitine transferase is easily reversible, the relation between carnitine and CoA can be an important factor in determining the metabolic pathway followed. Unpublished results from this laboratory have shown that an imbalance (within certain limits) of the above ratio in favor of carnitine results in an increased production of CO_2 from palmitic acid by muscle homogenates while an imbalance in the direction of CoA brings about an enhancement in the acylation of palmitic acid leading to an increased formation of triglycerides.

These carnitine concentrations were determined by the colorimetric method described in the Methods. Even though this technique was not ideal for this type of work because of the small amount of tissue available and because the recovery of carnitine was only about 80%, no differences were found between the total carnitine content in normal and dystrophic muscle. It is of interest to note that these results have been confirmed recently in this laboratory using a more precise enzymatic technique for the determination of carnitine (264).

In experiments related to the oxidation of acetyl-l-carnitine by isolated mitochondria of normal skeletal muscle it has been observed that

exogenous CoA is not required (261). This observation suggests that there exists an adequate intramitochondrial pool of CoA which is maintained through the impermeability of the inner mitochondrial membrane to CoA (265). The observed reduction (55%, see Table XXXVII) of CoA in mitochondria from dystrophic muscle is great enough to be a contributing factor to the observed decrease in the oxidation of acetyl-l-^{14}C-l-carnitine (253) as well as of palmitate and pyruvate (135) by these mitochondria. It is of interest to note that the CoA content in the muscle from dystrophic patients of the Duchenne type has been reported to be lower than in normal subjects (266).

CHAPTER VIII

DISCUSSION

After more than one hundred years from the time when Duchenne first described Muscular Dystrophy, and despite all the research that has been carried out in this field, we unfortunately do not as yet have any clear understanding of the biochemical mechanisms responsible for the morphological and functional alterations in myopathic muscle. At present the question remains unanswered as to whether muscular dystrophy is of myopathic or neurogenic origin, or whether it is the consequence of a circulatory failure in the capillary bed of muscle or due to an inmunological process. Whatever the origin of the disease (there is no conclusive evidence to support any one theory in preference to another), it is probable that many of the alterations in muscle will have to be explained in terms of events which are immediately secondary to biochemical changes going on in the muscle tissue. The finding of these changes could be of great importance in regard to the diagnosis of the disease.

Most of the biochemical data in which changes are seen to occur in this field have proven to be non-specific in relation to muscular dystrophy since the changes appear also in some types of non-hereditary myopathies. Among the more important changes reported may be included the accumulation of fat (neutral lipid or triglyceride) in dystrophic muscle and increases in enzymes of muscular origin in the

serum of animals or patients affected with the disease. Both types of changes might ultimately be related to some alteration in the metabolism of fatty acids in myopathic muscle. With triglyceride accumulation this relation is more evident than with the serum enzyme changes, where the involvement may relate to the lipids of the biological membranes that appear to show increased permeability in dystrophic animals. During the past several years the main concern of this laboratory has been the elucidation of the nature of triglyceride accumulation. Attempts have been made to determine whether associated with the accumulation there are specific changes in relation to particular enzymes of triglyceride metabolism or whether the accumulation reflects a more general effect on lipid metabolism.

Either a decreased utilization of triglycerides or an increased synthesis or a combination of both are the main factors that can lead to the accumulation of fat in a tissue. Alterations in both of these mechanisms (catabolic and anabolic) have been reported to occur for fatty acids being metabolised in the muscle of dystrophic mice (135). Of the two abnormalities reported, a decrease in the oxidation of long-chain fatty acid (palmitate) by isolated mitochondria has been considered to be of more quantitative importance. That such a defect does exist in the utilization of fat by dystrophic muscle in situ, is suggested by our in vivo experiments (Chapter III) with palmitate-1-^{14}C. The observed decreases in the radioactivity appearing in the expired CO_2 and in the cholesterol of liver, kidney and muscle of dystrophic mice along with an increase in the appearance of radioactivity in the total fatty acids isolated from the dystrophic muscle are consistent with a defect

in fatty acid oxidation. However, it should also be noted that mitochondria of muscle from dystrophic mice have been found to show similar defects in the utilization of certain other substrates such as pyruvate (135) and acetyl-l-carnitine (253). These observations led to extensive investigation of the metabolic pathways or cofactors which are common to the metabolism of these three substrates (long-chain fatty acids, pyruvate and acetyl-l-carnitine) in mitochondria from normal and dystrophic muscle. These include:

a) Transport mechanisms and compartmentalization in mitochondria:

The transport of acetyl groups to the respective loci for β-oxidation and its entrance into the tricarboxylic acid cycle requries the cofactor carnitine and either the enzyme long-chain acyl-CoA-carnitine acyltransferase (LCAT), or the enzyme acetyl-CoA carnitine transferase (CAT), respectively. Acetylcarnitine does not require carnitine but is still dependent on the presence of CAT for reconversion of the acetyl group to acetyl CoA in the interior of the mitochondria. In skeletal muscle the dependence of carnitine is absolute in respect to the transport of both acetate and long-chain fatty acid into the mitochondria (267).

A variety of experiments on gluconeogenesis and ketogenesis have led to the postulation of the existence of different compartments in relation to the utilization of fatty acid (260). One compartment contains the enzymes of β-oxidation and results in the end product acetyl CoA. The second compartment contains enzymes of the tricarboxylic acid cycle which utilizes acetyl CoA giving rise to CO_2. Furthermore, this scheme or postulate includes the assumption of two different

pools of CoA and carnitine and transfer between the two compartments is mediated by the CAT enzyme. Thus, according to this hypothesis CAT enzyme becomes a common factor in the metabolism of pyruvate, acetylcarnitine and fatty acid by mitochondria.

b) Intramitochondrial pool of CoA:

Because the inner membrane of the mitochondria is impermeable to CoA (265), two separate pools of CoA exist in the mitochondria: one localized in the outer compartment and the other in the inner compartment of the mitochondria. The second pool has been further divided into that portion available to α-ketoglutaric dehydrogenase and to succinyl-CoA synthetase, but not to the enzymes of the β-oxidation, and that associated with the enzymes of β-oxidation (268). There are a number of other important aspects to consider in this section. These relate to the possible different affinities of substrates for CoA, or to the influences through positive or negative feedback mechanisms that the accumulation of CoA esters of these substrates or products of the substrates may have. For example, it has been reported that long-chain acylcarnitines inhibit the decarboxylation of pyruvate (269) while long-chain acyl CoAs induce the synthesis of the enzymes of the β-oxidation and inhibit fatty acid synthesis (270) at the same time.

c) Tricarboxylic acid cycle, electron transport chain and oxidative phosphorylation:

These cellular processes should be considered as a unit because, even though each process or cycle has its own group of enzymes and its own localization within the mitochondria, they usually work in a synchronized manner to produce as end products CO_2, H_2O and ATP respectively. Three aspects need to be considered here: 1) the activities of individual

enzymes, 2) external mechanisms that change or regulate enzymatic systems responsible for products entering or leaving these cycles, e.g. the activities of pyruvate carboxylase or glutamate transaminase, and, 3) the interrelationships existing between these cellular processes, that is the state of coupling of the cycles and processes. Only the first of these three aspects has been studied in this thesis.

In addition to the above approaches a number of other investigations were undertaken to help in the explanation of the fat accumulation in dystrophic muscle. These included study of fatty acid synthesis, triglyceride synthesis, the pentose monophosphate shunt and the fatty acid-activating systems.

In Table XXXVIII is shown a summary of the changes observed in this laboratory for all the investigations undertaken.

The most significant changes that we have found in our research have been the decreases in CAT activity and in the intramitochondrial pool of CoA. Of these, the second finding appears to be of more quantitative significance in that it correlates more closely with the decreases in utilization of acetyl-l-carnitine and fatty acid observed in isolated mitochondria of dystrophic mice. This finding, (i.e. a reduced level of CoA in the mitochondria of skeletal muscle from mice affected with muscular dystrophy), even though it gives us a reasonable explanation for the reduced utilization of several substrates by mitochondria, must be further investigated in order to assess its real pathogenetic importance. Considerable investigation and independent verification is needed to establish whether the defect in CoA content reflects a part of the primary mechanism of the disease, or something

TABLE XXXVIII

A SUMMARY OF THE CHANGES OBSERVED IN A NUMBER OF BIOCHEMICAL PROCESSES RELATING TO NEUTRAL LIPID AND FATTY ACID METABOLISM IN NORMAL AND DYSTROPHIC SKELETAL MUSCLE

↑ an increased activity; ↓ a decreased activity; = no change in activity

	Reaction	Comparison with normal	Reference
Triglyceride synthesis	sn-^{14}C-glycerol-3-P ⟶ TG	=	This thesis
Fatty acid synthesis	^{14}C-acetate ⟶ FA	↑	(135)
	(Hexose monophosphate shunt)	↑	This thesis
Fatty acid activation	FA ⟶ Fatty-acyl CoA	=	(261)
Fatty acid oxidation	FA ⟶ CO_2	↓	(135)
a) Transport mechanism	LCAT activity	= ↓	(261)
	CAT activity		This thesis
	Carnitine content in muscle	=	This thesis
b) TCA cycle	Enzyme activities	=	This thesis
c) Electron transport chain	Enzyme activities	=	This thesis
d) Oxidative phosphorylation	ADP/O ratio	=	(201)
e) Intramitochondrial pool of CoA		↓	This thesis

secondary to the primary, or simply is an *in vitro* artefact that does not reflect the true *in vivo* situation.

In regard to either problem a number of questions must be examined. The first relates to whether the observed deficiency is confined to mitochondria or extends to other parts of the muscle cell or fibre or even to the animal as a whole. The answer to this question might provide some insight into whether muscular dystrophy is a localized or generalized disease even though muscle is mainly affected.

Secondly, there also exists the possibility that the deficiency in CoA could have originated from differences in permeability between the mitochondria from normal and dystrophic muscle, and as a consequence an increased leakage of CoA out of the mitochondria of dystrophic muscle may take place during the isolation procedure. An increased fragility of dystrophic mitochondria could represent a particular example of a more general defect, namely that of the involvement of membranes in the etiology of muscular dystrophy. If true, then the leakage of enzymes into serum would represent a clear manifestation of this defect. Such a defect in mitochondria is suggested by the paradoxical observation that the theoretical content of mitochondria in muscle (as calculated from the activities of succinic dehydrogenase in whole homogenates and in isolated mitochondria) (see Table XXIX) has a tendency to be increased in dystrophic muscle while the yield of mitochondria obtained after isolation is decreased (see Table XXX). However, no differences in permeability appear to exist between both isolated mitochondria as calculated from the latency of the NADH-cytochrome c reductase assay after osmotic shock of the mitochondria. Electron micrographs have provided some evidence in favor of alterations in

mitochondrial structure (38, 59).

The apparent increased content of mitochondria in dystrophic muscle could represent an increased biogenesis of mitochondria as part of a response of the affected muscle to carry out certain regenerative processes during its degeneration. However, as seems more probable the increase could result simply from a greater reduction in other components of the muscle cell.

If the deficiency in CoA in dystrophic animals proves to be genuine and of a generalized nature three main possibilities need to be examined. These are: (1) the possibility that some defect in the absorption of pantothenic acid may exist in the affected animals, (2) the possibility that some blockade may exist in the biosynthetic pathway leading to CoA formation, and, (3) the possibility that there may be a greater increase in the destruction of CoA to dephospho-CoA or that due to alterations in the oxido-reduction state of the muscle cell most of the CoA is converted to its oxidized form. Increased destruction could in some way be related to an increased activity of lysosomal enzymes in dystrophic animals together with some leakage of CoA from the mitochondria (271). Concerning the state of oxidation of CoA it is of interest to note that a decrease in the SH/SS ratio has been reported in dystrophic muscle (272).

In conclusion it may be stated that the deficiency in CoA may account for many of the abnormalities in lipid metabolism which result ultimately in the accumulation of fat in muscle and a decrease in the efficiency of muscle to generate energy. Both of these factors could contribute to or reflect a chronic process of muscle degeneration.

The affected muscle in its response to overcome such a process tends to increase its regenerative processes. These, to some extent may be evidenced by the increase in activity of the hexose-monophosphate shunt that has been reported and shown here to occur in dystrophic muscle, or by the increased incorporation of amino acids into proteins or of nucleotides into nucleic acids that have been observed and have been interpreted as indicative of alterations secondary to the primary mechanisms of the disease.

Even though the myopathic process that we have been studying in mice shows many characteristics resembling Duchenne Muscular Dystrophy (e.g., increased amount of lipids in the affected muscle and the elevation in serum enzymes) the disease in mice cannot be completely identified with the disease in humans. One important difference relates to the different genetic transmission of the defect (usually X-linked in Duchenne versus an autosomal recessive inheritance in mice). Nevertheless, it has been possible through the use of these experimental animals to gain considerable information to help us further understand the pathological processes which leads to the degeneration of muscle in general and to human dystrophic muscle in particular. It is hoped that continued studies of the type undertaken here together with data obtained from different approaches such as the study of muscle undergoing atrophy after denervation, study of muscle adaptation to different conditions or muscle development, will in time provide sufficient complementary information to be of help in the elucidation of the basic defect in Muscular Dystrophy.

CHAPTER IX

SUMMARY AND CONCLUSIONS

1. The utilization of palmitate-1-^{14}C by dystrophic mice (Bar Harbour Strain 129) and their littermate control was studied in vivo after intraperitoneal injection. The expired $^{14}CO_2$ and radioactivity incorporated into the fatty acids and cholesterol of brain, liver, kidney and muscle were determined. A reduction in the expired $^{14}CO_2$ (when expressed in terms of disintegrations per minute per cm^2 body area) and in the incorporation into cholesterol of liver, kidney and muscle was observed to occur in dystrophic animals together with an increased incorporation into the fatty acids of muscle.

2. Similar experiments were carried out after the injection of glucose-U-^{14}C into normal and dystrophic mice fed a high-carbohydrate diet. An increased incorporation of radioactivity was observed into the neutral lipid fraction from muscle of dystrophic mice. Most of the increase was observed in the glycerol moiety. In these experiments the daily food-intake for both normal and dystrophic mice was determined. An increased consumption of food was observed for the dystrophic animal when expressed in terms of calories per day per gram body weight.

3. The metabolism (both anabolism and catabolism) of triglycerides was examined in a series of <u>in vitro</u> experiments. The formation of triglycerides was assessed by studying the incorporation of pal-

mitate-1-^{14}C required ATP, CoA, Mg^{++}, glucose and NAD. After consideration of the dilution of the isotope by endogenous free fatty acids (previously calculated) a slight increase in incorporation was observed. The incorporation of sn-^{14}C-glycerol-3-phosphate was dependent on ATP, CoA and Mg^{++}. No differences in incorporation into triglycerides and phospholipids were observed between muscle from normal and dystrophic mice. Degradation of triglycerides was studied by determination of the activities of two different lipases: one using tributyrin as substrate and termed a "short-chain triglyceride lipase" and the second using tripalmitin as substrate which is termed a "long-chain triglyceride lipase". The first showed an increased activity in dystrophic muscle of about 160% while the second only increased by 50%.

4. The hexose-monophosphate shunt, an alternative pathway for the oxidation of glucose, was studied using as enzymatic preparation the supernatant obtained from muscle homogenates after centrifugation at 100,000 x g for 100 min. Glucose-U-^{14}C was used as substrate for the study of the formation of pentoses and of $^{14}CO_2$. Ribose-5-phosphate was used to evaluate the utilization of pentoses by the above high-speed supernatant of muscle. Dystrophic muscle showed an increased activity of the hexose-monophosphate shunt in two ways: these were both the formation and utilization of pentoses. It is postulated that this increased activity may be the consequence of regenerative processes taking place in the dystrophic muscle in response to the pathological process.

5. The intramitochondrial content of CoA and the level of carnitine in muscle from normal and dystrophic mice were determined. The

latter was found to be within the normal range, but a great reduction in the intramitochondrial pool of acid-soluble CoA (free CoA plus acetyl CoA) was found to exist in the mitochondria isolated from the dystrophic tissue.

6. The activities of several enzymes have been studied in isolated subcellular preparations and in whole homogenates. Normal values were found for the enzymes of the tricarboxylic acid cycle and electron transport chain systems in isolated mitochondria from dystrophic muscle. A slight increase in monoaminooxidase activity was found to exist in the isolated mitochondria, and microsomal fractions and in the whole homogenate from dystrophic muscle.

7. In muscle homogenates from patients affected with different myopathic diseases normal values of cytochrome oxidase, an increase in the activity of short-chain triglyceride lipase and a decrease in the activity of monoaminoxidase were observed.

8. In muscle from mice undergoing atrophy after denervation a large increase in short-chain triglyceride lipase was observed one day after denervation, and a slight increase in monoaminoxidase activity after 13 days denervation.

9. Even though the exact defect behind the etiopathogenesis of muscular dystrophy has not been elucidated some interesting observations have been obtained. The reduction in the intramitochondrial pool of CoA may in part or completely account for defects observed in the oxidation of fatty acid, pyruvic and acetyl-carnitine by isolated muscle mitochondria from dystrophic mice. A major point needing clarification is whether the defect is due to a leakage of CoA in dystrophic animals. If the latter does not apply then the defect

could exist at any point from the intestinal absorption of pantothenic acid to the biosynthetic pathway going from pantothenic acid to CoA. Defects in the oxidative pathways requiring CoA could lead to imbalances in favor of fat accumulation in muscle.

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APPENDIX A

Statistical Formulas Used:

1. The Normal Distribution

If there are n observations $X_1 \ldots\ldots\ldots\ldots X_n$ then the average $\bar{X} = \frac{1}{n} \sum_{1}^{n} X$, the standard deviation (S.D.)

$$S.D. = \sqrt{\frac{(X - \bar{X})^2}{n-1}}$$ and

the standard error of estimate of the mean value (S.E.M.)

$$S.E.M. = \frac{S.D}{\sqrt{n}}$$

2. Paired data comparison

If there are n observations from normal mice and their dystrophic littermates, only the differences within each littermate is considered. If one designates $X_1 \ldots\ldots\ldots\ldots X_n$ as data obtained from normal mice and $Y_1 \ldots\ldots\ldots\ldots Y_n$ from dystrophic mice, then: the difference between each pair $D = X - Y$ and the average difference of all observations, $\bar{M} = \frac{D}{n}$

The standard deviation $\hat{\sigma_D}$

$$\hat{\sigma_D} = \sqrt{\frac{(\leq D^2)}{n-1} - \frac{(\leq D)^2}{n(n-1)}}$$ and

the standard error of estimate of mean value (S.E.M.)

$$\hat{\sigma}_{\bar{X}D} = \frac{\hat{\sigma_D}}{\sqrt{n}}$$

Calculation of "P" values are based on the Student "t" test (for n-1 degrees of freedom). This test assesses whether the average difference is significantly different from zero.

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