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CARDIAC CARNITINE ACYLTRANSFERASE ACTIVITIES
IN EXERCISED HAMSTERS WITH GENETIC MUSCULAR
DYSTROPHY AND CARDIOMYOPATHY

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April A. Whitbeck

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ABSTRACT

CARDIAC CARNITINE ACYLTRANSFERASE ACTIVITIES IN EXERCISED HAMSTERS WITH GENETIC MUSCULAR DYSTROPHY AND CARDIOMYOPATHY

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Reports of human myopathies associated with deficient carnitine and/or carnitine acyltransferase, which are key components of lipid metabolic pathways; an hypothesis proposing a "generalized membrane defect" etiology for genetic muscular dystrophy; and the findings of an ameliorative effect of swimming exercise on the skeletal and cardiac muscle histopathology in hamsters with genetic muscular dystrophy and cardiomyopathy comprise the rationale basic to the present study.

Carnitine acetyl-, octanoyl-, and palmitoyltransferase were assayed spectrophotometrically in heart 500 x g supernate from normal and genetically dystrophic/cardiomyopathic, sedentary and twelve-week-swim-exercised hamsters. Heart and body weights and cardiac protein and water contents were also measured.

The mean specific activities (nmoles/min/mg protein) of all three cardiac carnitine acyltransferases in the dystrophic/cardiomyopathic animals were found to be significantly higher than those in normal hamster heart. Exercise had no effect on these enzyme activities in the normal or dystrophic/cardiomyopathic hamsters.

To

Dr. Jules Cohen

Dr. G. Marian Kinget

Sr. Agnes Sheehan, C.S.J.

GENEROUS AND INSPIRING MENTORS

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BACKGROUND

Introduction

Most of the amyotonic muscular dystrophies, which have been characterized and classified according to the involvement of various groups of muscles with differing rates of disease progression (121), are thought to be of genetic origin. Of the three major forms, that designated as "facioscapulohumeral" is now believed to be a clinical syndrome common to a number of unrelated disease processes (e.g., non-specific Type II fiber atrophy, spinal muscular atrophy, and mitochondrial myopathy), whereas the "limb-girdle" form appears, histologically, to be an example of chronic spinal muscular atrophy. The pathogenetic basis of the most severe form, Duchenne muscular dystrophy, remains obscure (118,121,122,124).

Duchenne dystrophy has been shown to be X-linked (123), though one-third to one-half of these cases seem to have arisen as a result of spontaneous mutation (116). In these patients, histopathological (119) and biochemical signs of the disease, i.e., increased serum levels of creatine kinase, are present at birth (31), while symptoms of muscle weakness become manifest at about age three. Progressive muscle degeneration usually leads to a wheel-chair existence by ten years of age and death, in the second decade, as a result of respiratory infection or congestive heart failure, from myocardial inclusion in the dystrophic process (40,59,87,114,123). Typically abnormal

electrocardiograms have been found in as many as 80% of Duchenne patients in some studies (88,125), and some individuals with muscular dystrophy may present with cardiac symptoms prior to any clinical manifestation of skeletal muscle involvement (79).

Effective treatment of the disease probably awaits elucidation of the primary biochemical expression of the basic genetic alteration. (To this end, animal models of this disease, i.e., genetically dystrophic strains of mice, chickens and hamsters [e.g., 47], are useful; but care must be taken in extrapolating to the human condition [124].) Pathological, morphological, histochemical, ultrastructural, and electrophysiologic studies of dystrophic skeletal and/or cardiac muscle reveal no information specific to any primary abnormality (e.g., 1,6,28,37,50,72,80). Present etiologic postulates suggest a primary muscle disorder, a primary nerve malfunction, an inadequate muscular blood supply, defective biogenic amine metabolism, autoimmune mechanisms, or a generalized membrane defect. It is my impression that the "generalized membrane defect" theory may be serviceable as a unifying concept in explaining many of the observations reported from studies of muscular dystrophy.

Discoveries of altered membrane structure, composition, and function and *in vitro* growth patterns in "dystrophic" tissues other than muscle and nerve are consistent with the latter hypothesis and/or offer argument against a primary muscle or nerve pathogenesis (41,60,86,94,95). The demonstration in tissue culture of normal regeneration of dystrophic mouse muscle explants under the influence of innervation by normal nerves also conflicts with the concept of a primary myogenic etiology (39). Additional results from a companion

experiment, showing anomalous regenerative attempts by normal muscle explants innervated by morphologically normal neurons from dystrophic mice, implicate neuronal dysfunction. Findings by others of decreased numbers of motor units (72) and mental retardation (90) in muscular dystrophy are also consonant with a nervous system irregularity, though direct interruption of neural influence on normal muscle has resulted in muscle fiber atrophy without the necrosis, phagocytosis, and regeneration characteristics of muscular dystrophy (20).

The data suggestive of neural impairment are not inconsistent with the "generalized membrane defect" hypothesis: electrical impulse generation and conduction, neuromuscular impulse transmission, synthesis and "export" of neurotransmitter and/or "trophic" substances, and generation of the energy required for these processes all presume adequate membrane function in the respective organelles of the neuron. Ultrastructural studies in young dystrophic mice have demonstrated the presence of abnormal mitochondria and decreased numbers of transmitter vesicles in motor nerve endings when the subjacent extrafusal or intrafusal muscle fibers appeared to be normal (50).

An inadequate blood supply to the skeletal muscles, advanced as a pathogenetic mechanism due to the "focal-grouped" pattern of muscle fiber necrosis in early Duchenne muscular dystrophy and the experimental reproduction of this topographic pattern by microarterial embolization (45), has not been substantiated. Light and electron microscopic studies of degenerating muscle from Duchenne patients revealed structurally normal skeletal muscle vasculature (58). Also, the majority of autopsy reports in progressive muscular dystrophy

cases cite the presence of normal coronary arteries, even in instances of severe cardiomyopathy (109,132). Arterial functional incompetence cannot be ruled out (36), but the histology of the dystrophic lesion is not that of true ischemic pannecrosis (2), and a direct study of muscular blood flow in murine dystrophy showed no evidence of ischemic foci (19).

Findings of fluorescent substances in muscle biopsies and impaired uptake of serotonin by platelets from patients with Duchenne muscular dystrophy led some investigators to postulate biogenic amine accumulation as the cause of the lesions in muscle. An experimental approximation of the metabolism that might obtain with impaired monoamine uptake resulted in lesions that were strikingly similar in distribution and histology to those seen in Duchenne patients (83). These facts, too, can be viewed as consistent with a membrane defect resulting in altered monoamine-specific receptor sites and/or permeability and a concomitant decrease in monoamine uptake.

An underlying hyperimmune mechanism in muscular dystrophy was suggested to explain the results of the following experiments: normal hamster muscle transplanted into dystrophic littermates was rejected, and dystrophic muscle transplanted into "carrier" and normal animals was tolerated, though the dystrophic muscle transplant maintained the characteristics of progressive dystrophy (53). (It should be noted that interpretation of these results is complicated by the fact that adequate innervation and vascularization of the transplants were not ascertained.) Others (113) have produced what they termed "muscular dystrophy-like lesions" in animals injected with homologous

or heterologous muscle homogenates with Freund's adjuvant. However, the interstitial connective tissue and lipid replacement of muscle seen in human dystrophy were found in only one of the experimental animals. If, indeed, a hyperimmune mechanism contributes to the pathogenesis of muscular dystrophy, that response might derive from membrane defects; the maintenance of cell membrane "self" antigenicity presupposes normal membrane structure and function, especially in the Golgi apparatus where membrane assembly and glycosylation occur (126).

Other Biochemical and Physiologic Findings in Genetic Muscular Dystrophy or Cardiomyopathy

Though many of the other biochemical and/or physiologic deviations from normal described in genetic muscular dystrophy and cardiomyopathy are theoretically explainable on other grounds, it seems plausible that they might be attributable or reducible to defective membrane function. Differences in membrane composition could affect the selectivity and permeability that effect transmembrane potentials and ion fluxes (11,62) and, consequently, electrical impulse generation and conduction, and could thereby cause the electrocardiographic alterations described in hamsters (4) and humans, including carriers of Duchenne dystrophy (e.g., 87). Alterations in membrane-dependent ion distribution may also underlie the abnormal erythrocyte morphology (70), deformability (70,86), and glycolytic activity (18) found in individuals with Duchenne dystrophy.

A depressed maximal systolic endocardial velocity in humans (59); subnormal work performance (as reflected in a decreased heart rate, peak developed tension, tension-time index, and tension time per minute) in the isolated perfused hamster heart (25); decreased

maximal twitch and tetanus production by mouse skeletal muscle (99); and decreased cell-free protein synthesis in hamster heart and skeletal muscle (12,29) may reflect inadequate energy production in those tissues. Diminished high-energy phosphate-producing capacities have been demonstrated in hamster heart mitochondria (25,102) and human skeletal muscle homogenate (52). Defective energy production may stem from abnormalities in mitochondrial membranes, since the constituents of the oxidative phosphorylation system are integral parts of the inner mitochondrial membrane (34).

The presence of mitochondrial enzymes in the serum of some Duchenne patients in the preclinical stage of the disease (26) and the discovery of increased activities of "brain cell" enzymes in the cerebrospinal fluid of Duchenne patients (57) may reflect leakage of these enzymes through mitochondrial and plasmalemmal membranes.

The findings of depressed maximal diastolic endocardial velocities in man, thought to indicate increased relaxation time in the cardiac cycle (59), and of a murine skeletal muscle relaxation period three times normal (99) are probably manifestations of sarcoplasmic reticulum dysfunction. This membranous organelle plays a well recognized role (133) in the calcium ion sequestration that is thought to initiate the relaxation phase of muscle contraction. Decreased calcium-accumulating capacity of sarcoplasmic reticulum has been demonstrated in hamster heart (47,102) and in hamster, mouse, chicken, and human skeletal muscle (85,98,108,130). An increased number of free polysomes in hamster cardiac and skeletal muscle (12) may also be the result of altered properties of the endoplasmic reticulum, which may associate with ribosomes actively engaged in the protein

synthetic process (82), or may reflect abnormal ribosomal activity, cited as a possible cause of the irregular apportionment of amino acid incorporated into cytoplasmic and structural proteins of skeletal muscle from individuals with progressive muscular dystrophy (74).

Freeze-fracture electron microscopic studies (101) of skeletal muscle plasma membrane from patients with Duchenne dystrophy have shown depletion and anomalous distribution of intramembranous particles, which are generally accepted to be proteins inserted in the lipid bilayer (115). The identification of these proteins with specific enzymes has not yet been accomplished, but alterations in multiple enzyme systems have been detected in genetic muscular dystrophy in several species. Skeletal muscle sarcoplasmic reticulum and actomyosin ATPase activities have been shown to be low in biopsies from Duchenne patients (98); Na^{+}-K^{+}-, Ca^{++}-, and Mg^{++}-stimulated ATPases from hamster skeletal muscle sarcolemma show increased activities (32); increased sarcolemmal Ca^{++}- and Mg^{++}-ATPase activities and decreased Na^{+}-K^{+}-ATPase activity have been described in two Duchenne patients (32); altered basal and/or stimulated activities of plasma membrane adenyl cyclase and ATPase have been found in muscle, liver, and erythrocytes of chicks (94); and increased membrane protein kinase activity in human red blood cells has been reported (95). These enzyme activity modifications may be the result of direct effects of the dystrophic process on catalytic units or non-specific changes accompanying several myopathic processes (84); but it is equally possible that changes in the membrane microenvironments of these enzymes are responsible for the adjusted activities (3,38,44,56,94,96).

Membranes, Lipid Metabolism, and Genetic Muscular Dystrophy and Cardiomyopathy

The fundamental framework of biological membranes is a lipid bilayer (23); therefore, essential to normal membrane assembly, structure, and function is normal lipid metabolism (22,42,89,91,96). The following is a selection of specific examples of lipid involvement in normal, membrane-related biochemical/physiological processes, and is one which relates to disorders described in genetic muscular dystrophy:

> -Calcium transport in the sarcoplasmic reticulum has been shown to be associated with a Ca^{++}-sensitive ATPase, the activity of which is absolutely dependent upon the presence of phospholipids (43,65). This phospholipid requisite may be due to the enzyme's partial lipid composition, which appears to be identical to that of the sarcoplasmic reticulum. In a highly purified state, the ATPase is capable of spontaneous vesicular membrane formation; the enzyme is believed to be a structural, as well as a functional, subunit of the sarcoplasmic reticulum (65). Strengthening this evidence of an essential relationship between lipid content and function of the sarcoplasmic reticulum are the results of recent studies of chick embryonic skeletal muscle development. Marked changes in the fatty acid composition of microsomal membrane phospholipids, toward a greater percentage of unsaturation, were found to correlate with the appearance of calcium transport activity in those vesicles (16). Spectroscopic probe studies (92) of mixed phospholipid dispersions have shown that the binding of calcium ion to certain phospholipids can cause aggregation and consequent separation of specific groups within the phospholipid mixture. If a similar motion and change in distribution or orientation of lipids within a membrane that binds calcium, such as the sarcoplasmic reticulum, has physiological significance, it becomes immediately apparent that any change in lipid species within that membrane could effect myriad functional consequences.
>
> -Lipid depletion and reconstitution experiments on the oxidative phosphorylation system of the inner mitochondrial membrane revealed a lipid requirement for activity of the components of the electron-transport chain and the ATPase which functions in the mitochondrial energy-coupling system (24,117). Phospholipid is essential for electron transfer at the sites for ubiquinone reduction and oxidation, for

the reduction and oxidation of cytochrome c, and for oligomycin-sensitive ATPase activity. Cytochrome oxidase and the oligomycin-sensitive ATPase complex have been shown to have the capability of forming membranes, the organization of which also requires the presence of phospholipids (73). These lipid requirements might be interpreted as an effect of lipid on the tertiary structure of the redox and ATPase proteins, since spectroscopic probe studies of submitochondrial particles have revealed conformational changes in the inner mitochondrial membrane when electron flow is coupled to energy conservation (93).

-The plasma membrane Na^+-K^+-activated ATPase, working against passive leaks of Na^+ and Ca^{++} inwards and K^+ outwards, functions in every cell of the human body to maintain the intracellular concentrations of sodium and potassium ions within normal limits. ATP is required in the process, which, consequently, acts as a metabolic pacemaker. Purified preparations of this enzyme also demonstrate a requirement for phospholipid for activity. Phosphatidylserine seems particularly interesting in this respect in that it may be selective in its affinity for Na^+ and K^+. ATP hydrolysis dependent on this phospholipid was found to be dependent on Na^+ and K^+, and was completely inhibited by ouabain. These characteristics have led some to consider the "sodium pump" to be a phosphatidylserine-protein complex, the phospholipid moiety of which may be necessary for the maintenance of the native configuration of the enzyme (128).

-The circulating erythrocyte normally maintains and renews its membrane lipid composition through active and passive mechanisms of exchange with lipids in the plasma. Membranes constituted via abnormal renewal reactions, originating *in vivo* from metabolic lesions or from *in vitro* manipulations of the suspending media, exhibit structural and/or functional abnormalities. Red blood cells from patients with a rare hemolytic anemia, characterized by a high red cell phosphatidylcholine content due to a block in the phosphatidylcholine catabolic pathway, show Na^+ and K^+ permeabilities above normal (104); and decreased erythrocyte membrane fluidity caused by an abnormal cholesterol-phospholipid ratio (as seen in spur cell anemia) or an abnormal sphingomyelin-lecithin ratio (seen in abetalipoproteinemia) is associated with a folded and scalloped cell contour and decreased erythrocyte deformability (30).

The apparently central role of lipid in the normal function of a representative sample of membrane types makes lipid metabolism a

potentially fertile area in which to search for sources of membrane anomalies.

Direct studies of lipid metabolism in genetic muscular dystrophy and cardiomyopathy have revealed irregularities. Elevated rates of fatty acid synthesis from acetate are reported as discernible in homogenates of mouse liver and muscle (110), and changes in murine muscle phospholipid composition and total lipid, neutral lipid, plasmalogen, and cholesterol contents have been shown to occur; no changes appeared in muscle free fatty acid composition (81). In the hamster, linoleic acid is not present in the serum, and the fatty acid composition of myocardial free fatty acid, triglyceride, and cholesterol ester fractions shows deviation from controls. No variations in total lipid, phospholipid, cholesterol, or triglyceride contents were detectable in the hamster serum, liver, or heart; and no change in heart phospholipid fatty acid composition was observed (111). Fatty acid composition of, and incorporation into, erythrocyte lipids are altered in humans (60); and muscle biopsies from Duchenne patients have revealed an increase in oleic acid and a decrease in the percentage of linoleic acid in lecithin (112), a major phospholipid in human skeletal muscle. Cultured lipocytes showed a decreased triglyceride content and myoblasts showed increased cytoplasmic triglyceride with decreased nuclear phospholipid upon cytochemical assay of human explants (41). A suggestion of retarded or abated developmental maturation of lipid metabolism has been proposed to explain findings in Duchenne dystrophy of muscle characteristics similar to those seen in the normal human fetus (51).

Carnitine Acyltransferase

The carnitine acyltransferases play a primary role in normal lipid metabolism in the beta-oxidation of free fatty acids, a process which occurs in the mitochondrial matrix and from which the myocardium derives most of its energy.

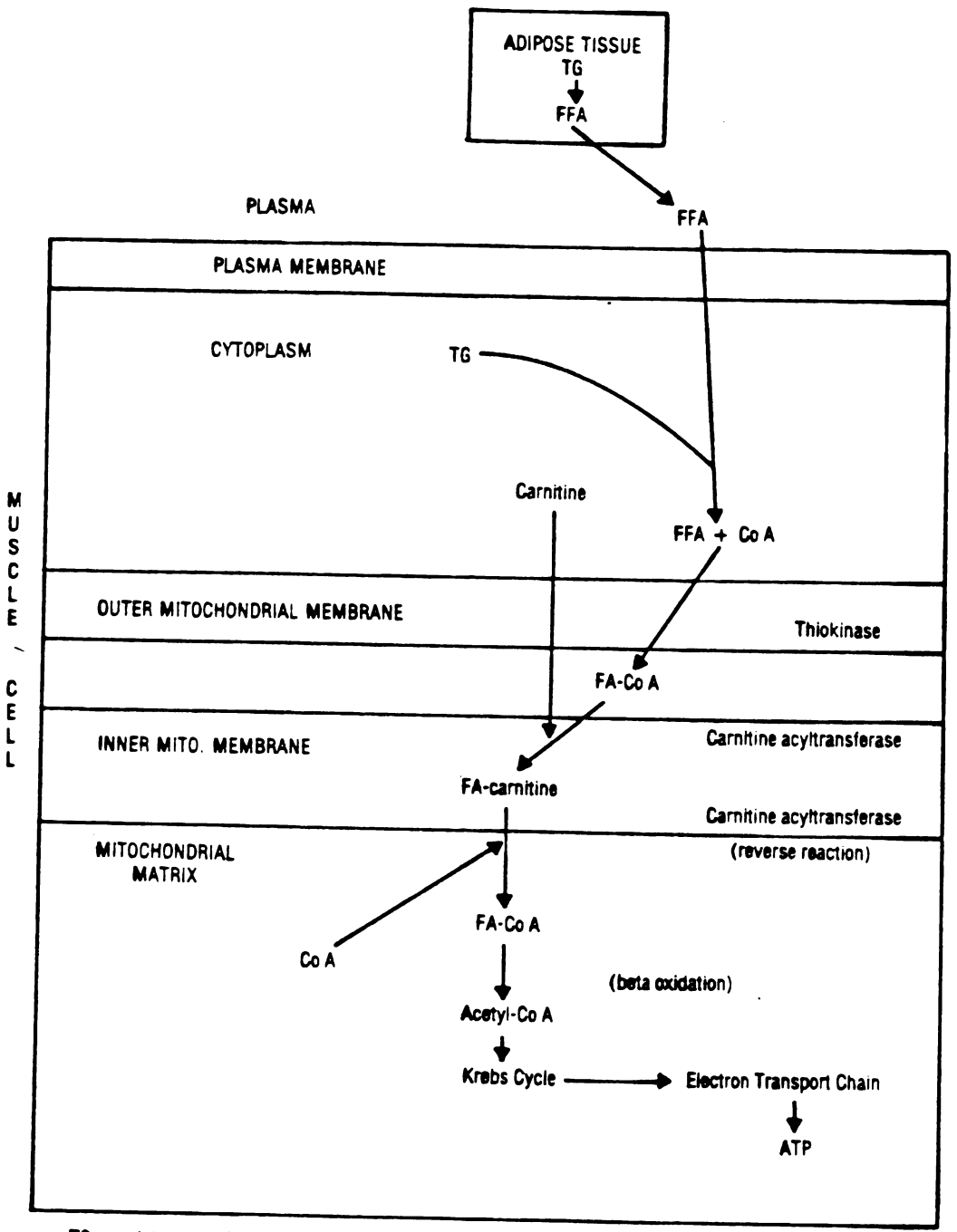
Free fatty acids in the cytoplasm are "activated" (i.e., esterified with extra-mitochondrial Coenzyme A) by thiokinases in the outer mitochondrial membrane, but the fatty acid-Coenzyme A ester cannot easily cross the inner mitochondrial membrane. The fatty acid is subsequently transferred from the Coenzyme A to carnitine through the action of a carnitine acyltransferase (Figure 1). The resultant fatty acyl-carnitine molecule is able to cross the inner mitochondrial membrane into the matrix where the fatty acyl moiety is transferred from carnitine to intra-mitochondrial Coenzyme A through the reverse reaction catalyzed by a carnitine acyltransferase. The acyl-Coenzyme A can then undergo beta-oxidation yielding acetyl-Coenzyme A, which can be oxidized via the tricarboxylic acid cycle and the electron transport system to generate ATP.

Through the action of carnitine acetyltransferase and acetyl-carnitine the cell is buffered against rapid changes in acetyl-Coenzyme A level, and this influence upon acetyl-Coenzyme A levels contributes to the regulation of fatty acid and cholesterol biosynthesis and ketogenesis (63).

Carnitine acetyltransferase may also be important in biological acetylations, such as in the formation of acetylcholine (21,71). There is some evidence that, in the heart, choline may serve as a

Figure 1. Carnitine acyltransferase catalyzes the reversible transfer of fatty acyl groups between Coenzyme A and carnitine. These reactions occur at the inner mitochondrial membrane and allow the transport of fatty acids, in the form of carnitine esters, across that membrane into the mitochondrial matrix, where they undergo beta-oxidation.

CARNITINE ACYLTRANSFERASE: LIPID METABOLISM



TG = triglyceride; FFA = free fatty acid; Co A = Coenzyme A;
FA = fatty acyl moiety

FIGURE 1.

substrate, albeit a rather poor one, for carnitine acetyltransferase (127).

The formation in heart mitochondria of branched-chain acylcarnitines from the 2-oxoacids corresponding to valine, leucine, and isoleucine also suggests a possible function of the carnitine acyltransferases in amino acid metabolism (107).

Recent reports (67,68) of the subcellular localization of carnitine acetyl- and octanoyltransferase in organelles other than mitochondria indicate other, as yet undefined, roles for those enzymes in the cell. Carnitine palmitoyltransferase was found to be strictly a mitochondrial enzyme, and there is evidence for its occurrence in two mitochondrial locations (49).

In some cases of human skeletal myopathy, muscle carnitine (5,35,55) or carnitine palmitoyltransferase (8) have been shown to be deficient. In dystrophic murine skeletal muscle mitochondria, significant depressions in acetyl-l-^{14}C-L-carnitine and palmitate-1-^{14}C oxidations and carnitine acetyltransferase activity have been detected (54,110). (No alterations in muscle mitochondrial carnitine acetyltransferase localization [66] or muscle carnitine palmitoyltransferase activity [110] were found in these mice.) Although urinary (33), serum (15), and muscle (35) carnitine levels have been reported to be normal in Duchenne muscular dystrophy, no carnitine acyltransferase activities in humans with any form of genetic muscular dystrophy have been recorded.

In normal animals, the existence of a myocardial carnitine acetyltransferase has been reported (69,71,127), but there appear to be no data pertaining to carnitine octanoyltransferase or carnitine

palmitoyltransferase activities in the heart of any species. No references are available regarding cardiac carnitine acyltransferase activity in genetic muscular dystrophy and cardiomyopathy, although the hamster cardiomyopathy has been extensively investigated (7). The results of this study, therefore, will add to the general information regarding cardiac metabolism, as well as to that specifically focused on genetic muscular dystrophy and/or cardiomyopathy.

Effect of Exercise in Genetic Muscular Dystrophy and Cardiomyopathy

Pharmacological treatment of patients with progressive muscular dystrophy has generally been ineffective (122). The use of physical methods of therapy has yielded varied results, though some studies have shown beneficial effects of chronic exercise programs (120), and it is widely held by clinicians that patients should be encouraged to exercise because of the rapidly deteriorative effect of inactivity (122,124).

The effects of exercise on dystrophic experimental animals have also varied, and it is becoming increasingly apparent that the exercise type, duration, intensity, and time of initiation of exercise within the disease course contribute to this variation (27,28,48,120,129).

An animal exercise (swim) program has been developed that appears to ameliorate the morphological and histopathological changes that occur with progression of the dystrophic process. In exercised, dystrophic hamsters, areas of myocardial inflammation were fewer and smaller, and calcification was more confined than in sedentary, dystrophic animals (46). Less severe pathological change was also demonstrated in the skeletal muscle of these exercised animals

(personal communication from Dr. Rexford Carrow). These experimental results provide the rationale for the inclusion of exercised animals in the present study; the possibility exists that the ameliorative exercise effect may be mediated by, or reflected in, changes in carnitine acyltransferase activities (75,105).

MATERIALS AND METHODS

Exercise Programs

Hamsters (*Mesocricetus auratus*) were 35 days old when separated into five groups according to the presence or absence of genetic muscular dystrophy and cardiomyopathy and subjection to a specific exercise regimen. These groups were designated normal, sedentary; normal, high-exercise; dystrophic (Syrian hamster, BIO 53.58), sedentary; dystrophic, moderate-exercise; and dystrophic, high-exercise. All exercised animals swam in 34°C water five days a week. By the twelfth exercise day, those in the "moderate" program were swimming with an attached weight equal to 1% of their body weight for one hour per day. This schedule was continued through twelve weeks. By the eleventh day of the "high" program, animals swam with an attached weight equal to 3% of their body weight. The duration of the exercise period was gradually increased until the hamsters were swimming one hour per day on the thirty-seventh exercise day. This schedule was maintained through twelve weeks. All animals were given food and water *ad libitum* throughout the study. For two days prior to decapitation, none was subjected to exercise.

Tissue Preparation

At the time the hamsters were killed, body weights were obtained. The heart was excised, trimmed of fat, blotted, and weighed. A

piece of apex was removed and weighed and used for a dry weight/wet weight determination, after being dried at 65°C for forty-eight hours. The remainder of the heart was minced in cold buffer (0.25 M sucrose, 0.0025 M HEPES, and 0.00025 M EDTA at pH 7.5) and homogenized. (The carnitine acyltransferases are stable in heart mince, in buffer, on ice for four hours.) The final tissue concentration was one gram of heart per 60 ml of buffer. Aliquots of whole homogenate were frozen (-20°C) and assayed for lactic dehydrogenase (10) (used in determining the percentage of cell breakage with homogenization) within two weeks. The remaining homogenate was centrifuged at 500 x g for twelve minutes, and the supernatant fluid was aliquoted and frozen for lactic dehydrogenase and carnitine acyltransferase assays. Protein determinations were made on the same samples using the method of Lowry et al. (64).

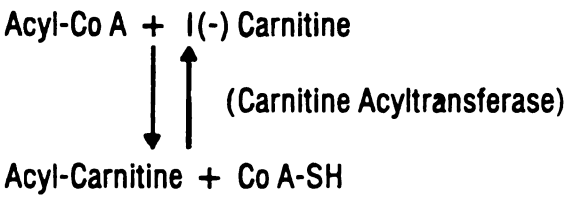
Carnitine Acyltransferase Assay

Carnitine acetyltransferase, carnitine octanoyltransferase, and carnitine palmitoyltransferase activities were assayed spectrophotometrically (14,68) using acetyl-Coenzyme A, octanoyl-Coenzyme A, and palmitoyl-Coenzyme A, respectively, as substrates (Figure 2). The concentrations of reagents in the cuvette were 116 mM Tris Cl, pH 8.0, 0.1% Triton X-100, 1.25 mM NaEDTA, 0.25 mM 5,5'-dithiobis(2-nitrobenzoate), 1.25 mM L(-)-carnitine (when present), and either 0.1 mM acetyl-Coenzyme A, 0.1 mM octanoyl-Coenzyme A, or 0.038 mM palmitoyl-Coenzyme A. The final volume was 0.2 ml.

The reaction was started by adding no more than 0.1 ml of enzyme solution (500 x g supernate). (If less than 0.1 ml of enzyme was used, water was added to yield a total volume added equal to 0.1 ml.)

Figure 2. Acetyl-, octanoyl-, and palmitoyl-Coenzyme A were used as substrates in the assays for the carnitine acyltransferases. The enzyme specific for each substrate catalyzed the transfer of the respective acyl group from Coenzyme A to carnitine with the concomitant release of Coenzyme A. The rate of this reaction was measured by spectrophotometric monitoring of the rate of appearance of TNB, a product of the reaction of thiol groups with DTNB. Specificity of the assay was obtained via measuring TNB production in the absence and in the presence of carnitine (see text).

CARNITINE ACYLTRANSFERASE ASSAY: PRINCIPLE



Co A-SH + DTNB* ⟶ TNB** + mixed disulfide

The rate of TNB production (which is directly proportional to the rate of Co A-SH release) is monitored at 412 nm.

* DTNB = 5,5' - dithiobis (2-nitrobenzoate = general thiol reagent

** TNB = 5-thio-2-nitrobenzoate; molar extinction coefficient = 13,600

FIGURE 2.

The rate of Coenzyme A-SH release at 25°C was recorded at 412 nm within the initial five minutes. A second, similar cuvette without L(-)-carnitine was used to determine the hydrolase activity (14) in the 500 x g supernate. The difference in reaction rates with and without L(-)-carnitine was assumed to be the respective actyltransferase activity.

All chemicals were reagent grade. Acetyl-Coenzyme A, octanoyl-Coenzyme A, and palmitoyl-Coenzyme A were purchased from the Sigma Chemical Company, St. Louis, Missouri. L(-)-Carnitine was a gift from the Otsuka Pharmaceutical Factory, Naruto, Tokushima, Japan. Hamsters were purchased from TELACO (Trenton Experimental Laboratory Animal Company), Bar Harbor, Maine.

Pilot experiments revealed the carnitine acyltransferases in all animal groups to be stable in frozen 500 x g supernate for seven weeks and to give optimal assay linearity after the first three weeks of that freezing time (data not shown).

To ascertain assay linearity and reproducibility for each sample, four different volumes of 500 x g supernate were used as quadruplicate determinations.

Due to the design of the experiment, not all samples were able to be assayed at once. Therefore, one sample from each group was assayed each day to control for daily variations in assay conditions.

The data obtained were analyzed using several one-way analysis of variance tests. The Student-Newman-Keuls procedure was used to evaluate pair-wise comparisons whenever a significant F-ratio was obtained for more than two group means (78,106).

RESULTS

Carnitine Acyltransferase Activity

Tables 1, 2, and 3 list the specific activities of cardiac carnitine acetyl-, octanoyl-, and palmitoyltransferase, respectively, in the normal and dystrophic/cardiomyopathic, sedentary hamsters. The mean specific activities of all three carnitine acyltransferases were significantly higher than normal in the hearts of the genetically dystrophic and cardiomyopathic animals. Figures 3, 4, and 5 illustrate the effect of exercise on the mean specific activities of the three cardiac carnitine acyltransferases measured in each of the five animal groups. There was no statistically significant effect of exercise on the mean specific activity of cardiac carnitine acetyltransferase in the dystrophic/cardiomyopathic hamsters, when compared to the dystrophic/cardiomyopathic, sedentary control animals. The mean ($\pm$ standard error of the mean) specific activity of that enzyme in the dystrophic/cardiomyopathic, sedentary group was 9.40 $\pm$ 0.71 nmoles/min/mg protein; that in the dystrophic/cardiomyopathic "moderate" and "high" exercise groups was 8.77 $\pm$ 0.71 nmoles/min/mg protein and 8.88 $\pm$ 0.65 nmoles/min/mg protein, respectively. There was no detected[1] significant effect of exercise on the mean specific

---

[1]The power ($1-\beta$) of the F-test at $\alpha = 0.05$ is low; i.e., the probability of failing to detect a true treatment effect is greater than .10 (G. W. Snedecor and W. G. Cochran, *Statistical Methods* [Ames, Iowa State University Press, 1967]).

TABLE 1.

CARNITINE ACETYLTRANSFERASE
SPECIFIC ACTIVITY

(nmoles/min./mg. protein)

	NORMAL HAMSTER HEART	CARDIOMYOPATHIC (Strain 53.58) HAMSTER HEART
	7.01	11.5
	7.94	12.0
	6.65	13.9
	10.5	6.82
	10.3	9.66
	8.33	9.14
	6.20	7.29
	6.35	6.04
	10.3	9.52
	4.02	8.23
	5.06	9.25
	4.38	
MEAN :	7.25	9.40
S.E.M. :	0.65	0.71
No. of Animals :	12	11

$p < 0.025$

TABLE 2.

CARNITINE OCTANOYLTRANSFERASE
SPECIFIC ACTIVITY

(nmoles/min./mg. protein)

	NORMAL HAMSTER HEART	CARDIOMYOPATHIC (Strain 53.58) HAMSTER HEART
	9.51	16.4
	8.48	15.2
	9.86	15.7
	14.7	13.4
	12.0	15.0
	11.2	12.8
	9.07	12.4
	7.97	10.8
	13.2	11.0
	6.51	11.5
	10.3	12.0
	7.86	
MEAN :	10.1	13.3
S.E.M. :	0.69	0.60
No. of Animals :	12	11

$p < 0.005$

TABLE 3.

CARNITINE PALMITOYLTRANSFERASE
SPECIFIC ACTIVITY

(nmoles/min./mg. protein)

	NORMAL HAMSTER HEART	CARDIOMYOPATHIC (Strain 53.58) HAMSTER HEART
	8.45	14.8
	7.24	12.8
	8.75	13.7
	12.2	12.5
	11.1	13.1
	9.50	11.6
	6.56	10.0
	7.23	10.2
	11.2	6.36
	6.55	7.27
	7.83	6.96
	5.28	
MEAN :	8.49	10.8
S.E.M. :	0.62	0.88
No. of Animals :	12	11

$p < 0.025$

Figure 3. The effects of the "moderate" (MOD.) and "high" (HIGH) swim exercise program on the mean specific activity of cardiac carnitine acetyltransferase (CAT) are shown. Comparison is made to the sedentary (SED.) animals in the normal and cardiomyopathic hamster groups. The "lined" bars represent the significant difference in mean CAT specific activity between the normal and cardiomyopathic, sedentary hamsters shown in Table 1.

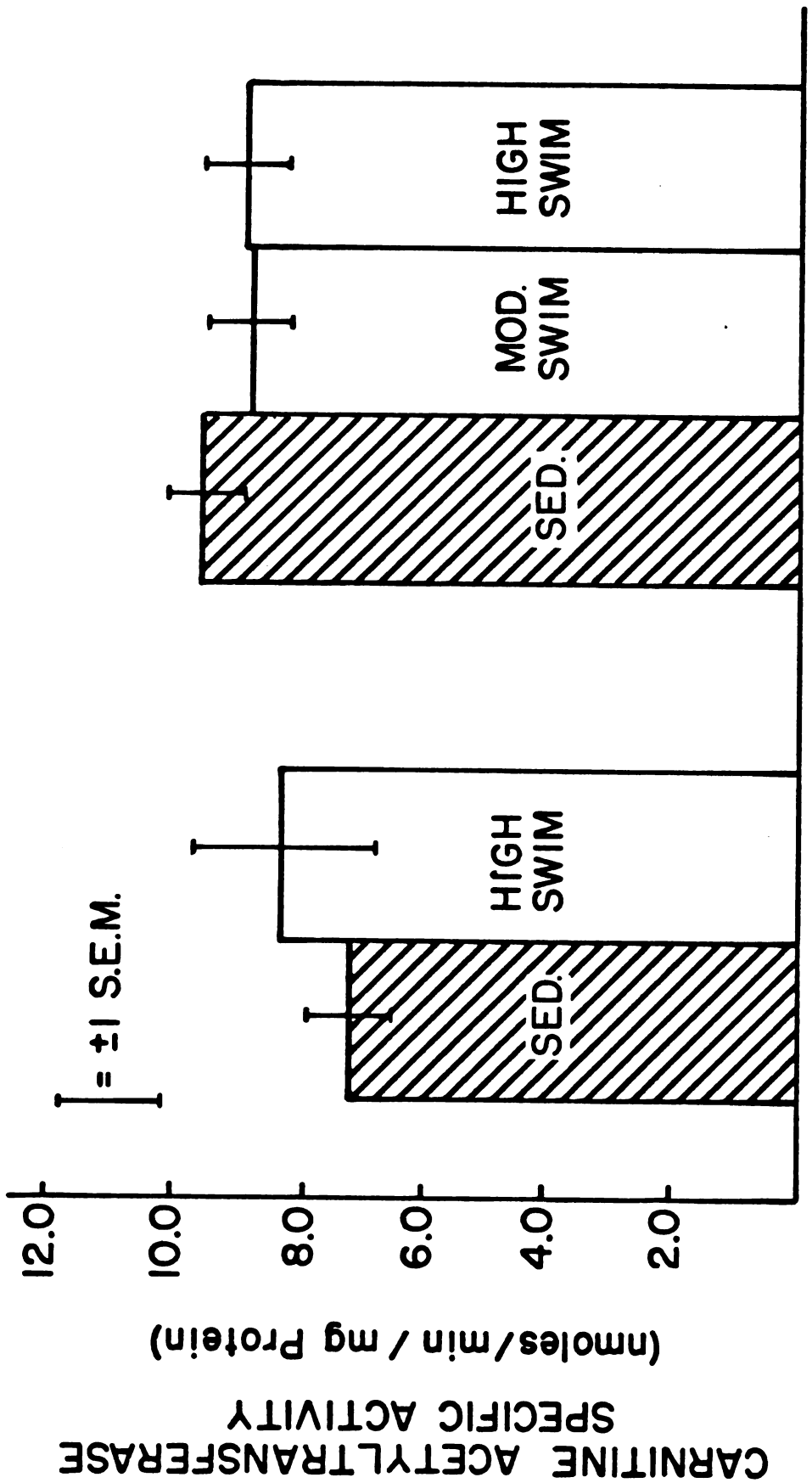

CARNITINE ACETYLTRANSFERASE
SPECIFIC ACTIVITY
(nmoles/min / mg Protein)
12.0
10.0
8.0
6.0
4.0
2.0
= ±1 S.E.M.
HIGH SWIM
SED.
NORMAL
Hamster Heart
MOD. SWIM
HIGH SWIM
SED.
CARDIOMYOPATHIC
Hamster Heart
(Strain 53.58)

Figure 3.

Figure 4. The effects of the "moderate" (MOD.) and "high" (HIGH) swim exercise programs on the mean specific activity of cardiac carnitine octanoyltransferase (COT) are shown. Comparison is made to the sedentary (SED.) animals in the normal and cardiomyopathic hamster groups. The "lined" bars represent the significant difference in mean COT specific activity between the normal and cardiomyopathic, sedentary hamsters shown in Table 2.

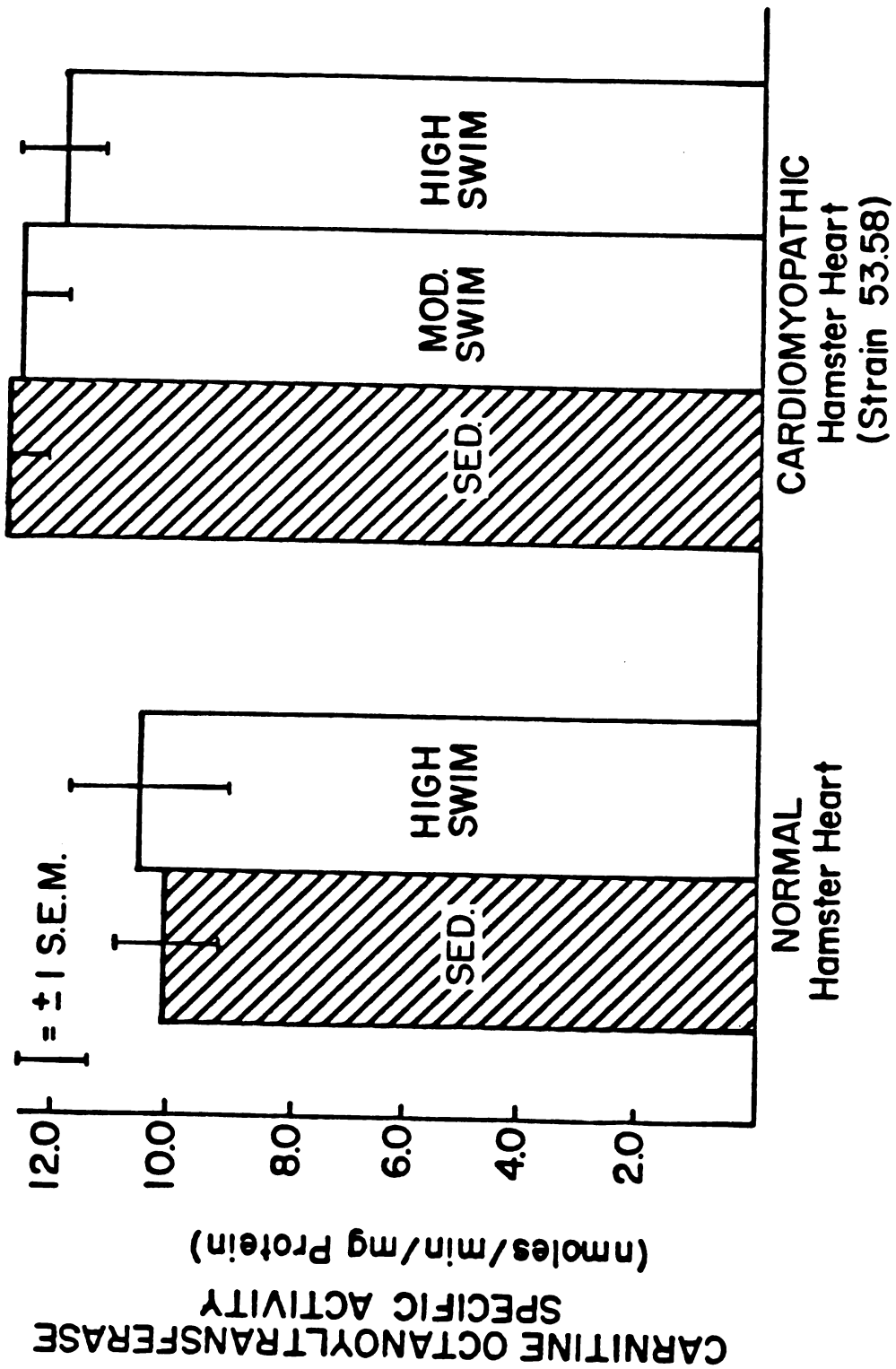

CARNITINE OCTANOYLTRANSFERASE
SPECIFIC ACTIVITY
(nmoles/min/mg Protein)
12.0
10.0
8.0
6.0
4.0
2.0
= ± 1 S.E.M.
NORMAL
Hamster Heart
SED.
HIGH
SWIM
CARDIOMYOPATHIC
Hamster Heart
(Strain 53.58)
SED.
MOD.
SWIM
HIGH
SWIM

Figure 4.

Figure 5. The effects of the "moderate" (MOD.) and "high" (HIGH) swim exercise programs on the mean specific activity of cardiac carnitine palmitoyltransferase (CPT) are shown. Comparison is made to the sedentary (SED.) animals in the normal and cardiomyopathic hamster groups. The "lined" bars represent the significant difference in mean CPT specific activity between the normal and cardiomyopathic, sedentary hamsters shown in Table 3.

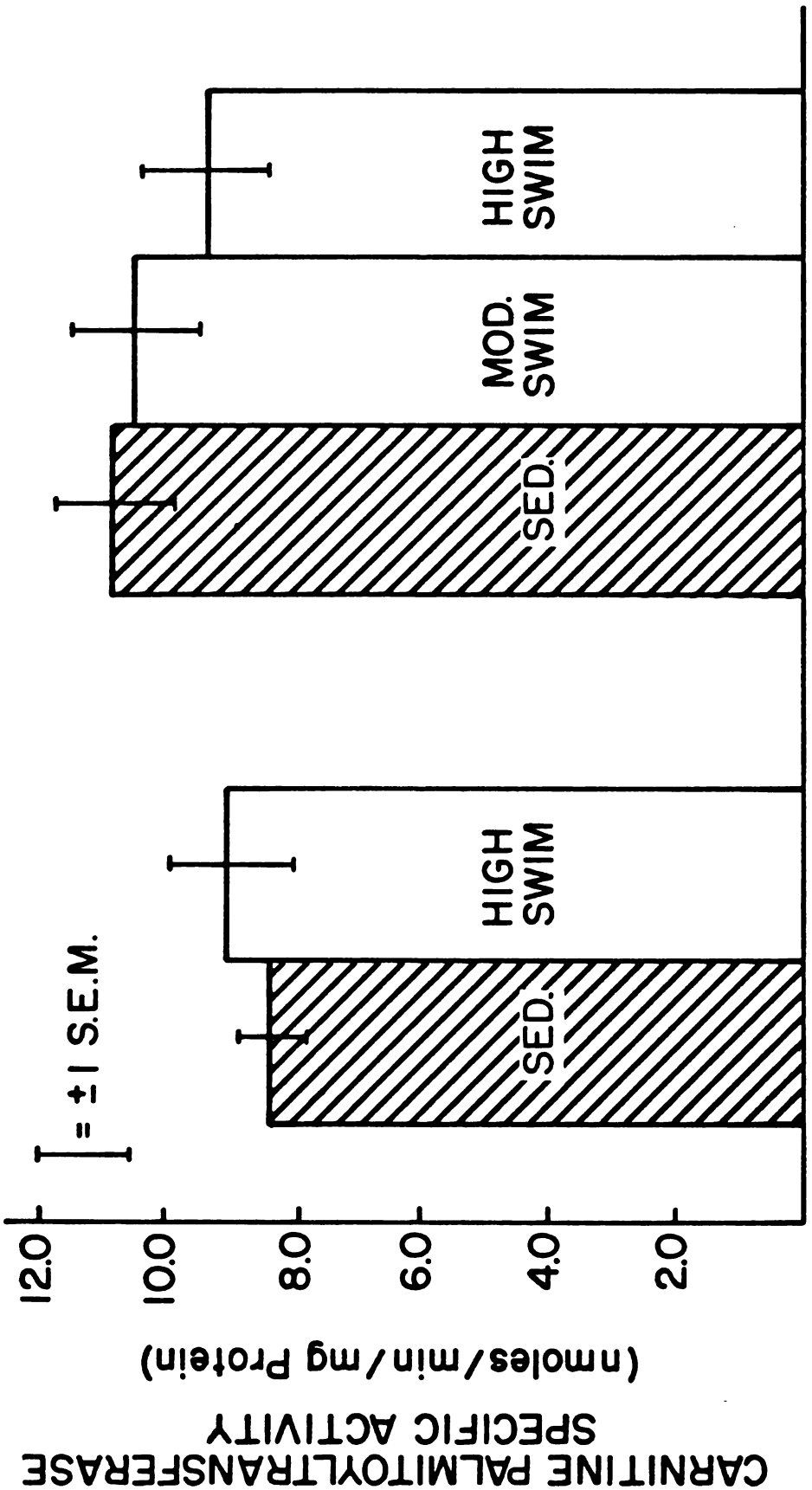

CARNITINE PALMITOYLTRANSFERASE
SPECIFIC ACTIVITY
(nmoles/min/mg Protein)
12.0
10.0
8.0
6.0
4.0
2.0
= ± 1 S.E.M.
NORMAL
Hamster Heart
HIGH
SWIM
SED.
CARDIOMYOPATHIC
Hamster Heart
(Strain 53.58)
MOD.
SWIM
HIGH
SWIM
SED.

Figure 5.

activities of cardiac carnitine octanoyl- or palmitoyltransferase in the diseased animals; and there was no detected[1] significant effect of exercise on the mean specific activity of any of the three cardiac carnitine acyltransferases measured in the normal animals.

A comparison of the activities of the three cardiac enzymes within each animal group (Figures 3 through 5) revealed a mean carnitine octanoyltransferase specific activity significantly higher (at most, $P<0.05$) than that of either carnitine acetyltransferase or carnitine palmitoyltransferase in each group except the normal, exercised hamsters. "High" exercise obliterated the significant differences in specific activity detected in the normal, sedentary animals.

Heart Weight, Body Weight, and Heart Weight/Body Weight Ratio

The mean heart weight/body weight ratio of each animal group is shown in Figure 6. There was no detected[2] significant difference in heart weight/body weight ratio between the normal and dystrophic/cardiomyopathic, sedentary animals. The exercised hamsters in both the normal and dystrophic/cardiomyopathic groups had significantly ($P=0.00$) elevated mean heart weight/body weight ratios compared to the respective sedentary controls. A supranormal heart weight/body weight ratio is generally considered to be indicative of cardiac hypertrophy.

Averages of the data used to calculate the heart weight/body weight ratios are also shown. The mean absolute heart weight of each

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[1] *Idem*

[2] *Idem*

Figure 6. The mean heart weight/body weight ratio of each hamster group is represented. Comparison is made between sedentary (SED.) animals in the normal and cardiomyopathic groups, and between sedentary and swim-exercised animals within the normal and cardiomyopathic groups. The "lined" bars represent statistically significant increases in the heart weight/body weight ratios with exercise in both the normal and cardiomyopathic hamster groups.

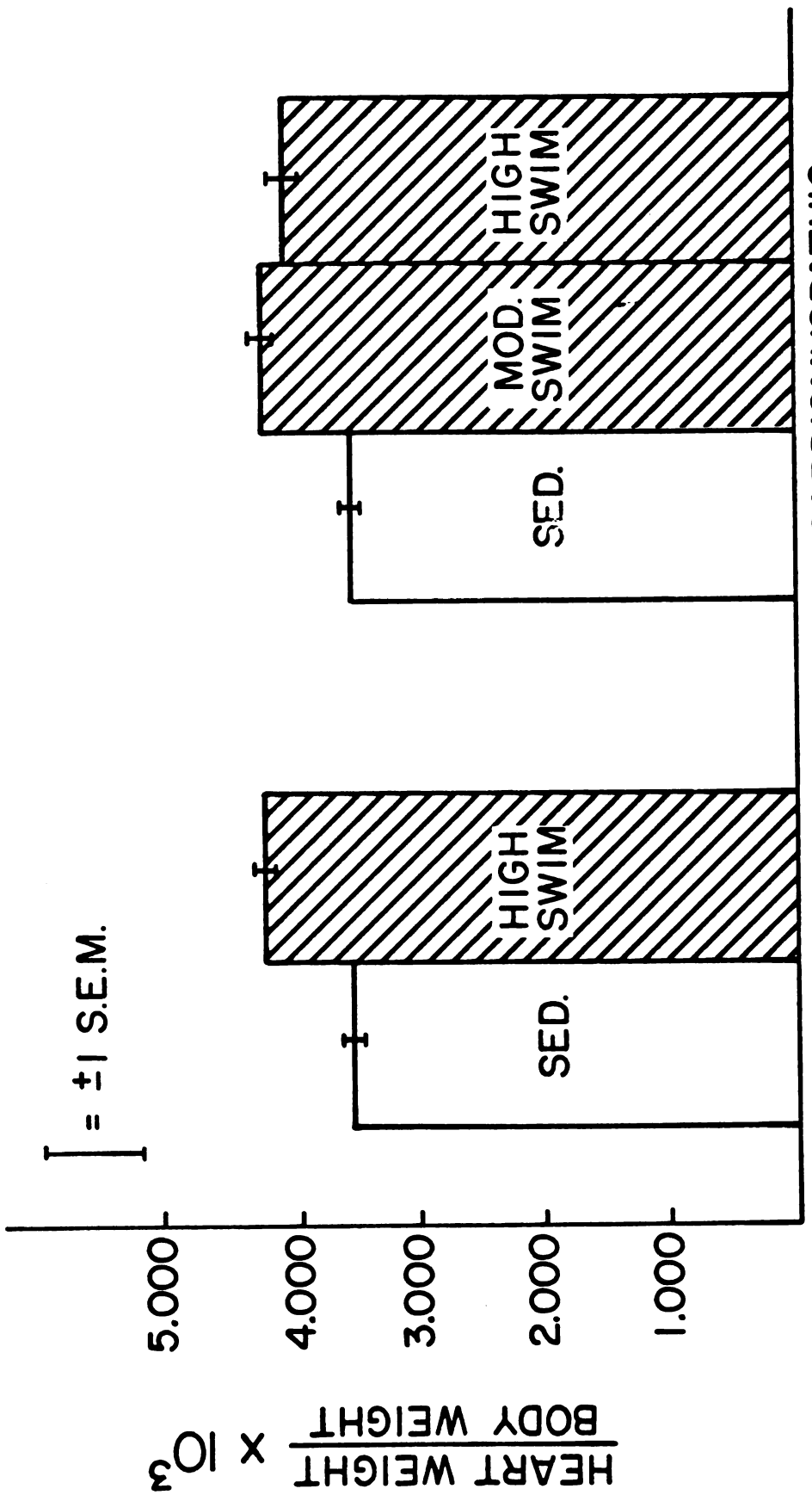
HEART WEIGHT / BODY WEIGHT x 10^3
5.000
4.000
3.000
2.000
1.000
= ±1 S.E.M.
NORMAL HAMSTER
SED.
HIGH SWIM
CARDIOMYOPATHIC HAMSTER (Strain 53.58)
SED.
MOD. SWIM
HIGH SWIM

Figure 6.

Figure 7. The mean heart weight of each hamster group is represented. Comparison is made between sedentary (SED.) animals in the normal and cardiomyopathic groups, and between sedentary and swim-exercised animals within the normal and cardiomyopathic groups. The "lined" bars represent statistically significant increases in absolute heart weight with exercise in the cardiomyopathic animal group.

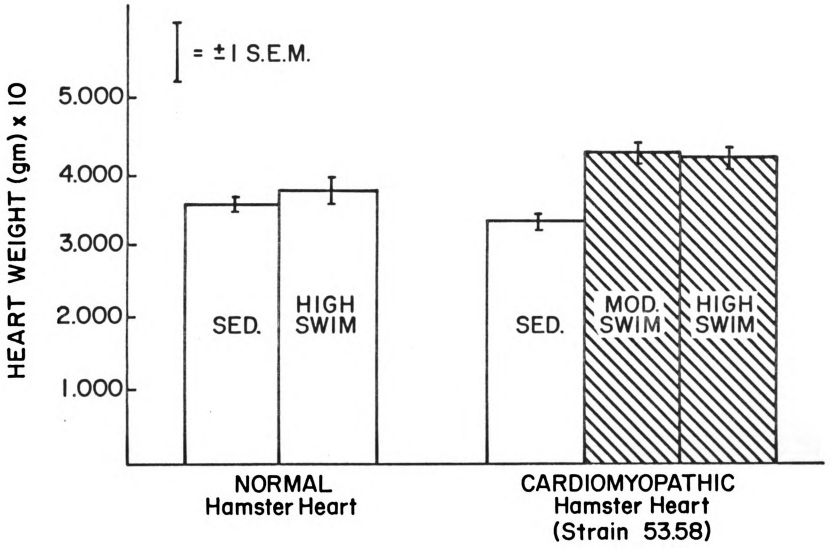
= ±1 S.E.M.
HEART WEIGHT (gm) x 10
5.000
4.000
3.000
2.000
1.000
SED.
HIGH SWIM
SED.
MOD. SWIM
HIGH SWIM
NORMAL
Hamster Heart
CARDIOMYOPATHIC
Hamster Heart
(Strain 53.58)

Figure 7.

Figure 8. The mean body weight of each hamster group is represented. Comparison is made between sedentary (SED.) animals in the normal and cardiomyopathic groups, and between sedentary and swim-exercised animals within the normal and cardiomyopathic groups. The "lined" bars represent a statistically significant decrease in normal hamster mean body weight with exercise and a statistically significant increase in mean body weight with "high" exercise in the cardiomyopathic hamster group.

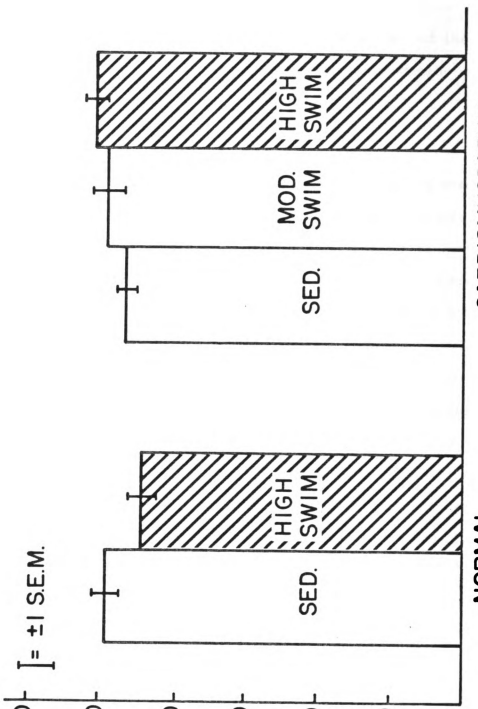
BODY WEIGHT (gm)
120
100
80
60
40
20
I = ± 1 S.E.M.
SED.
HIGH SWIM
NORMAL HAMSTER
SED.
MOD. SWIM
HIGH SWIM
CARDIOMYOPATHIC HAMSTER
(Strain 53.58)

Figure 8.

the hamster hearts are shown in Figure 9. No detected[1] significant differences in these mean ratios appear among any of the groups, i.e., with exercise or with genetic muscular dystrophy and cardiomyopathy.

Protein Concentration

The distribution, among the five groups, of the mean total protein concentration of the heart is represented in Figure 10. A significantly ($P<0.01$) lower mean cardiac protein concentration, 170 $\pm$ 5 mg Lowry protein/gm heart, was found in the dystrophic/cardiomyopathic, sedentary hamsters compared to 192 $\pm$ 5 mg Lowry protein/gm heart in the sedentary normal controls. With the "high" swim exercise regimen, the mean protein concentration in the hearts of the dystrophic/cardiomyopathic animals increased significantly ($P<0.05$) above that of the sedentary dystrophic/cardiomyopathic controls to a value (187 $\pm$ 7 mg Lowry protein/gm heart) not significantly different from that measured in normal hamster heart. Exercise did not significantly change the mean cardiac protein concentration in the normal animals.

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[1] *Idem*

Figure 9. The mean cardiac dry weight/wet weight ratio of each hamster group is represented. Comparison is made between sedentary (SED.) animals in the normal and cardiomyopathic groups, and between sedentary and swim-exercised animals within the normal and cardiomyopathic groups.

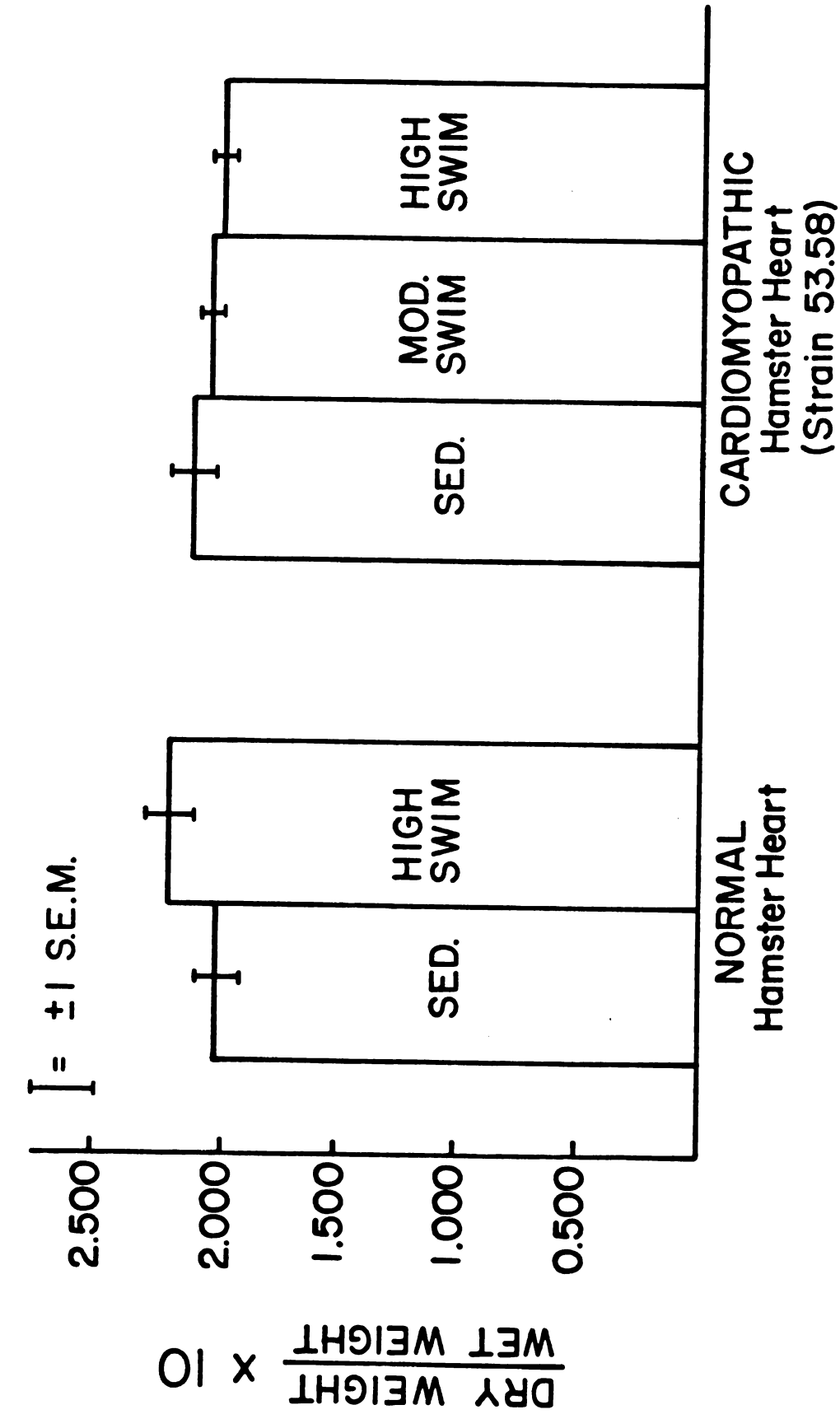
DRY WEIGHT / WET WEIGHT x 10
2.500
2.000
1.500
1.000
0.500
I = ±1 S.E.M.
SED.
HIGH SWIM
NORMAL Hamster Heart
SED.
MOD. SWIM
HIGH SWIM
CARDIOMYOPATHIC Hamster Heart (Strain 53.58)

Figure 9.

Figure 10. The mean cardiac protein concentration of each hamster group is represented. Comparison is made between sedentary (SED.) animals, where a statistically significant decrease in mg Lowry protein/gm heart is observed in the cardiomyopathic group. A statistically significant increase in protein concentration in the cardiomyopathic hearts is seen with the "high" swim exercise program.

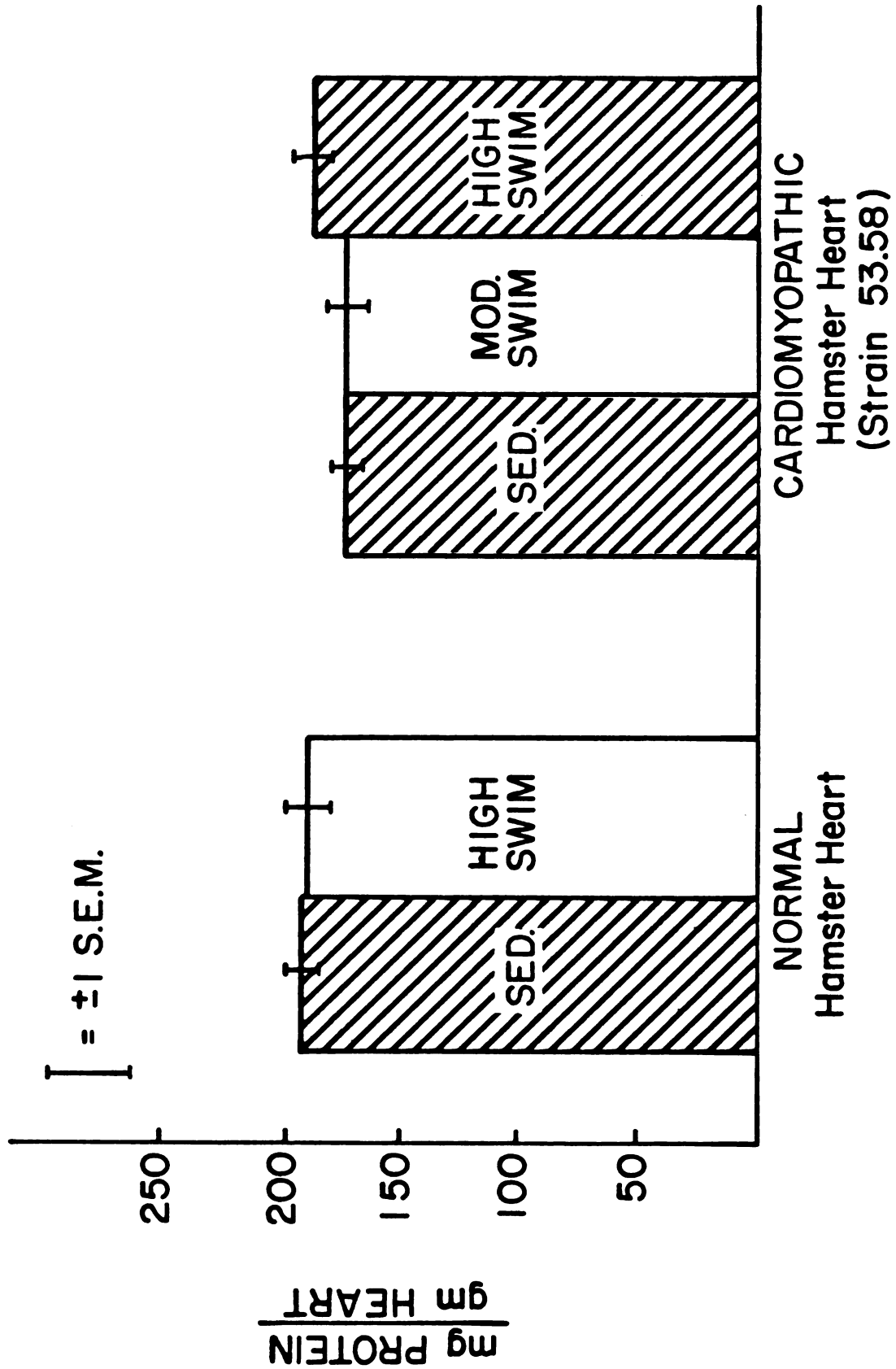
mg PROTEIN / gm HEART
250
200
150
100
50
I = ±1 S.E.M.
NORMAL Hamster Heart
HIGH SWIM
SED.
CARDIOMYOPATHIC Hamster Heart (Strain 53.58)
MOD. SWIM
SED.
HIGH SWIM

Figure 10.

CONCLUSIONS AND DISCUSSION

The data generated from this study show, for the first time, that carnitine acyltransferases which utilize acetyl-, octanoyl-, and palmitoyl-Coenzyme A as substrates are part of the enzymic profile in the normal, and in the dystrophic/cardiomyopathic (B10 53.58), hamster heart.

Findings of enhanced carnitine acetyl-, octanoyl-, and palmitoyl-transferase specific activities in the hearts of the dystrophic/cardiomyopathic hamsters lend themselves to multiple interpretations.

Consideration must, first of all, be given to the possibility of the occurrence of differential protein degradation during progression of the disease, with a resultant enhanced relative concentration, and, therefore, elevated specific activity, of carnitine acyltransferase. This type of situation might obtain, for example, if myofibrillar proteins were preferentially destroyed during the cardiomyopathic process. (Results of further experimentation, in which the carnitine acyltransferase assays are done on isolated organellar fractions, and which show no changes [with dystrophy] in specific activities, expressed per unit of organellar protein, will be required to support the validity of this explanation.) However, the data, from this study, which demonstrate a statistically significant "high"-exercise-induced increase in mean cardiac protein concentration in the dystrophic/cardiomyopathic hamsters with a lack of any concomitant

change in cardiac carnitine acetyltransferase activity might be cited as evidence against this interpretation of the results, if one assumes that the newly synthesized protein represents a specific regeneration of that which was destroyed.

As emphasized previously with reference to other enzymes, the modification of cardiac carnitine acyltransferase activity in genetic muscular dystrophy and cardiomyopathy may also derive from direct effects of the disease(s) on enzyme protein or non-specific changes that characterize myopathic processes generally.

Carnitine acetyl- and palmitoyltransferase are known to be membrane-associated enzymes. Carnitine octanoyltransferase may also be associated with the membranes of the organelles in which it is found. The concept, then, of altered membrane microenvironments causing the altered activities of these enzymes must be entertained as the basis for another interpretation of the data.

Finally, the elevated specific activities of the carnitine acyltransferases measured may be due to absolute increases in these enzyme proteins in the hearts of the dystrophic/cardiomyopathic hamsters.

As previously mentioned, these animals eventually die as a result of congestive heart failure, a condition in which the blood-pumping performance of the heart is insufficient to meet systemic metabolic needs. Prior to the onset of failure, however, cardiac hypertrophy occurs, becoming evident in other strains after the third month of life and presumably provoked by the increased work load imposed on remaining normal cardiac fibers, as the dystrophic process progressively encroaches upon larger areas of myocardium. This stage of cardiac

hyperfunction is characterized by increased ribonucleic acid (RNA) polymerase activity and synthesis of ribosomal RNA and protein (77), increased adenyl cyclase activity (77), altered sympathetic nervous system responses (9,97), and abnormalities in calcium ion transport (61,103) and the contractile apparatus (103). Studies of mitochondrial function during this period have yielded varied results (103). However, under optimal isolation and assay conditions, it appears that mitochondria from hearts undergoing non-failing compensatory hypertrophic changes exhibit augmented respiratory activity with no defect in oxidative phosphorylation. This enhancement of energy-generating mechanisms might be expected to depend on a concomitant increase in fatty acid oxidation in the predominantly "fat-burning" myocardium. The elevations in cardiac carnitine acyltransferase activity may, therefore, be interpreted as fitting reasonably into this scheme of hyperfunctional, compensatory events.

The calculated high probabilities of Type II statistical errors,[1] occurring with regard to the dry weight/wet weight ratios of the hearts, complicate interpretation of the heart weight/body weight ratios. However, it seems highly unlikely that such significant elevations in mean heart weight/body weight ratio, as those observed with exercise, would be due, solely, to an undetected increase in water content of the tissue. Also, light microscopic examination of cardiac sections, obtained from hamsters randomly selected from each animal group expressly for histopathological evaluation, revealed increased heart diameter and ventricular wall thickness in animals

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[1] *Idem*

that had been subjected to exercise (personal communication from Dr. Rexford Carrow). The elevated mean heart weight/body weight ratios in the exercised hamsters are, therefore, viewed as indicators of cardiac hypertrophy.

Cardiac hypertrophy developed in all exercised animal groups, normal and dystrophic/cardiomyopathic. Of potential significance, also, is the fact that the degree of increase in heart weight/body weight ratio attained over twelve weeks of exercise in the dystrophic/cardiomyopathic hamsters was similar to that seen in the normal hamsters. Considered with the histological findings of (46) decreased frequency and severity of areas of myocardial degeneration in dystrophic/cardiomyopathic exercised hamsters, these data indicate that the potential for net, histologically normal myocardial tissue growth, in response to appropriate stimuli, is still retained by the diseased hearts.

No definitive conclusions[1] can be drawn from this study regarding the effect of exercise on the specific activities of the cardiac carnitine acyltransferases measured, except with reference to carnitine acetyltransferase in the dystrophic/cardiomyopathic hamsters. Neither the "moderate" nor "high" swim exercise programs evoked any statistically significant change, compared to sedentary controls, in carnitine acetyltransferase mean specific activity in the diseased animals. That the exercise was effective as a metabolic stimulus is attested to by the significant increase above sedentary values in heart weight/body weight ratios in both the normal and dystrophic/

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[1] *Idem*

cardiomyopathic exercised hamsters. The metabolic alterations which must underlie the improved cardiac histopathology seen in the dystrophic/cardiomyopathic hamsters subjected to this exercise program (46) are, therefore, not reflected in measurable changes in carnitine acetyltransferase specific activity and, hence, are probably not mediated via this enzyme. The possibility does remain, however, that maintenance of the elevated carnitine acyltransferase activity seen in the sedentary dystrophic/cardiomyopathic animal hearts is required to support those metabolic pathways which are directly responsible for the exercise-induced improvement in myocardial architecture.

Cardiac metabolism has been analyzed in terms of three phases: (a) energy liberation, consisting of catabolic reaction sequences such as those found in glycolysis, the hexose monophosphate shunt, fatty acid oxidation, and the Krebs citric acid cycle; (b) energy conservation, involving the reactions of oxidative phosphorylation and the subsequent transfer of energy from ATP to creatine, yielding another high-energy reservoir, creatine phosphate; and (c) energy utilization, referring mainly to the series of steps coupling excitation with cardiac muscular contraction (76). The present data indicating a lack of an exercise effect on cardiac carnitine acetyltransferase mean specific activity in the dystrophic/cardiomyopathic hamsters are consistent with studies of exercise effects on cardiac metabolism in other, normal animal species: exercise-induced improvements in cardiac performance appear to be related to altered contractile protein activity and not to any change in energy liberation mechanisms (13,100,131).

A meaningful interpretation of the pattern of specific activities found among the three cardiac carnitine acyltransferases, analyzed per animal group, must await the discovery of specific functions for carnitine octanoyltransferase.

The statistically significant decrease, compared to normal, in mean mg Lowry protein/gm heart, found in sedentary hamsters with genetic muscular dystrophy and cardiomyopathy, might be anticipated in view of the areas of myocardial degeneration and calcification observed, histologically, in these animals.

The statistically significant increase in mean cardiac protein concentration to normal values, in the dystrophic/cardiomyopathic hamsters subjected to the "high" exercise program, may imply the stimulation of cardiomyopathic reparative and/or retardative processes which are superior, quantitatively and/or qualitatively, to those provoked by the "moderate" exercise program. This interpretation is supported by observations of improved cardiac histopathology in both exercised groups of dystrophic/cardiomyopathic hamsters (compared to sedentary animals), with the "high swim" group showing greater improvement than the "moderate swim" group of hamsters (personal communication from Dr. Rexford Carrow).

A difference between normal and dystrophic/cardiomyopathic hamsters in the response of body weight to exercise has been reported previously (46) with use of another (B10 14.6) strain of dystrophic/cardiomyopathic hamsters. In the present and previous studies, the mean body weight in normal hamsters significantly decreased (compared to normal, sedentary hamster controls) upon exercise while that of the dystrophic/cardiomyopathic hamsters significantly increased above

the mean body weight values of sedentary dystrophic/cardiomyopathic hamsters.

Recently it was shown (17) that voluntary disc exercise in adult hamsters in the asymptotic phase of growth caused accelerated skeletal growth and an increase in body weight to a new, lasting (after retirement from exercise) plateau. The increase in body weight was associated with an increase in body length, rather than an increase in the percentage of body fat, and appeared to represent growth increases in all body compartments.

Growth in normal rodents is characterized by an early, exponential phase and a later, asymptotic phase, the initiation and maintenance of which appear to be regulated by integrated neural and neuroendocrine mechanisms. The results from the above experiment were interpreted as representing a disc-exercise-induced reinstatement of an earlier phase of hamster ontogeny (17).

The discordance in exercise-induced body weight changes in the chronological-age matched normal and dystrophic/cardiomyopathic hamsters used in this study may reflect differences in developmental maturation between the two groups at the time of initiation of exercise, or may be another manifestation of altered neural or general growth-regulating processes in the genetic muscular dystrophic and cardiomyopathic hamsters.

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