The effects of exhaustive exercise on (tritium) PHE incorporation into heart muscle contractile proteins.

James Robert. Swartman

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS RÉCU
THE EFFECTS OF EXHAUSTIVE EXERCISE ON \(^{(3)H}\)PHE INCORPORATION INTO HEART MUSCLE CONTRACTILE PROTEINS.

by

James Robert Swartman

A Thesis submitted to the Faculty of Graduate Studies through the Faculty of Human Kinetics in Partial Fulfillment of the requirements for the Degree of Master of Human Kinetics at The University of Windsor

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To Jan
ABSTRACT

THE EFFECTS OF EXHAUSTIVE EXERCISE ON (3H)PHE INCORPORATION INTO HEART MUSCLE CONTRACTILE PROTEINS

by

James Robert Swartman

To establish the responsiveness of protein synthesis to acute exercise and recovery, female Wistar rats (200-220 gm) were swum to exhaustion or allowed to recover for one, two, or four hours. Hearts were removed and perfused for 30 minutes with a Krebs-Henseleit bicarbonate buffer containing normal levels of plasma amino acids, 15mM glucose and (3H)Phenylalanine (0.1 uCi/ml perfusate). Hearts were fractionated into total tissue, myofibrillar, actomyosin and myosin protein. Myosin was further fractionated into heavy chain (HC) and light chains (LC₁ and LC₂) using SDS polyacrylamide gel electrophoresis. Phenylalanine incorporation continued to increase from 15 to 60 minutes of perfusion. In total, tissue protein amino acid incorporation was reduced by 30 per cent at exhaustion, returned to normal by one hour of recovery and showed no further change four hours post exercise. Amino acid incorporation into myofibrillar
protein showed a nonsignificant decrease at exhaustion and increased 57 per cent following two hours of recovery. Myosin purified electrophoretically showed an immediate 84 per cent increase in amino acid incorporation at exhaustion. Following two hours of recovery \((^{3}\text{H})\text{Phe}^+\) incorporation into total myosin increased 112 per cent. Of the three protein components of myosin, \(L_{C_1}\) and \(L_{C_2}\) accounted for most of the increased rate of incorporation. These data demonstrate two synthetic responses in heart muscle following an acute bout of exhaustive exercise. The increased incorporation was associated with the small sub-units of myosin suggesting that an early response to repeated bouts of exercise may be a proliferation of the contractile proteins with the subsequent induction of tissue hypertrophy.
ACKNOWLEDGEMENTS

The author is indebted to Dr. P. B. Taylor, Dr. R. T. Hermiston and Dr. M. J. P. Dufresne for invaluable guidance and assistance throughout the investigation.

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CHAPTER I

INTRODUCTION

Generally, it has been found in biological systems that functional overload of an organ will result in its growth (22). This phenomenon appears to be related to an imbalance between the functional demands placed on the organ and the capacity of that tissue to meet those demands. A number of conditions have been found which may cause an imbalance between the functional capacity of cardiac tissue and the physiologic demands. Among these are various forms of hypertension (6, 7, 13, 21, 51), anoxia (26), catecholamine stimulation (48) and chronic exercise (53, 15).

Three types of growth are postulated to account for cardiac hypertrophy: an enlargement of pre-existing muscle cells (hypertrophy); an increase in the number of muscle cells (hyperplasia); or a combination of both. In the fully differentiated adult heart, myocyte growth occurs by the first mechanism only (22), indicating that the mitotically static muscle cell must adapt by synthesizing specific subcellular components.

A great deal of evidence exists linking a prolonged increase in cardiac function to an increase in heart size.
Steinhaus (52) has shown that 18 months of swimming resulted in a 17 per cent increase in the heart weight to body weight ratio. Meerson (34) has clearly shown that 24 hours after surgically induced aortic constriction, there was a 10 per cent increase in myocardial mass that reached a maximum of 40-50 per cent after eight days. Furthermore, Schrieber (45) has found that three hours of moderate pressure overload in an isolated perfused heart was a sufficient stimulus to increase amino acid incorporation into protein, indicating that the adaptive response was initiated shortly after the application of the stimulus and that the mechanisms for ventricular growth were extremely sensitive to slight alterations in the homeostatic state of the cell.

Studies that have utilized chronic pressure overload (6, 7, 12, 13, 21, 51), hypoxia (5), polycythemia (11) or catecholamine stimulation (48) to elicit myocardial growth have demonstrated a significant increase in muscle mass but have often been criticized for a possible pathological response. Frequently, these hearts show evidence of cellular necrosis and a failure to respond to an increased hemodynamic challenge (18). These forms of compensatory hypertrophy are pathologic and may not be appropriate to study normal cardiac adaption to physiologic stress. In contrast, exercise-induced
myocardial hypertrophy shows no evidence of tissue damage and possesses increased muscle contractility (15) with an enhanced capacity to hydrolyze adenosine triphosphate (9).

During the early phase of compensatory cardiac growth some doubt exists as to whether the various subcellular components, which contribute to cardiac performance, proliferate in constant proportion. Previously, the relative size of the mitochondria (35) and their capacity for oxidative phosphorylation (39, 40) were used as markers to potentially explain the increased performance of cardiac muscle. However, the respiratory capacity of cardiac tissue does not adapt to physical training (39). The recent discovery that myosin adenosine triphosphatase activity (8, 9) increased in response to chronic exercise suggests that fundamental alterations may occur in the structure and/or function of contractile proteins and that these myofibrillar proteins may be involved with the initial adaptive response of cardiac tissue. Furthermore, the increased contractile capacity appears to be related to the severity and the duration of the training program (8).

The synthesis of contractile proteins has been shown to be initiated within the first few hours of pressure overload in the isolated perfused rat heart (46). These findings seem to suggest that a single bout of
exercise may be sufficient to stimulate protein synthesis. In the present study, an acute bout of exhaustive exercise was used as a physiologic stress, in an effort to establish a model for the future study of control mechanisms involved in exercised-induced cardiac hypertrophy. The ability of the heart to synthesize, total tissue, myofibrillar, actomyosin, myosin and myosin heavy and light chains protein was investigated.
CHAPTER II

METHODS

Animal Care and Handling

Female Wistar rats (200-220 grams) were individually housed and provided with Purina rat chow and water ad libitum. The animals were divided into control and exercised groups. Exercised animals were exhausted by swimming in a 17 litre barrel, filled to a depth of 45 centimeters with water maintained at 33°C-35°C. To facilitate exercise to exhaustion, weights corresponding to approximately five per cent of the animals' body weight were tied to their tails. Exhausation was identified by the loss of the righting reflex when the animal was placed on its back. In preliminary experiments this procedure resulted in severe glycogen depletion in skeletal muscle, liver and heart muscle (Figure 1).

Removal and Perfusion of the Heart

Following the appropriate treatment, each animal was anesthetized with Nembutal (15-20 mg/animal I.P.) and the abdominal cavity opened by making a midline incision with scissors. Two hundred units of heparin were

5.
Figure 1: The Effects of Exhaustive Exercise on Body Glycogen Reserves
injected into the inferior vena cava. The hearts were then quickly removed and placed in ice cold saline to arrest the contractions. The hearts were removed from the saline with fine tipped forceps and the aorta slipped onto a grooved perfusion cannula and secured with a thread ligature (Figure 2). After five minutes of preliminary washout perfusion, the heart was transferred to a recirculating system in which the perfusion pressure was adjusted by a peristaltic pump to 60 mmHg. The heart was perfused for 30 minutes as determined from the kinetics of phenylalanine incorporation. The perfusion medium was an amino acid fortified Krebs-Henseleit bicarbonate buffer (36) containing 15mM glucose and (3H) phenylalanine, 0.1μCi/ml perfusate. The perfusate was maintained at 37°C and gassed with 95 per cent oxygen and five per cent carbon dioxide gas mixture equilibrated with water at 37°C. Following each perfusion, the heart was cut from the cannula, wrapped in tin foil and frozen in liquid nitrogen.

**Isolation of Myofibrils**

Myofibrils were isolated and purified by the method of Solaro (50). Individual hearts were thawed to 0-4°C, trimmed of fat and excess connective tissue, weighed and cut into small pieces with scissors. The tissue mince was homogenized in a Potter Elvehjem tissue homogenizer
Figure 2: Diagrammatic Representation of the Perfusion Apparatus
in 25ml of solution A, (0.3M sucrose; 10mM imidazol pH 7.0) with 10 passes of the pestal. The homogenate was centrifuged at 17,300xg for 20 minutes. The resulting pellet was suspended to the original homogenate volume using solution B, (60mM KCl; 30mM imidazol; 2mM MgCl₂; pH 7.0) referred to as standard buffer solution and centrifuged for 15 minutes at 750xg. The pellet was resuspended and centrifuged four times in solution B. The myofibrils were then suspended in eight pellet volumes of solution C, (standard buffer solution containing 2mM ethylenediamine tetraacetate (EDTA)) and centrifuged for 15 minutes at 750xg. Further purification of the myofibrils was achieved by suspending the EDTA treated, washed myofibrils in eight pellet volumes of solution D, (standard buffer solution containing one per cent tritonX-100) for 15 minutes and centrifuged at 750xg. This treatment was repeated once. The resulting pellet was resuspended four times in eight pellet volumes of solution B and centrifuged at 750xg for 15 minutes.

**Extraction of Actomyosin**

Actomyosin was extracted from purified myofibrils by suspending the myofibrillar pellet in 25ml of 0.6M KCl for 20 hours at 0-4°C(33). The solution was centrifuged at 2000xg for 10 minutes. The actomyosin was
precipitated from the supernatant by a 15 fold dilution with ice-cold distilled water for three hours at 0-4°C. The cloudy precipitate was recovered by centrifugation at 2000xg for 10 minutes.

**Extraction of Myosin**

Myosin was extracted by suspending the actomyosin pellet in 10ml of 0.47M KCl, 0.01M NaPO₄ and 0.02M pyrophosphate (pH 6.8) at 0-4°C for 30 minutes (9). The viscous solution was then centrifuged for 30 minutes at 13,000xg and the supernatant diluted 10 fold with ice-cold distilled water for three hours. The resulting cloudy myosin precipitate was recovered by centrifugation at 2000xg for 10 minutes.

**Electrophoretic Fractionation of Myosin**

Total myosin was solubilized in 0.3 to 0.5 ml protein solubilizing solution (1 per cent SDS; 1 per cent B mercaptoethanol; 0.01 M NaPO₄) and fractionated into myosin heavy chain (HC), myosin light chain one (LC₁) and myosin light chain two (LC₂) by the method of Weber and Osborne (56), using a five per cent SDS polyacrylamide gel. The gels were pre-electrophoresed for one hour at four mA per gel. After pre-electrophoresis 100µg of protein, containing three or four crystals of sucrose, five µl of bromphenol blue and five µl of B
mercaptoethanol, were electrophoresed for six hours under the same conditions as pre-electrophoresis. The gels were removed and stained for two to three hours at room temperature with a solution containing 0.0025 per cent Coomassie brilliant blue, 50.4 per cent methanol and 9.2 per cent acetic acid (55). Gels were destained electrophoretically (30V; 1mA) for six minutes on each side with 7.5 per cent acetic acid in 10 per cent isopropyl alcohol followed by diffusion destaining for three to four days with several changes of destaining solution.

**Molecular Weight Determination**

Myosin sub-units \((LC_1 \text{ and } LC_2)\) were identified by molecular weight determinations using Lysosome (14000), Aldolase (40000) and assuming myosin heavy chain \((200,000)\). Swelling of gels during staining and destaining was corrected for by the use of the following equation:

\[
\text{Mobility} = \frac{\text{Protein migration}}{\text{length after staining}} \times \frac{\text{length before staining}}{\text{Marker dye migration}}
\]

**Total Myocardial Protein Preparation**

Two ml of total tissue homogenate were precipitated overnight with two ml of 10 per cent trichloro acetic acid (TCA). The precipitate was recovered by centrifugation for 10 minutes in a table top centrifuge and washed once
in five per cent TCA. The protein pellet was then boiled for 15 minutes in five per cent TCA and washed twice in five per cent TCA. The pellet was then washed twice with chloroform, methanol, ether, 2:1:1 and 1:1:1 (vol/vol) respectively to remove lipid contamination. The protein was then washed twice with ether and solubilized in four ml of 1N NaOH.

**Protein and Radioactive Determination**

Appropriate aliquots (100μl) from total tissue, myofibrillar, actomyosin and myosin protein fractions were suspended in 10ml of toluene-based scintillation solution for radioactivity measurements (4gm PPO; 50mg POPP per litre of toluene). Vials were counted in a Beckman LS100 liquid scintillation spectrometer. The protein in each sample was quantified according to Lowry et al. (32). The specific radioactivity in each sample was expressed as either counts per minute (CPM) per mg protein or disintegrations per minute (DPM) per mg protein by using the external channel correction ratio in conjunction with a quench correction curve.

Myosin for electrophoretic fractionation was quantified using a protein dye binding assay (10). Bands corresponding to molecular weights of approximately 200,000; 27,000 and 18,500 daltons were sliced, digested in hydrogen peroxide (30 per cent) and suspended in Beckman
high performance liquid scintillation cocktail. The specific radioactivity was expressed as counts per minute per 100μg of starting protein.

Statistics

The data were analyzed by a one-way analysis of variance followed by the Duncan multiple-range test (31). A p-value of 0.05 or less was considered significant in this study.
CHAPTER III

RESULTS

Protein Yields

The recovery of various proteins isolated from heart muscle is shown in Table 1. Yields of actomyosin and myosin from exhausted hearts were about half those obtained from other time points. This indicates that exhaustive exercise may decrease the amount of protein recoverable using these procedures. Furthermore, poor yields may have masked any significant change in the rate of amino acid incorporation in the tissues.

In general, the yields of actomyosin were about half that found by Bladwin et al. (3). However, their isolation procedure extracted actomyosin from total tissue homogenate whereas the present study extracted actomyosin from purified myofibrils. Myosin yields were comparable to those of Morgan et al. (36).

Myosin Purity

The purity of each myosin preparation was checked by fractionating 100μg of protein on five per cent SDS polyacrylamide gels followed by a densitometric scan (Figure 3). Good preparations produced three major
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<td>Exhaustion</td>
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<td>1 hour Recovery</td>
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<td>11.9 ± 0.43</td>
<td>6.6 ± 0.49</td>
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<td>21.9 ± 1.2</td>
<td>11.9 ± 0.69</td>
<td>4.2 ± 0.50</td>
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protein bands, identified as heavy chain (HC), light chain one (LC₁) and light chain two (LC₂) which have been previously identified (9). Myosin preparations that possessed altered protein banding or showed evidence of possible protein degradation were not used in this study. Molecular weight determinations using aldolase (40,000) and lysozyme (14,000) indicated that LC₁ had a molecular weight of 27,000 while LC₂ was 18,500 which has been previously reported for cardiac myosin sub-units (53).

**Kinetics of (³H)Phe Incorporation**

To ensure the incorporation of phenylalanine into isolated perfused heart was increasing, and to select an appropriate perfusion time to pulse label heart tissue, the kinetics of this amino acid was first determined (Figure 4). Amino acid incorporation was increasing from 15 to 60 minutes of perfusion (Total tissue, myofibrillar, actomyosin, and myosin fractions). For the subsequent experiments, 30 minutes of perfusion was selected as a constant perfusion time since the rate of amino acid incorporation was increasing and produced appreciable amounts of radioactivity.

**Effects of Exhaustive Exercise on (³H)Phe Incorporation**

Exhaustive exercise reduced the rate of (³H)Phe
Figure 4: Kinetics of \(^{3}\text{H}\)Phe Incorporation
incorporation into all protein fractions. However, this decrease was only significant \((P<.05)\) for total tissue protein. Amino acid incorporation was reduced by 31 per cent in this fraction. In the contractile protein fraction myofibrillar, actomyosin and myosin was reduced by 33, 22 and 22 per cent respectively (Figure 5a).

**Effects of Recovery from Exhaustive Exercise on \(^{(3)}\text{H}\)Phe Incorporation**

During the recovery phase amino acid incorporation into total tissue protein returned to normal after two hours of recovery with no further change four hours post exercise (Figure 5a). For the contractile proteins a one-hour recovery resulted in a return to normal rates of incorporation. However, two hours after exhaustive exercise \(^{(3)}\text{H}\)Phe incorporation increased 57 per cent \((P<.05)\) in myofibrillar protein, 23 per cent in the actomyosin fraction and 16 per cent in the myosin protein extract (Figure 5a and 5b). Amino acid incorporation into myofibrillar protein remained 20 per cent above resting values after four hours of recuperation.

**\(^{(3)}\text{H}\)Phe Incorporation into Myosin Purified Electrophoretically**

For these experiments, myosin was fractionated electrophoretically into heavy and light chain components, removed from the gels by slicing and analyzed for radio-
Figure 5a: (3H)Phe Incorporation into Total Tissue and Myofibrillar Protein (R: Rest; E: Exhausted; 1: One Hour Recovery; 2: Two Hour Recovery; 4: Four Hour Recovery)
Figure 5b: \( ^3\text{H} \)Phe Incorporation into Actomyosin and Myosin Protein (R: Rest; E: Exhausted; 1: One Hour Recovery; 2: Two Hour Recovery; 4: Four Hour Recovery)
activity. The amino acid incorporation into total purified myosin was estimated from the sum of the radioactivity in the heavy and light chain fractions. At exhaustion, phenylalanine incorporation into myosin increased 50% per cent (P<.05) and after two hours of recovery was 84% per cent above resting rates. When the rate of incorporation was expressed per myosin sub-unit it was found that no significant change was seen in the synthesis of HC. However, amino acid incorporation into the light chains accounted for almost all of the increased radioactivity of total myosin (P>.05). Following four hours of recovery, the increased rate of phenylalanine incorporation was nearly normal (Figure 6).
Figure 6: (³H)Phe Incorporation into Total Myosin, Heavy Chain (HC) and Light Chains (LC₁ and LC₂)
CHAPTER IV

DISCUSSION

Compensatory growth of cardiac tissue can be stimulated by a number of methods (6, 7, 12, 13, 15, 21, 26, 30, 44, 53). In general, these involve overloading of the functional capacity of the heart. The most common stimulus for bilateral cardiac hypertrophy (right heart and left heart) is chronic strenuous exercise; a condition that creates demands on the right ventricle to perfuse the pulmonary capillary bed, as well as on the left ventricle to deliver blood to the peripheral tissues (15, 54). The present study was designed to establish a model for the future study of the biochemical correlates of exercised induced myocardial growth. A number of in vivo and in vitro techniques have been used to investigate control mechanisms involved in cardiac enlargement. In vivo methods, using various forms of hypertension (6, 7, 13, 21, 51) to induce hypertrophy have been criticized for poor control over factors responsible for tissue growth such as thyroxine (7, 16, 17, 49), growth hormone (1, 6, 7, 19, 24), insulin (37, 57), substrate supply (42), tissue oxygenation (27), amino acid supply (36), and mechanical activity of the
heart (29, 45). Alternatively, in vitro techniques, using increased afterloading of an isolated perfused heart have failed to show an increase in tissue mass but are adequate for studies on the initial biochemical response to acute pressure overload (1, 27, 36, 37, 42, 45). Hjalmarson (25) have shown that protein synthesis is stimulated during pressure overload in the isolated heart and remains elevated after normalization of aortic pressure. Since repeated bouts of exercise ultimately results in cardiac hypertrophy (15, 54) it may be possible to study, using the isolated perfused heart preparation, the initial phase of adaptive growth following a single bout of exercise. In this study, an isolated perfused heart was used because it permits rigid control of myocardial hemodynamic parameters while hormonal and neurotrophic factors are excluded. Furthermore, this approach allows the use of a defined medium that is delivered to the tissue via its own capillary bed.

Studies using swimming as a method to develop and study myocardial compensatory growth have usually standardized the exercise bout by controlling the length of the swimming period (15). In the present study, we have kept the length of the time to exhaustion relatively constant at 2 1/2 to 3 hours. In addition, the loss of the righting reflex when the animals were placed on their backs, was used as additional evidence for fatigue.
Using this exercise protocol, muscle (gastrocnemius), liver and heart glycogen stores were decreased by 87, 96 and 58 per cent respectively. These findings indicate that the animals in the present study were exhausted and that any change in the rate of amino acid incorporation was probably not due to differential levels of fatigue.

Following exhaustive exercise, amino acid incorporation into total tissue protein was significantly reduced. However, in the contractile protein fraction (myofibrillar, actomyosin and myosin), this decrease was not statistically significant. The possibility exists that a decrease in protein synthesis may reflect (a) a dilution of the intracellular amino acid pool, or (b) oxidation of available amino acids. Either of these processes could decrease amino acid incorporation into protein by reducing the available intracellular amino acids for t-RNA acylation. However, there are several lines of evidence that indicate this may not be the case in these experiments. Phenylalanine is especially convenient for the study of heart protein synthesis because it has been shown to rapidly reach a stable intracellular equilibration within 10 minutes of perfusion (14, 36, 47) and maintains a stable intracellular specific activity with time (36). Furthermore, phenylalanine is not converted to other amino acids in heart muscle (36) since phenylalanine hydroxylase is lacking in this tissue (55). While
phenylalanine is a very stable marker for protein synthesis, conditions of physiologic overload may dilute the specific activity of phenylalanine in some other way. Schrieber (47) has found no significant change in the rate of phenylalanine release in the isolated perfused heart with moderate pressure overload, indicating that intracellular phenylalanine is not diluted by amino acids arising from the degradative process. It is possible that exercise to exhaustion may produce tissue edema that could increase the intracellular water compartment with the resultant dilution of the intracellular amino acid pool. Recently, Hjalmarson (25) has shown that after two hours of increased aortic pressure, a change in the tissue water was associated with the extracellular space, while the intracellular compartment remained stable. Subsequently, he showed that amino acid transport during pressure overload was significantly increased (2).

Collectively, these experiments indicate that intracellular pools of phenylalanine would be maintained at normal levels following acute work overload. If this is the case following exercise, then the apparent decrease in amino acid incorporation must result from a change in protein synthesis.

The hypothesis that free amino acids are oxidized during exercise to supplement other energy sources in the heart has not been verified experimentally. Indeed,
severe metabolic interventions such as extreme forms of fasting or untreated diabetes are not associated with cardiac atrophy or acute congestive heart failure (28), as would be expected if amino acids were to act as substrates for oxidative metabolism. This has been attributed to elevated plasma free fatty acids and ketone bodies which are capable of maintaining and/or stimulating protein synthesis in isolated myocardial tissue (25). Following acute exhaustive exercise, plasma free fatty acids increase 1.8 fold while glycogen is only 50 percent depleted and endogenous triglyceride stores are only slightly decreased (43). This strongly suggests that blood borne substrates are sufficient to maintain the energy supply of the myocardium during exercise and that amino acid oxidation probably does not play an important role in cardiac ATP generation.

During recovery from exercise, amino acid incorporation into total tissue protein returned to normal by two hours with no further increase after four hours, indicating that amino acid incorporation was not stimulated in total tissue protein. This is in direct contrast to studies using work overload in isolated perfused hearts (25, 45), which clearly shows an increased rate of protein synthesis after three hours of hemodynamic work. If specific subcellular fractions are considered, there was a significant increase in phenylalanine incorporation into myofibrillar
proteins. This finding supports Schrieber (46), who found that the myofibrillar-nuclear fraction and specifically myosin appeared to be the protein preferentially synthesized in the acutely overloaded ventricle. In the present study, further fractionation of myosin into heavy and light chains revealed that, of the total amino acid incorporated into total myosin, most was located in the myosin light chains. Since the light chain proteins are essential for the hydrolysis of ATP it seems reasonable to speculate that any structural change in the myosin enzyme may be initiated with the first bout of exercise. This is quite interesting since Scheuer (9) has found that structural changes, as measured fluorophotometrically, and manifested by increased sulfhydryl group availability, seemed to underline the increased myosin ATPase activity with training. Unfortunately, these experiments were unable to discern whether the increased myosin ATPase activity was due to an increased amount of LC protein or an unmasking of available sites for ATP hydrolysis. Data from the present study suggest that in the heart this adaptive response occurs, at least partially, through a synthesis of LC components. This supports the theory that mechanical stimulation of muscle, whether due to cross-innervation (4) or long-term electrical stimulation (41) is paralleled by an increase in myosin ATPase activity and an increase
in myosin LC protein content. Thus, the present study indicates that one of the earliest responses to acute work overload is a synthesis of the polypeptides involved in the catalytic portion of the myosin molecule.

When total myosin was electrophoretically separated into purified fractions and the total radioactivity determined as the sum of the myosin heavy chain, myosin LC$_1$ and myosin LC$_2$ components the rate of phenylalanine incorporation increased at exhaustion and continued to increase during two hours of recovery. It is difficult to interpret these data and the results from iso-electrically prepared myosin. These results suggest that protein contamination may be masking the synthetic response in myosin isolated from the myofibrillar protein pellet.

This study may indicate the existence of two protein synthetic responses to physiologic overload in the heart. Phenylalanine incorporation into total tissue protein decreased in response to exhaustive exercise while in myosin light chains, a highly purified protein with specialized function, synthesis occurred at almost double the normal rate. At present, it is not yet clear the role a dichotomous response in protein synthesis would play in the adaptive process in cardiac muscle. The enhanced rate of amino acid incorporation into myosin light chains may be interpreted as reflecting a fundamental
mechanism of cellular adaption, through a phasic increase in the synthesis of one specific group of proteins. It may then be quite reasonable to suggest that with repeated bouts of exercise, the synthesis of more proteins become actively involved in the synthetic response until hypertrophy, at the histological level, is apparent.

In summary, these data indicate that exhaustive exercise induces an initial decrease followed by an increase in the incorporation of phenylalanine into myofibrillar fraction of the heart muscle with most of the incorporation localized in the myosin light chains. This suggests that one of the initial responses to training may be a synthesis of cardiac contractile proteins with the later induction of tissue hypertrophy. Although the present set of experiments provides no direct evidence for possible control mechanisms of increased or decreased protein synthesis in the heart in response to acute exercise, future work should provide this information.
References


5. Barnard, P. J. "Experimental Anoxic Cardiac Enlargement." Laboratory Investigation, 1958, 7, 81-90.


41. Pette, D., Muller, W., Leisner, E., and Vrbova, G. "Time Dependent Effects on Contractile Properties, Fibre Population, Myosin Light Chains and Enzymes of Energy Metabolism in Intermittently and Continuously Stimulated Fast Twitch Muscle of the


Perfusion Buffer

1. Krebs-Henseleit Bicarbonate Buffer

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<th>Molar Conc.</th>
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3. **Procedure for Buffer Preparation**

- Add 27g NaCl
- 11.6g Glucose
- .0891 Tyrosine
- 27 ml amino acid mixture
- Appropriate volume of salt sol'n gas with 95% O₂ & 5% CO₂
- Add 8.1459 gm NaHCO₃

Amino acid fortified Krebs-Henseleit bicarbonate buffer
APPENDIX B
Isolation and Extraction Procedure for
Myofibrils, Actomyosin and Myosin

1. **Buffers:**
   - Sol'n A  - 3 M Sucrose
     - 10mM imidazole
   - Sol'n B  - 60mM KCl
     - 30mM imidazole
     - 2mM MgCl₂
   - Sol'n C  - Sol'n B
   - Sol'n D  - Sol'n B
     - 1% tritonX-100
   - Sol'n E  - 0.6 M KCl
   - Sol'n F  - 0.47 M KCl
     - 0.02 M Na₄P₂O₇·H₂O
     - 0.01 M Na₂H₂PO₄·H₂O
     - pH 6.8

2. **Procedure:**
   - Pulse label hearts for 30 min.
   - Trim atria, fat, and connective tissue and weigh T.P.
   - Mince and homogenize heart in 4 vol. of Sol'n A, spin 17,300 20 min.
     - (4x) Suspend pellet in 4 vol. of Sol'n B, spin 750g 15 min.
     - suspend in Sol'n C, spin 750g 15 min.
     - (2x) suspend in Sol'n D, spin 750xg 15 min.
     - (4x) suspend in Sol'n 8 vol. Sol'n B
     - suspend pellet in 25 ml buffer E for 20 hr.
     - M.E.P.
     - spin 2000g 10 min.
     - Dilute sup. 10 fold with cold distilled water (wait 3 hr.)
     - Aspirate clear sup. and pellet cloudy precipitate 2000g 10 min.
suspend pellet in 10 ml of Sol'n F for 30 min.

A.M.P.

spin 13,000g for 30 min.

dilute sup. 10 fold let stand for 3 hr.

aspirate clear sup. and pellet cloudy precipitate spin 2000 10 min.

M.P.

prepare myosin pellet for electrophoretic fractionation
Total Myocardial Protein Preparation

Precipitate whole homogenate (2 ml) 1:1 with 10% TCA, spin 10 min., aspirate
↓
wash with 5% TAC (2 ml), spin 10 min., aspirate
↓
boil 15 min. in 5% TCA, spin 10 min., aspirate
↓
wash (2x) in 5% TCA, spin 10 min.
↓
precipitate (2x) chloroform: methanol: ether 2:1:1, 1:1:1 (vol:vol)
↓
wash (2x) in ether
↓
dissolve to 4 ml of 1N NaOH
↓
remove aliquotes for protein determination (50:1) and radioactive determination (100:1)
Protein Quantification

1. Lowry (32)

   a. Chemicals: 1) \(2\% \text{ Na}_2\text{CO}_3\) in \(.1\text{N NaOH}\)
                  2) \(1\% \text{ CuSO}_4 \cdot 5\text{H}_2\text{O}\)
                  3) \(2\% \text{ Na-K Tartrate (cold)}\)
                  4) Phenol folin reagent

   b. Standard: BSA \(1\text{mg/ml sol'n}\)

   c. Sol'n "C": prepare fresh daily in this order
                  1) \(.5\text{ml} 1\% \text{CuSO}_4\)
                  2) \(.5\text{ml} 2\% \text{ NaK Tartrate}\)
                  3) \(50\text{ml} 2\% \text{ Na}_2\text{CO}_3\) in NaOH

   d. Assay: 1) make sample vol. up to .4ml with \(.1\text{N NaOH}\)
                  2) add 2ml Sol'n "C"
                  3) add .2ml Phenol folin reagent
                  4) read O.D. at 750nm

   e. Standard Curve:

         \[\begin{array}{cc}
         \text{Vol.} & \text{Abs.} \\
         20 & .3 \\
         40 & .2 \\
         60 & .1
         \end{array}\]

2. Protein Binding Assay (10)

   a. Sol'n "A": 100 mg coomassie brilliant blue G-250
dissolve in 50ml 95% ethanol
add 100ml of 85% phosphoric acid
dilute to 1 liter and filter

   b. Assay: bring final volume to .1ml with
                  \(.15\text{ M NaCl}\)
add 5ml Sol'n "A"
mix & wait 2 min.
read O.D. at 595nm
Blank .1ml \(.15\text{ M NaCl}\)
c. Standard Curve:

O.D.

Protein g
Electrophoresis Stock and Procedures

1. **Stock Acrylamide Sol'n for 5% Gel System**
   
   11.1 gm acrylamide  
   .3 gm Bis  
   dilute to 100ml with double distilled water

2. **Phosphate Buffer System (Running Buffer)**
   
   38.6 gm Na₂HPO₄·7H₂O  
   7.7 gm NaH₂PO₄·H₂O  
   2 gm SDS  
   -dissolve to 1 litre

3. **Protein Solubilizing Sol'n (PS²)**
   
   .05ml B mercaptoethanol diluted to 50ml  
   running buffer diluted 1:1 with water  
   = 1% SDS  
   1% BME  
   .01M NaPO₄ buffer

4. **Preparation of Gels (casting)**
   
   a) mark all tubes 7.5 cms  
   b) take 15ml of stock PO₄ buffer and 13.5ml of stock acrylamide sol'n and mix by swirling  
   c) add .045ml TEMED - deairate  
   d) place on ice to slow polymerization  
   e) add 1.5ml of .075gm/5ml (freshly made) ammonium persulfate  
   f) add gel sol'n to 7.5 cm mark with pastur pipette  
   f) add 100 1-200 l of H₂O to flatten gel

5. **Preparation of Protein for Electrophoresis**
   
   a) suspend sample in .047 MKCl  
   b) repellet sample in small venous tube  
   c) add .5ml PS² and cap tube  
   d) incubate samples at 80°C for 20 min.  
   e) spin 2000g to remove insoluable material  
   f) quantify protein using dye binding assay  
   g) layer on 100g of protein
6. **Preparation of Protein Aliquote**
   a) mix in test tube: 3-4 crystals sucrose
   5 l 0.05% bromphenol blue dye
   5 l BME
   100g protein sample
   b) layer above sol'n on top of gel

7. **Staining Sol'n**
   1.25gm coomassie brilliant blue in 450ml of 50% methanol
   46 glacial acetic acid: filter through #1 whatman filter

8. **Destaining**
   7.5% acetic acid
   10% isopropal alcohol
### Statistical Analysis System

**General Linear Models Procedure**

Source variables: TP

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### Statistical Analysis System

**General Linear Models Procedure**

**Duncan's Multiple Range Test for Variable TP**

Means with the same letter are not significantly different.

**Alpha Level = .05**

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**General Linear Models Procedure**

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**Statistical Analysis System**

**General Linear Models Procedure**

**Duncan's Multiple Range Test for Variable HFP**

**Means with the same letter are not significantly different.**

**Alpha Level**: 0.05

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**General Linear Models Procedure**
**STATISTICAL ANALYSIS SYSTEM 9:33 MONDAY, OCTOBER 2, 1978**

**GENERAL LINEAR MODELS PROCEDURE**

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**STATISTICAL ANALYSIS SYSTEM 9:33 MONDAY, OCTOBER 2, 1978**

**GENERAL LINEAR MODELS PROCEDURE**

**DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE AMP**

**MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.**

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### Statistical Analysis System

**General Linear Models Procedure**

**Dependent Variable: Mass**

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<th>C.V.</th>
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<tbody>
<tr>
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<td>0.81433272</td>
<td>0.1161</td>
<td>0.2244</td>
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</tr>
<tr>
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**Statistical Analysis System**

**General Linear Models Procedure**

**Huncan's Multiple Range Test for Variable Mass**

**Means with the Same Letter are Not Significantly Different.**

**Alpha Level = .05**

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### Statistical Analysis System

#### General Linear Models Procedure

**Dependent Variable:** IIC

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<th>R-Square</th>
<th>C.V.</th>
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<tr>
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<td>17309.68447017</td>
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<td>0.6496</td>
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<td>27076.4958521</td>
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### Statistical Analysis System

#### General Linear Models Procedure

**Duncan's Multiple Range Test for Variable IIC**

**Means with the same letter are not significantly different.**

**Alpha Level:** 0.05

**df = 21**  **MS = 27470.44**

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<td>A''''</td>
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<tr>
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*Page 77*
**STATISTICAL ANALYSIS SYSTEM**

**GENERAL LINEAR MODELS PROCEDURE**

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<th>SOURCE</th>
<th>DF</th>
<th>SUM OF SQUARES</th>
<th>DF</th>
<th>MEAN SQUARE</th>
<th>F VALUE</th>
<th>PR &gt; F</th>
<th>R-SQUARE</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MODEL</td>
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<td>59,147.0620919</td>
<td>4</td>
<td>14,786.9228</td>
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**STATISTICAL ANALYSIS SYSTEM**

**GENERAL LINEAR MODELS PROCEDURE**

**DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE LCI**

Means with the same letter are not significantly different.

**ALPHA LEVEL=.05**

**DF=21**

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<td>C</td>
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<tr>
<td>D</td>
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<td>E</td>
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## Statistical Analysis System

**General Linear Models Procedure**

### Source Table 1

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<th>Mean Squares</th>
<th>F Value</th>
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<th>C.V.</th>
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### Statistical Analysis System

**General Linear Models Procedure**

Duncan's Multiple Range Test for Variable LC2

Means with the same letter are not significantly different.

**Alpha Level**: .05  
**DF**: 21  
**RS**: 0.00241

### Grouping Table

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## General Linear Models Procedure

### Statistical Analysis System

### General Linear Models Procedure

#### DEPENDENT VARIABLE: HP (TOTAL)

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<th>MEAN SQUARE</th>
<th>F VALUE</th>
<th>PH &gt; F</th>
<th>R-SQUARE</th>
<th>C.V.</th>
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<tr>
<td>MODEL</td>
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#### SOURCE

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<th>F VALUE</th>
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<th>F VALUE</th>
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### Duncan's Multiple Range Test for Variable HP

Means with the same letter are not significantly different.

**Alpha level**: 0.05

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<th>TYPES</th>
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<td>881.11/426</td>
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### Additional Notes

- The analysis was conducted on **Thursday, October 26, 1978**.
- The system used is the Statistical Analysis System (SAS).
- The report includes a general linear models procedure with model and error details.
- Duncan's multiple range test is applied to variable HP with an alpha level of 0.05.
- Means with the same letter are not significantly different.
## Individual Data (DMP/mg protein)

### Resting

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<th>AMP</th>
<th>MP</th>
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<td>7994.1</td>
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<td>7495.5</td>
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<td>6575.8</td>
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### Exercise Exhausted

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### 1 Hour Recovery

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<td>3931.6</td>
<td>3466.9</td>
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<td>4494.9</td>
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<td>6616.3</td>
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### 4 Hour Recovery

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### Myosin Purified from SDS Page

#### Resting

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#### Exhaustion

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Vita Auctoris

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August 27, 1953

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