Fernando R. Puente. SOME EFFECTS OF ENDURANCE TRAINING ON PROTEIN METABOLISM. (Under the direction of Dr. G. Lynis Dohm) Department of Biology, August, 1977.

This study was conducted to investigate the influence of endurance training on several aspects of protein metabolism (amino acid transport, protein synthesis, tissue protein levels, and nitrogen balance). After a six week training period, a significant decrease was found in amino acid transport into heart tissue of trained rats. The extracellular space of the different tissues was analyzed and an increase in the extracellular space was found in the gastrocnemius muscle of trained rats. In vivo incorporation of [^{14}C]-leucine into tissue proteins showed significantly lower values for heart, liver and stromal fraction of gastrocnemius muscle from trained rats. Similarly, lower protein levels were found for sarcoplasmic and myofibrillar fractions of trained muscle. Training also resulted in a decrease in nitrogen absorption and a concurrent increase in nitrogen excretion. These observations demonstrate that endurance exercise training results in a shift to a more protein catabolic state; i.e., protein synthesis is depressed, nitrogen excretion is increased, and muscle protein levels are lowered.

SOME EFFECTS OF ENDURANCE TRAINING ON PROTEIN METABOLISM

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INTRODUCTION

Heavy work induces hypertrophy of skeletal muscle (1) and this phenomenon is also accompanied by various changes in cellular mechanisms such as an increase in amino acid transport (1) and protein synthesis (2). Endurance training also appears to induce a number of physiological and biochemical alterations in skeletal muscle and other organs, ranging from a reduction in food consumption and body weight (3) to increases in amino acid oxidation in skeletal muscle and urea excretion (4). Although previous studies suggest that endurance training may alter protein metabolism, there is a general lack of information concerning the changes that occur and the mechanisms involved. As a result, this study was undertaken to elucidate changes in amino acid transport, protein synthesis, tissue protein levels and nitrogen balance that occur in response to endurance training.

Amino acid transport and protein synthesis: The importance of protein metabolism in muscle can be appreciated when one considers the fact that muscle constitutes 45% of the total body weight (4) and that 80% of the dry weight of muscle is protein (5). In addition, the protein composition of all tissues is not static but is rapidly turning over; i.e., being degraded and resynthesized. This dynamic process has been known for years (6). In order for muscle to maintain this homeostatic condition (or to hypertrophy if growth is taking place), it is essential for its cells to be able to absorb amino acids. Amino acids are actively transported across cell membranes by a process that requires ATP expenditure (7). This mechanism appears to be coupled with Na^+ transport;

the greater the external concentration of the ion, the higher the capacity of the transport system of amino acids (7). Specific transport systems have been found depending on the relative charge of the amino acid (7). The neutral amino acid system being the largest in number, is subdivided into two systems; the A or alanine-preferring system which favors the smaller and more polar amino acids and the L or leucine-preferring system which favors larger and more hydrophobic amino acids (8).

For our measurement of amino acid transport, we will use [^{14}C]-α-aminoisobutyric acid (AIB), a non metabolized amino acid analogue. Christensen et al. (9) showed that AIB, even though it is not metabolized, is transported into tissue cells by the same mechanisms which transport natural amino acids. Thus, this particular amino acid analogue is a model for studying the ability of the cells to take up and utilize natural amino acids.

Goldberg (10) showed that muscle tissue cells absorb amino acids at different rates depending on the type of muscle fiber. Red muscle fibers absorbed more [^{14}C]-AIB and [^{14}C]-leucine than pale muscle fibers, while muscle with both types of fibers absorbed an intermediate amount of the respective amino acids. Goldberg (10) also showed that the RNA content of the red skeletal muscle is higher than white muscle and the rate of protein catabolism per mg of muscle is higher in red fibers. Brown (unpublished data) showed that red quadriceps muscle in rat had a higher rate of amino acid oxidation than white quadriceps muscle. These differences in protein metabolism in skeletal muscle show that muscle tissue is not homogeneous in protein metabolism. These differences should be kept in mind when doing related studies.

Once the amino acids are in the precursor pool of free amino acids they can become incorporated into the protein of the tissue. There is much to be learned about this mechanism since there are still many questions open for speculation in regard to whether intracellular or extracellular amino acids are the source of free amino acids for protein synthesis. Martin et al. (11) have shown that the extracellular amino acid pools serve as precursors for protein synthesis during the early stages after injection of a labeled amino acid. Hider et al. (12) have demonstrated that the labeled amino acid incorporated into protein may come from the extracellular pool without equilibrating with the total intracellular amino acid pool. Jeanne et al. (13) believe that the intracellular pool of free amino acids is the source for protein synthesis. There has also been evidence (14,15,16) that both pools (extracellular and intracellular) serve as the precursor sources for protein synthesis.

Studies investigating protein metabolism in work-induced hypertrophy have shown a significant increase in protein synthesis (2). Goldberg (2) found that tenotomy of the gastrocnemius resulted in extra weight to be moved by the synergyst plantaris and soleus muscles. Due to this increased demand on strength they increased their mass and thus their protein synthesis to adjust to the increase in power requirement. Zimmer et al. (17) reported that protein synthesis in rat heart and muscle was lowered by exercise. This decrease in protein synthesis occurred during and immediately after exercise but it rapidly increased back to normal levels 2-4 hours after exercise. Thus it seems evident that protein synthesis is altered by exercise and the increase in power

requirement. This study will investigate the effects, if any, of endurance training on protein synthesis.

Protein catabolism: There has been much controversy about whether protein catabolism is increased by exercise. It is well established that carbohydrate and fat are the main sources of fuel during exercise, but the fact that endurance exercise induces so many alterations in protein metabolism, including catabolism, has stimulated further investigation to demonstrate the changes in protein metabolism during exercise. Older literature tended to discount protein as fuel for muscular work when caloric supply was adequate (18). They sited observations in which there were no changes in total nitrogen excretion even after exhaustive work. More recent studies have demonstrated the opposite. It has been reported (19) that exercise does increase protein catabolism and that urinary nitrogen excretion is significantly increased as a result of exercise in men who were under adequate caloric intake. It was also demonstrated (20) that exercise decreases nitrogen balance in individuals on a diet which provided adequate caloric supply. This information provided additional support to the hypothesis that exercise increases protein catabolism.

The liver has been known to be the major site of amino acid oxidation in mammals (21). More recent data (22-25) have demonstrated that amino acids may be oxidized in skeletal muscle. Dohm et al. (26) showed that even though heart and liver oxidize amino acids at a rate 40-50 times faster than skeletal muscle, it plays a significant role in total amino acid oxidation when it is considered that muscle constitutes 45% of the total body weight.

Endurance training induces changes in amino acid catabolism; Dohm et al. (4) showed that in gastrocnemius muscle homogenates, trained rats had a significantly greater leucine oxidation rate than untrained rats. Additional data that strongly substantiates the hypothesis of an increase in protein catabolism was given by Dohm et al. (4) who demonstrated that urea excretion was significantly increased in trained rats. Haralambie et al. (27) found that in exercising men, there was a decrease in serum α-amino nitrogen, a rise in urea and an increase in serum tyrosine levels. These changes occurred at a point at which liver glycogen and muscle glycogen are substantially lowered if not exhausted. Both the increase in urea and tyrosine serum levels strongly suggest the possibilities of protein utilization and protein degradation as a result of exercise.

Because of the need for further information concerning protein metabolism and its importance as fuel during exercise, the present study was undertaken. The aims of this research were to investigate the effect of endurance training on: 1) amino acid transport, 2) protein synthesis, 3) tissue protein levels, and 4) nitrogen balance.

MATERIALS AND METHODS

Radioactive materials: The following radioisotopes were obtained from Amersham/Searle Company, Arlington Heights, Ill.: [^{14}C]-α-aminoisobutyric acid, 250 μCi, 60 mCi/mMole; [^{3}H] inulin, 1 mCi, 750 mCi/mMole, (Hydroxy [^{14}C]-methyl) inulin, 100 μCi, 18.2 mCi/mMole. Leucine [Carboxyl ^{14}C], 57 mC/mMole was purchased from Schwarz Mann, Orangeburg, New York. Each of these isotopes was dissolved in physiological saline, pH 7.4, at a concentration of 100 μCi/ml, with the exception of [^{3}H] inulin whose concentration was 40 μCi/ml.

Corrections for quench: All radioactive samples show some degree of quench which causes a decrease in the counts obtained from the sample under investigation. To solve this problem, a quench calibration was made using the least quenched sample (muscle) and the most quenched sample (blood). The isotopes used for the quench curves were standard solutions of [^{14}C] toluene (8.12×10^{5} dpm/g) and [^{3}H] toluene (1.295×10^{6} dpm/g) purchased from Amersham/Searle Company, Arlington Heights, Ill. A constant number of counts from the standard radioactive solutions was added to a series of counting vials. Varying volumes of solubilized tissue extracts were also added to these vials. A constant total volume was maintained by decreasing the amount of scintillating liquid in accordance to each increment in tissue volume. The result was that the vials with the least amount of tissue sample had the most counts and the highest S-number (external standard-channels ratio) while the vials with the most amount of solubilized tissue had the lowest counts and S-number and hence were the most quenched samples. From these values, a quench

curve was plotted by graphing percent efficiency versus the S-number (Figure 3). To determine percent efficiency, the following formula was used:

$$\% \text{ efficiency} = \frac{\text{standard cpm-background x 100}}{\text{standard dpm}}$$

Background was determined by counting the same type of samples being analyzed in scintillation liquid without any radioisotopes. The scintillation cocktail used was 6 g 2,5-Diphenyloxazole (PPO)/1 toluene and all samples were counted in a Beckman LS-233 Liquid Scintillation Counter. Tissues were solubilized with NCS (Nuclear Chicago Solubilizer), Amersham/Searle, Arlington Heights, Ill.

Methods for determination of extracellular space: Inulin was used to establish the amount of extracellular space in tissues. This compound is too large to enter the tissue cells and thus it only occupies the extracellular portion of the tissue under analysis. In the preliminary work, rats were injected with [^{3}H] inulin (4 μCi/100 g body weight) intraperitoneally, and were sacrificed at different time periods after the radioisotope injection in order to find the time period at which the inulin content in the tissue was maximum. The time periods were: 10, 30 and 45 minutes after the injection. In the actual experiment, the animals were injected by way of the tail veins with (Hydroxy [^{14}C] methyl) inulin, (1 μCi/100 g body weight). The animals were sacrificed 30 minutes after the prescribed dose of isotope and the tissues were removed.

To determine the extracellular space in each tissue, the following formula was used:

$$\text{Extracellular space} = \frac{\text{dpm/g of wet weight of tissue}}{\text{dpm/ml of plasma}}$$

Muscle water was measured as the difference between wet and dry weights of the tissues. The latter was obtained by heating tissues in tared glass counting vials at 100° centigrade until the dry weights were constant.

Methods for determining AIB uptake: [^{14}C]-α-aminoisobutyric acid (AIB) is an amino acid analog that is not metabolized (9). In the preliminary studies, AIB was injected (1.0 μCi/100 g body weight) intraperitoneally in a group of rats which were sacrificed at different time periods after the injection. The time periods were 30, 45, 60 and 90 minutes after the [^{14}C] AIB injection. In the actual experiment, unanesthesized, restrained rats were injected with the labeled amino acid via tail veins (1.0 μCi/100 g body weight) and the animals were sacrificed 60 minutes after the AIB injection. In order to determine the net AIB uptake by the intracellular space of each tissue, it was necessary to use the average extracellular space value obtained from the tissues analyzed. The following formula was used:

$$\text{Net AIB uptake} = \frac{\text{dpm AIB}}{\text{gram tissue}} - \text{extracellular space (ml/g)} \times \frac{\text{dpm AIB}}{\text{ml plasma}}$$

The result is expressed in dpm AIB/g dry weight.

The counts (cpm) obtained from each tissue were corrected to dpm by correcting for quench and background by the following formula:

$$\frac{\text{cpm-background}}{\text{\% efficiency}} = \text{dpm}$$

Animals used: Male rats from the Holtzman Company (Madison, Wisconsin) were utilized for the studies. The animals weighed approximately 200 grams at the start of training; they were housed one per cage and received at diet of Wayne Lab Blox. Half of the rats were randomly chosen as runners and were trained for a period of six weeks as outlined in Table 1. During the sixth and final week of training, the animals were running 1 hr/day, 6 days/week, at 35 m/min, 8% grade. This group of animals was designated as trained. Since it has been found that trained rats consume about 10% less food than the untrained ad libitum fed rats, it was necessary in some studies to set up an additional group of animals. This untrained group, designated pair fed, was fed to the food consumption of the trained rats.

Surgical procedures: The animals were injected with their corresponding radioisotopes intraperitoneally or by way of the tail veins. Ten minutes before the sacrifice, the animals were anesthesized with sodium pentabarbitol (5 mg/100 g body weight) injected intraperitoneally. An incision was made along the linea alba, approximately 5 ml of blood were drawn from the inferior vena cava using a heparinized plastic syringe and a 20 gauge needle. The heart, liver, red and white quadriceps and gastrocnemius were removed, quickly rinsed in 1.15% KCl and blotted. The tissues were pressed through a tissue press (Harvard Inst. Co., Mills, Mass.). The blood drawn from each animal was placed in heparinized plastic centrifuge tubes. To separate the plasma, the blood in

Table 1. The Speed, Time and Grade for the Treadmill Training Regimens

Week*	Time (min)	Grade (%)	Treadmill Speed (m/min) Trained
1	30	0	20
2	40	0	30
3	50	0	35
4	50	8	35
5	60	8	35
6	60	8	35

* The speed and time are given for the last day of the week. Rats ran 6 days per week.

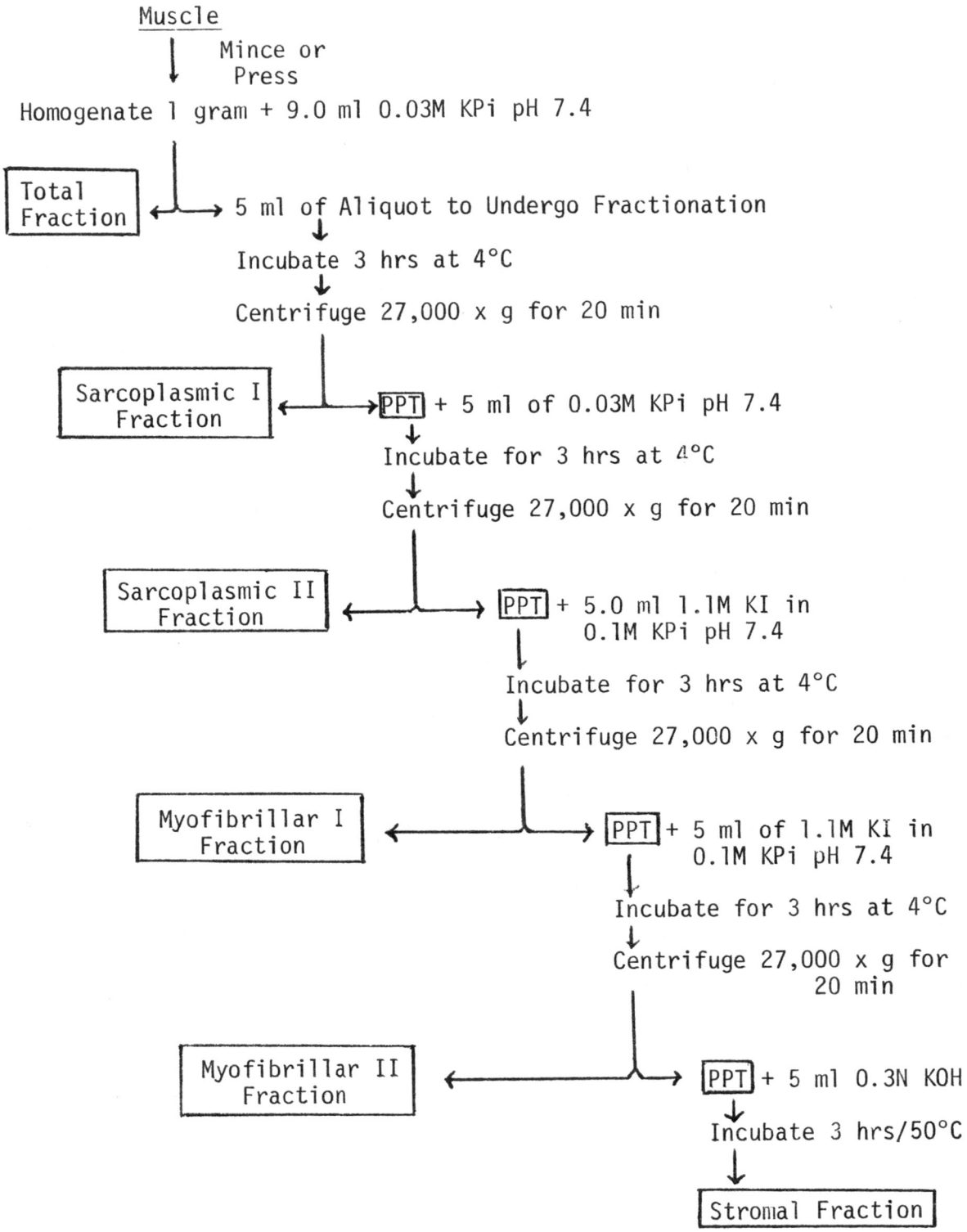
the tubes was allowed to sit in ice for two hours and then it was centrifuged at 1000 x g for 30 min using an International centrifuge, model UV.

Preparation of tissues: Nuclear Chicago Solubilizer (NCS) Amersham/ Searle, Arlington Heights, Ill. was used to solubilize the samples. Blood was solubilized by mixing 0.05 ml of blood and 0.8 ml of NCS in a glass counting vial, shaken vigorously and incubated in a water bath (American Optical Corp. Scientific Products) for 20 minutes at 50°C. After allowing the samples to cool to room temperature, 0.20 ml of 30% hydrogen peroxide were slowly added to each vial and then heated for an additional 30 minutes at 50 degrees. For plasma, a 0.1 ml aliquot was added to 1.0 ml of NCS in a glass counting vial and was allowed to sit for 30 minutes at room temperature. For the muscle tissues, 0.1 g of the pressed tissue was mixed with 1.0 ml of NCS in a counting vial; for the liver and heart samples, 0.06 g of the pressed tissue was mixed with 1.0 ml of NCS in a glass counting vial. All of these solid samples were then incubated in a water bath at 50°C until the sample was dissolved in its entirety by NCS. After complete solubilization, all of the vials were allowed to cool to room temperature and 10 ml of the scintillating cocktail (6 g PPO/1 toluene) were added to each vial. The vials were left in the dark until constant counts were obtained from them and then were counted. The samples were counted in a Beckman LS-233 liquid scintillation counter using the wide window (^{3}H+^{14}C).

Methods for measuring protein synthesis: Unanesthesized restrained rats were injected with leucine [^{14}C Carboxyl] via the tail vein (10 μCi/100

g body weight). The animals were sacrificed 60 minutes after the isotope injection. Ten minutes prior to sacrificed, the rats were anesthesized with sodium pentabarbitol (5 mg/100 g body weight), injected intraperitoneally. After opening the animals on the ventral side, about five ml of blood were drawn from the inferior vena cava using a heparinized plastic syringe and a 20 gauge needle. To separate the plasma, the blood collected was placed in heparinized plastic centrifuge tubes and centrifuged at 1000 x g for 30 min. The heart, liver, quadriceps and gastrocnemius muscles were removed from each animal and were rinsed in 1.15% KCl solution. Tissues were pressed in a tissue press (Harvard Inst. Co., Mills, Mass.). Each tissue was homogenized 1:9 (weight:volume) in .03 M KH_2 PO_4 (pH 7.4) in a glass-teflon Potter-Elvehjem homogenizer. For the gastrocnemius muscle homogenates, the sarcoplasmic, myofibrillar and stromal protein fractions were separated following a modified method described by Helander (28), (Figure 1). Proteins from all the tissue homogenates, including the fractions from the gastrocnemius muscles, were precipitated with 10% Trichloroacetic acid (TCA). Two ml of each homogenate were added to 8 ml of 10% TCA; for plasma, one ml of plasma and 9 ml of 10% TCA. All samples were centrifuged at 1000 x g for 15 min. The initial supernatant of the total fractions designated the soluble fraction was saved for analysis, and it was used to determine the specific radioactivity for each tissue. The precipitated pellets were resuspended with 10% TCA and centrifuged. This wash procedure was repeated and a final wash was done with a saturated solution of sodium acetate in 95% ethanol to get rid of any remaining TCA. The final pellet was dissolved in 4 ml of

Figure 1. Method used for separation of muscle protein fractions

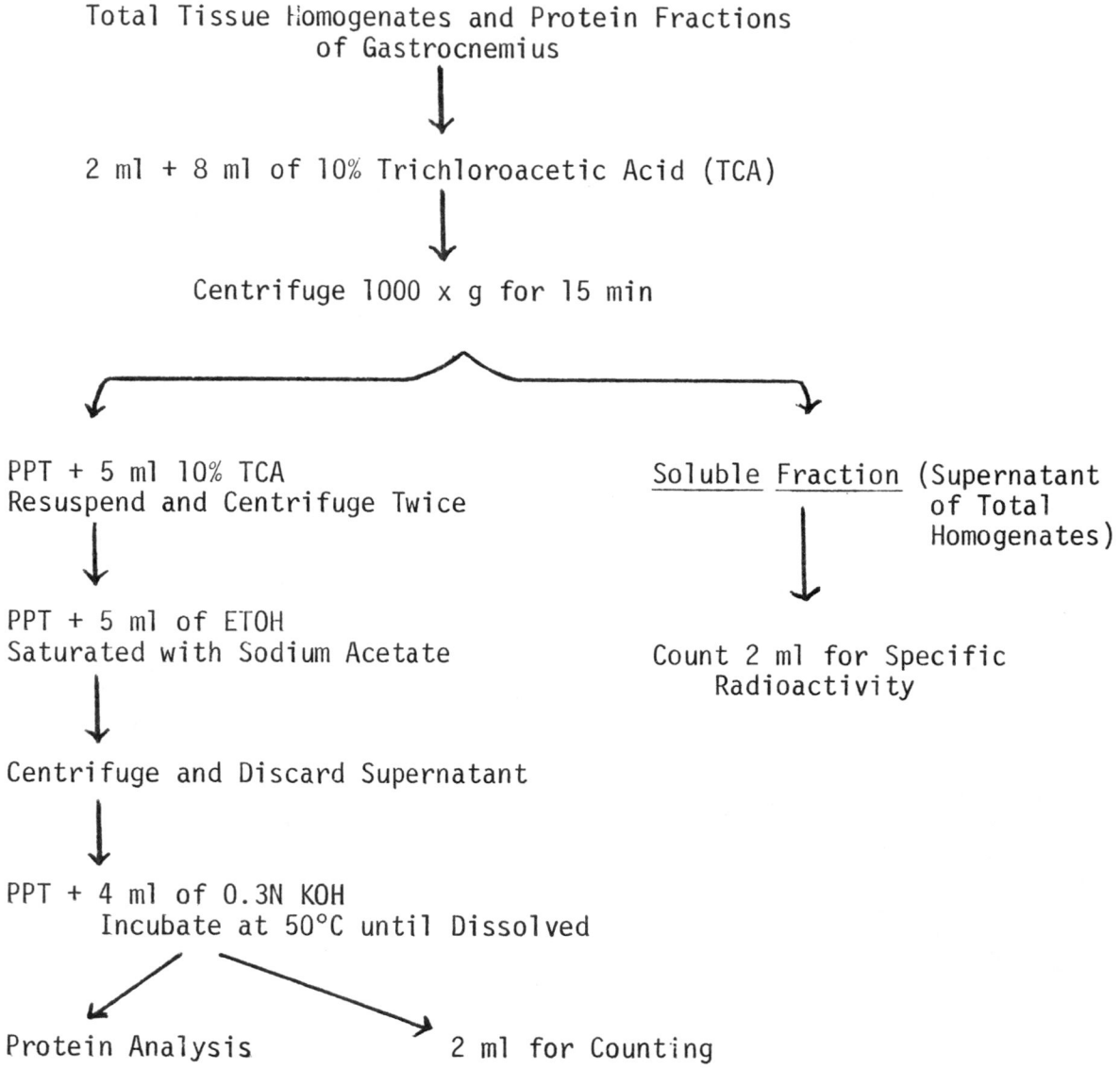

Muscle
Mince or Press
Homogenate 1 gram + 9.0 ml 0.03M KPi pH 7.4
Total Fraction
5 ml of Aliquot to Undergo Fractionation
Incubate 3 hrs at 4°C
Centrifuge 27,000 x g for 20 min
Sarcoplasmic I Fraction
PPT + 5 ml of 0.03M KPi pH 7.4
Incubate for 3 hrs at 4°C
Centrifuge 27,000 x g for 20 min
Sarcoplasmic II Fraction
PPT + 5.0 ml 1.1M KI in 0.1M KPi pH 7.4
Incubate for 3 hrs at 4°C
Centrifuge 27,000 x g for 20 min
Myofibrillar I Fraction
PPT + 5 ml of 1.1M KI in 0.1M KPi pH 7.4
Incubate for 3 hrs at 4°C
Centrifuge 27,000 x g for 20 min
Myofibrillar II Fraction
PPT + 5 ml 0.3N KOH
Incubate 3 hrs/50°C
Stromal Fraction

0.3 N KOH by incubating it in a water bath at 50°C until dissolved (Figure 2). From the final 0.3 N KOH aliquots, 2.0 ml of each were counted in glass counting vials with 8.0 ml of counting solution (Toluene, Triton and PPO) and 2.0 ml of Nuclear Chicago Solubilizer. All samples were counted in a Beckman LS-233 liquid scintillation counter using the wide window (^{3}H+^{14}C). After correcting for quench, the results were expressed as dpms/ml. Using the same KOH aliquots, protein analyses were done using the Lowry method (29) and the results were expressed as mg/ml. To determine the amount of leucine incorporation into protein, the following calculation was performed:

$$\frac{\text{dpms/ml}}{\text{mg/ml}} = \text{dpm/mg of protein}$$

Nitrogen determination: Food, feces and urine were analyzed for total nitrogen content by the method of Beecher and Whitten (31). A 0.05 g (or .05 ml) sample of food, feces, or urine was placed in kjeldhal flasks with 2 ml of concentrated sulfuric acid. The flasks were then placed in a kjeldhal heater (Lab. Con Co.) and were heated slowly for a period of twenty minutes and then they were heated at maximum temperature for one hour. If, after one hour the solution was not colorless, which was the case for the food and feces samples, 2 ml of 30% hydrogen peroxide were added slowly to each kjeldhal flask. These flasks were then heated for an additional 40 minutes at low temperature. Each final aliquot was quantitatively transferred to a 100 ml volumetric flask with 50 ml of deionized water. Five ml of 13 N NaOH were added to each volumetric flask to raise the pH to 12.0, and the solutions were brought

Figure 2. Method used to precipitate tissue proteins with Trichloroacetic acid


Total Tissue Homogenates and Protein Fractions of Gastrocnemius
2 ml + 8 ml of 10% Trichloroacetic Acid (TCA)
Centrifuge 1000 x g for 15 min
PPT + 5 ml 10% TCA
Resuspend and Centrifuge Twice
PPT + 5 ml of ETOH
Saturated with Sodium Acetate
Centrifuge and Discard Supernatant
PPT + 4 ml of 0.3N KOH
Incubate at 50°C until Dissolved
Protein Analysis
2 ml for Counting
Soluble Fraction (Supernatant of Total Homogenates)
Count 2 ml for Specific Radioactivity

up to volume with distilled water. A 0.2 ml aliquot of each diluted sample was mixed with 0.8 ml of deionized water, 5 ml of reagent A (5 g phenol, 25 mg of sodium nitroprusside/500 ml of water) and 5 ml of reagent B (2.5 g of NaOH, 4.2 ml of sodium hypochlorite/500 ml of water) and mixed well. The mixture was then heated at 37°C for 15 minutes and allowed to cool to room temperature for 10 minutes. The samples were then read at 630 nm in a spectrophotometer (Gilford 2000). The amount of nitrogen in each sample was determined by comparing the values to a standard curve of ammonium sulfate.

Methods for determination of nitrogen balance: In the nitrogen balance study, trained and untrained rats were placed in plastic metabolism cages (Maryland Products) during the sixth week of training. Urine was collected every twelve hours for a period of twenty-four hours, centrifuged at 100 rpm for 15 min (International Centrifuge model UV) to remove any food particles that may have gotten in the urine. The supernatant volumes were recorded and refrigerated to prevent growth of bacteria. Feces were also collected during the last week of training for a 24 hr period. Special pads were placed directly beneath the rat cages to collect the fecal pellets. The feces samples were then dried by heating them at 100°C for 48 hrs and the dry weights were recorded. Food consumption was measured for a three day period during the sixth week of training. The amount of nitrogen absorbed was determined by the total grams of nitrogen uptake/24 hrs - total grams of nitrogen in feces/24 hrs. These values were then divided by the metabolic body size to get N absorbed (g/24 hrs/metabolic body size). Nitrogen balance was obtained

by subtracting the total amount of nitrogen in urine/24 hrs from the amount of nitrogen absorbed in grams/24 hrs.

$$\text{Nitrogen balance} = \frac{\text{g of nitrogen absorbed-g of nitrogen excreted}}{\text{Metabolic body size}}$$

Determination of protein levels: Animals were decapitated and blood samples were collected from the neck. The gastrocnemius muscle, heart, and a lobe of liver were removed and rinsed in cold 1.15% KCl. Liver and heart were minced while the gastrocnemius was pressed through a tissue press (Harvard Inst. Co., Mills, Mass.). Each tissue was homogenized 1:9 (weight:volume) in 0.03 M KH_2 PO_4 (pH 7.4) in a glass-teflon Potter-Elvehjem homogenizer. For the heart and gastrocnemius, total homogenate, sarcoplasmic myofibrillar and stromal protein fractions were extracted following a modified method described by Helander (28). Five ml aliquots of each homogenate were placed in a shaking water bath at 4°C for three hours. The samples were then centrifuged at 27,000 x g for 20 min, and the supernatant was saved. The pellet was extracted again as above for two hours. The precipitate was centrifuged, and the combined supernatants were the sarcoplasmic fraction. The precipitate was extracted twice with five ml of 1.1 M KI plus 0.1 M KH_2 PO_4 (pH 7.4) for 3 and 2 hours respectively in a shaking water bath at 4°C. The supernatants were the myofibrillar fraction. The final precipitate was suspended in five ml of 1.1 M KI plus 0.1 M KH_2 PO_4 (pH 7.4) and extracted in a shaking water bath at 4°C for 3 hours. Each sample was centrifuged at 27,000 x g for 20 min, and the pellet was suspended in 5 ml of 0.3 N KOH and incubated in a water bath at 50°C until dissolved. This was designated as the stromal fraction.

The protein content of each fraction was measured by the biuret method (30).

Statistics: The data was subjected to one-way analysis of variance, and group comparisons were made by the Neuman-Keuls Test.

RESULTS

Quench curve: Figure 3 shows a graph of the quench curve obtained by plotting the percent efficiency versus the external standard channels ratio (S-number). This standard curve was a means for correcting raw counts to the unquenched counts in the specimen under analysis. Using the wide window (^{3}H + ^{14}C), the background counts averaged 57 cpm.

Extracellular space determination: [^{3}H] inulin was used during the preliminary studies to establish the correct parameters of inulin accumulation in the tissues. Nine rats were used ranging in weight from 430 g - 490 g. These animals were injected with the radioisotope intraperitoneally and were sacrificed at three different time periods: 10, 30 and 45 minutes after the injection. The tissues were solubilized and radioactivity was measured in a Beckman LS-233 liquid scintillation counter. It was found that with the exception of liver, the samples analyzed (including blood and plasma) showed a maximum concentration of inulin at 30 minutes. Figures 4, 5, 6 show the graphs of inulin accumulation in the samples (expressed as dpm ^{3}H inulin/gram wet weight) versus the three time periods. For blood and plasma, these values were expressed as dpm ^{3}H inulin/ml. These results concur with similar work done by Goldberg and Goodman (1).

Using the corresponding values for plasma and tissues, the extracellular space was calculated and expressed in ml/gram wet weight. This was done for each of the three time periods as shown on Table 2.

In the actual experiment (Hydroxy [^{14}C] Methyl) inulin was used for the extracellular space determination and it was injected via the tail

Figure 3. Quench curves for ^{14}C (o) and ^{3}H (□) counted with a wide window (^{3}H+^{14}C)

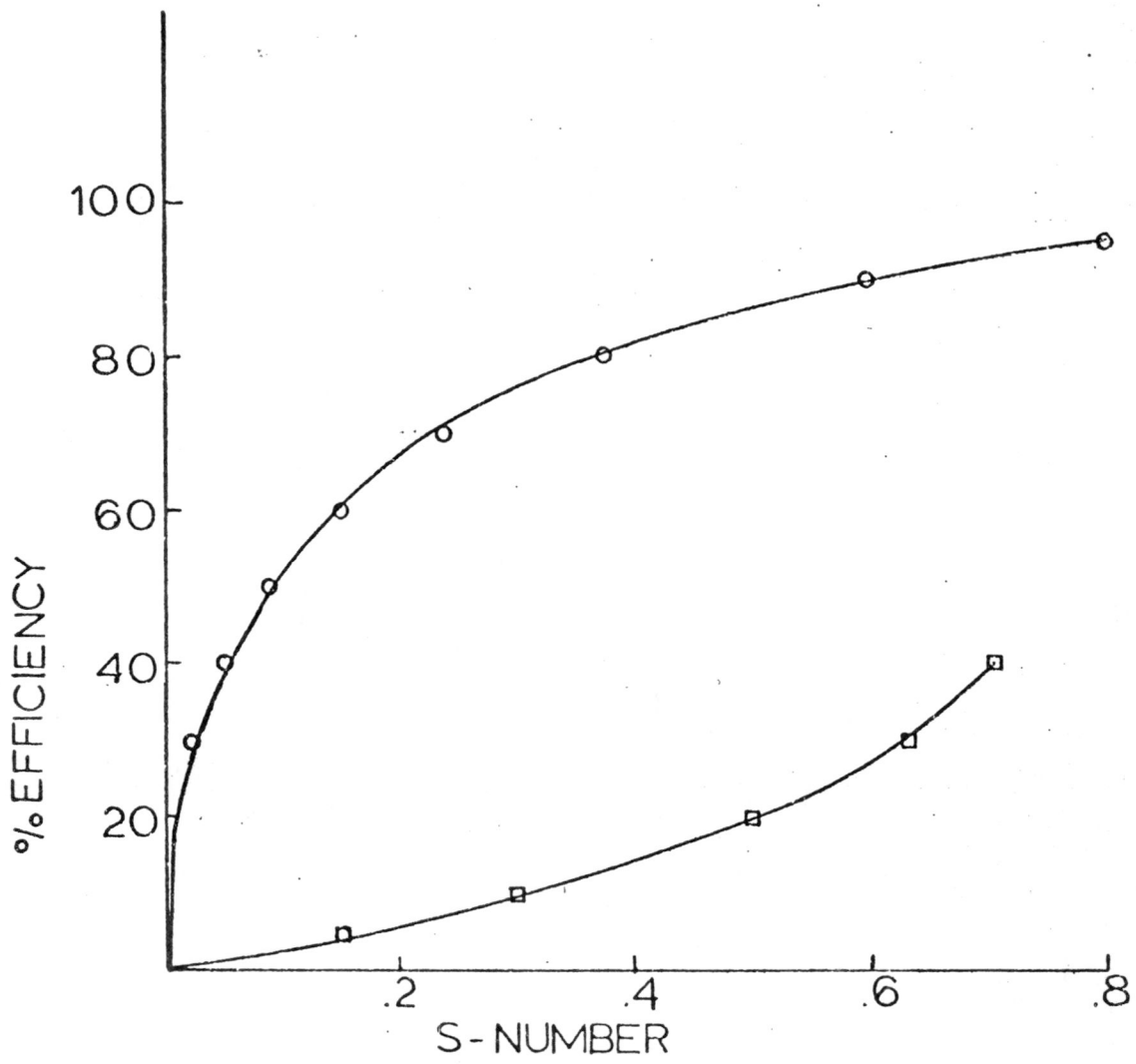

100
80
60
40
20
% EFFICIENCY
.2
.4
.6
.8
S - NUMBER

Figure 4. Concentration of ^{3}H Inulin versus time after injection in white quadriceps (Δ), red quadriceps (o), and gastrocnemius (●).

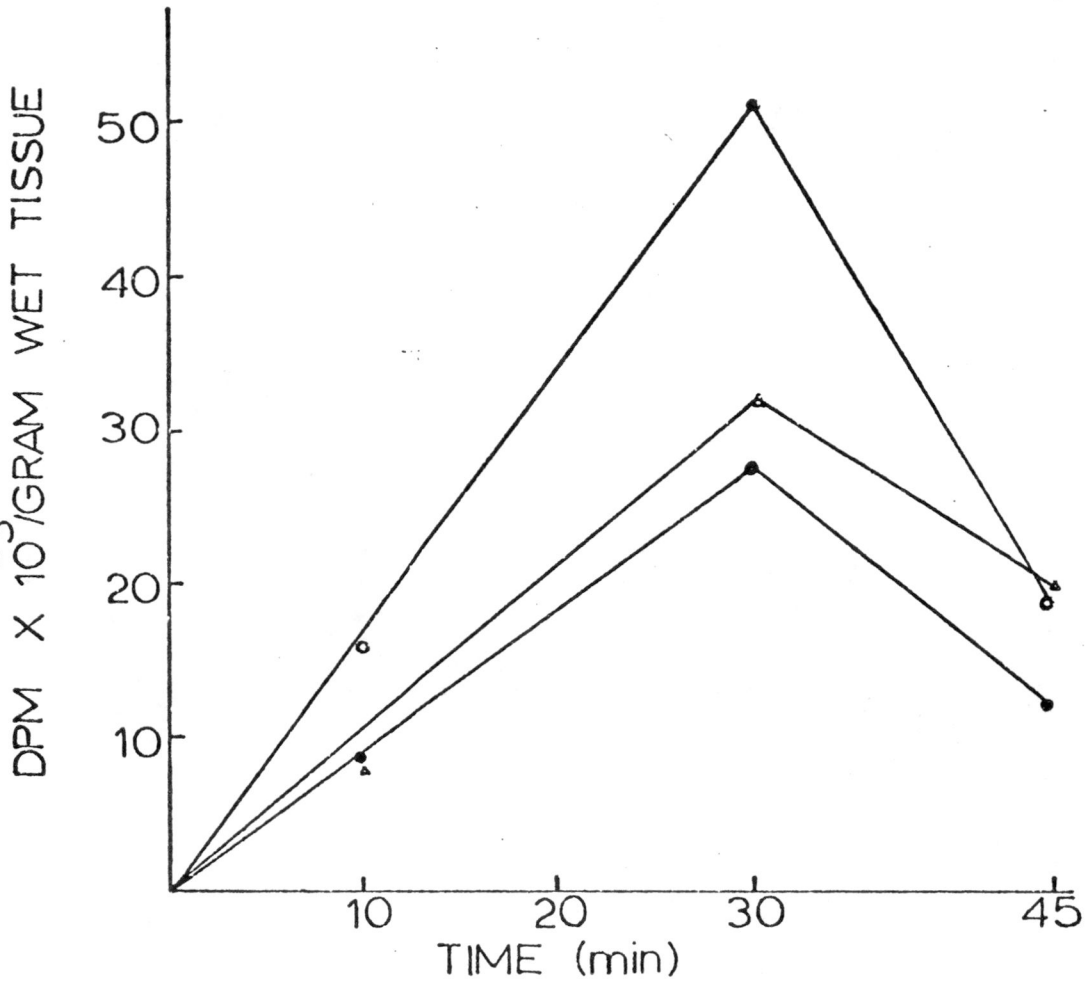

DPM X 10^{-3}/GRAM WET TISSUE
50
40
30
20
10
10
20
30
45
TIME (min)

Figure 5. Concentration of ^{3}H Inulin versus time after injection in liver (●) and heart (o)

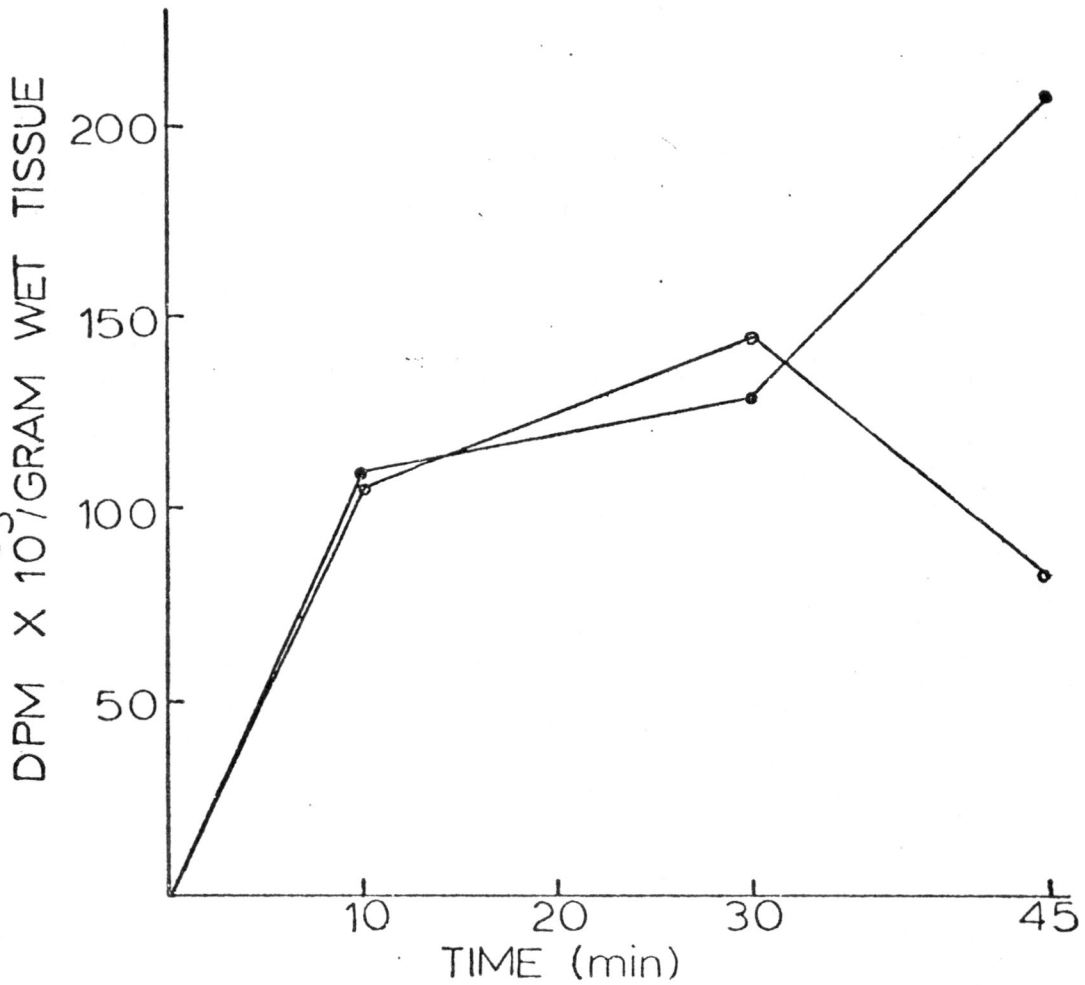

DPM X 10^{-3}/GRAM WET TISSUE
200
150
100
50
10
20
30
45
TIME (min)

Figure 6. Concentration of ^{3}H Inulin versus time after injection in plasma (o) and blood (●)

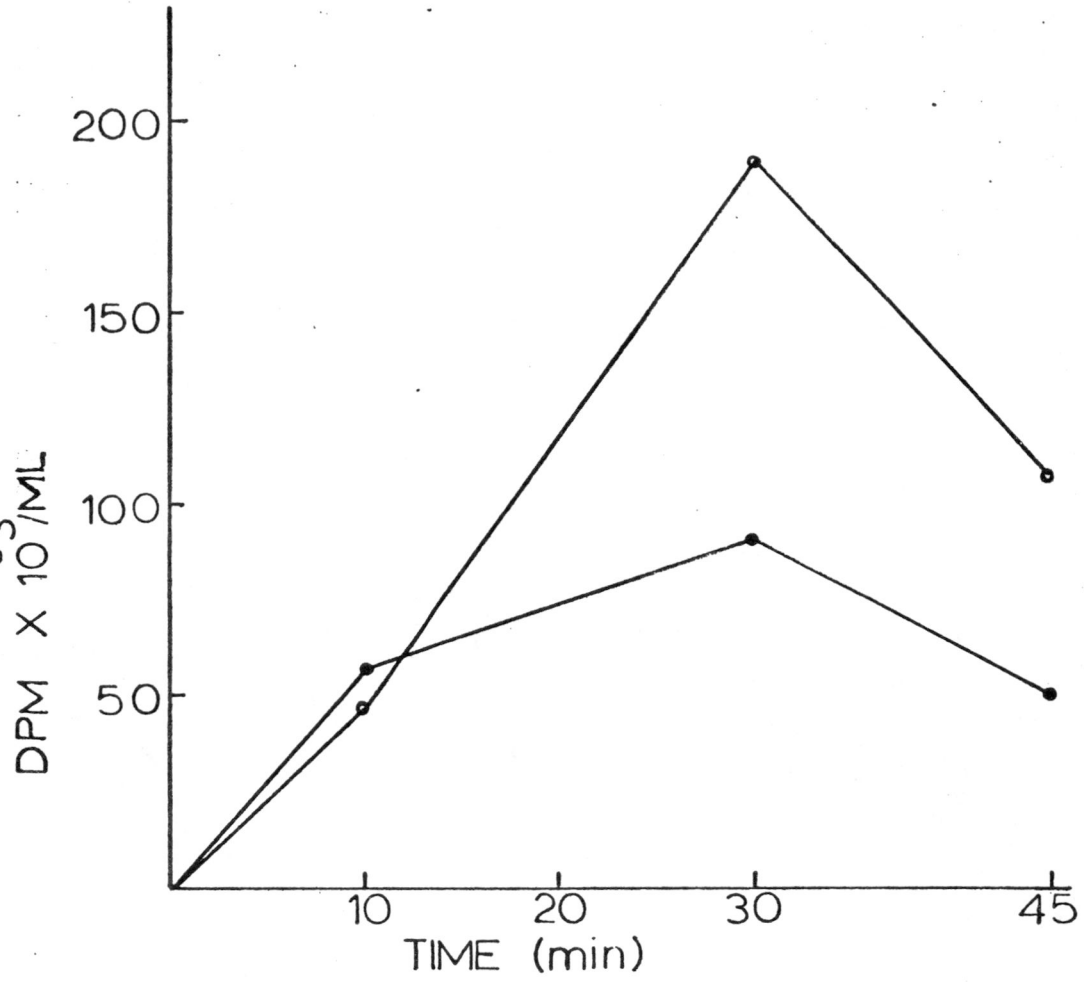

200
150
100
50
DPM X 10^{-3}/ML
10
20
30
45
TIME (min)

Table 2. Extracellular Spaces of Muscle Tissues at 10, 30, and 45 Minutes After the Injection of [^{3}H] Inulin

Tissue	Time (min)	Extracellular Space (ml/g wet weight)
Gastrocnemius	10	0.25
	30	0.15
	45	0.19
Red Quadriceps	10	0.42
	30	0.32
	45	0.22
White Quadriceps	10	0.43
	30	0.20
	45	0.21

vein (1.0 μCi/100 g body weight). Ten untrained control rats and seven trained rats were used in this experiment. After sacrifice (30 minutes after the injection), the proper tissues were removed and underwent the various steps involved in the solubilization and analysis of each sample. The results are shown in Table 3.

A problem was encountered which involved the presence of chemoluminescence in the prepared vials. The initial radioactivity was high and kept dropping with time. Thus, the vials were kept in the dark until constant counts were obtained. For some samples (such as liver), this was not achieved until 25 days after the preparation of the vials.

Tissue water was expressed in grams of water/gram of tissue, and the values were obtained as the difference between wet weight and dry weight. The values tabulated are shown in Table 3.

There were no changes found in the amount of tissue water between trained and untrained animals. The extracellular space was significantly greater in the gastrocnemius of trained rats than the untrained animals. No significant differences were found in the other tissues although the same trend was found in the white quadriceps as shown in Table 3.

Assay for amino acid transport: Amino acid transport was measured by using [^{14}C]-α-aminoisobutyric acid (AIB) a non metabolized amino acid analog. During the preliminary studies, twelve rats were used in order to establish the optimal point of AIB concentration in the tissues. These rats ranging in weight from 380 g - 420 g were injected with radioisotope intraperitoneally (1 μCi/100 g body weight). The animals were sacrificed at four time periods after the isotope injection; 30, 45, 60

Table 3. Effect of Endurance Training on Tissue Water and Extracellular Volume

Tissue	Tissue Water ($g\ H_2O/g$ tissue)	Extracellular Volume (ml/g tissue)
Gastrocnemius		
Untrained	.761±.003[a] (9)	.096±.005 (8)
Trained	.758±.003 (9)	.116±.006* (7)
Red Quadriceps		
Untrained	.768±.003 (9)	.093±.006 (9)
Trained	.763±.003 (9)	.080±.005 (6)
White Quadriceps		
Untrained	.765±.007 (9)	.069±.004 (8)
Trained	.758±.005 (9)	.081±.005 (6)
Heart		
Untrained	.734±.002 (9)	.163±.004 (10)
Trained	.729±.005 (9)	.155±.008 (7)
Liver		
Untrained	.704±.005 (9)	.134±.008 (10)
Trained	.710±.005 (9)	.135±.011 (6)

[a] Values are of the mean ± SEM
* Values are significantly different from Untrained ($P < .05$)

and 90 minutes. After the proper solubilization, preparation and counting of all the samples, the results were expressed in dpm/gram of tissue versus time. For blood and plasma, these values were expressed in dpm/ml. It was found that all tissues show a linear increment in [^{14}C] AIB accumulation (Figures 7, 8, 9, 10). Blood and plasma dropped in counts after 30 minutes and then gradually increased in concentration of AIB; this may be due to an equilibrium phenomenon (Figure 11).

Once the parameters were established for the actual experiment, 20 rats were sacrificed 60 minutes after the injection of the radioactive AIB. Half of these animals were untrained-control rats and the others were trained. After analysis of the different samples, the results were expressed in dpm/gram or dpm/ml as shown in Table 4. To correct these figures to the amount of radioactive amino acid which actually entered the tissues intracellularly, the extracellular spaces obtained from the inulin assay were applied to each corresponding tissue for untrained and trained rats. The result of this was a lower value which constituted the net amino acid entering the tissues (Table 4).

There were no differences in amino acid transport to gastrocnemius, white quadriceps, red quadriceps and liver. Heart showed a significant decrease in amino acid transport in trained animals.

Protein synthesis: There was a significant decrease in leucine incorporation into heart and liver proteins (Table 5). This decrease in amino acid incorporation suggests a decrease in protein synthesis in heart and liver of trained rats. There were no differences in [^{14}C]-leucine incorporation into total proteins of quadriceps and gastrocnemius,

Figure 7. Concentration of ^{14}C-alpha-aminoisobutyric acid versus time after injection in gastrocnemius (o) and white quadriceps (●)

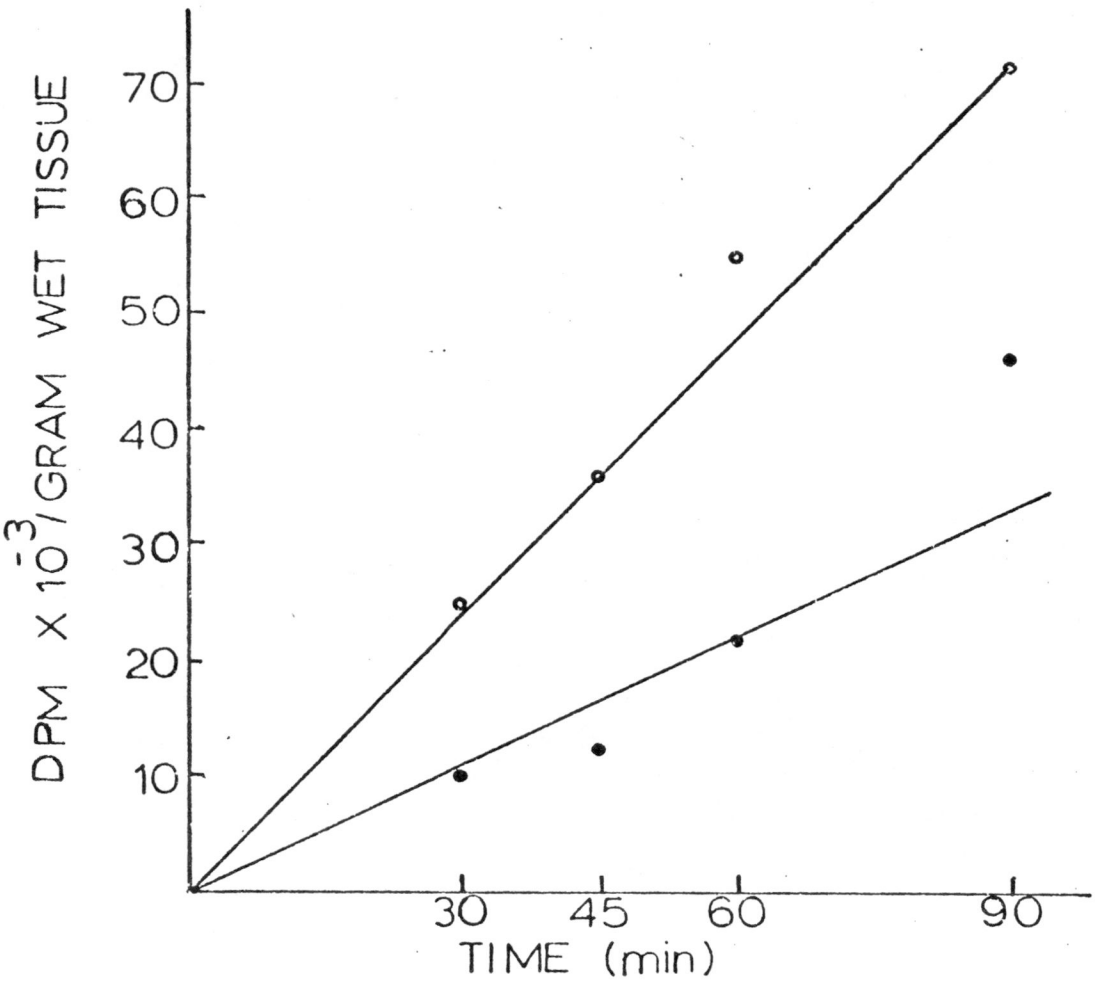

DPM X 10^{-3}/GRAM WET TISSUE
70
60
50
40
30
20
10
30
45
60
90
TIME (min)

Figure 8. Concentration of ^{14}C-alpha-aminoisobutyric acid versus time after injection in red quadriceps

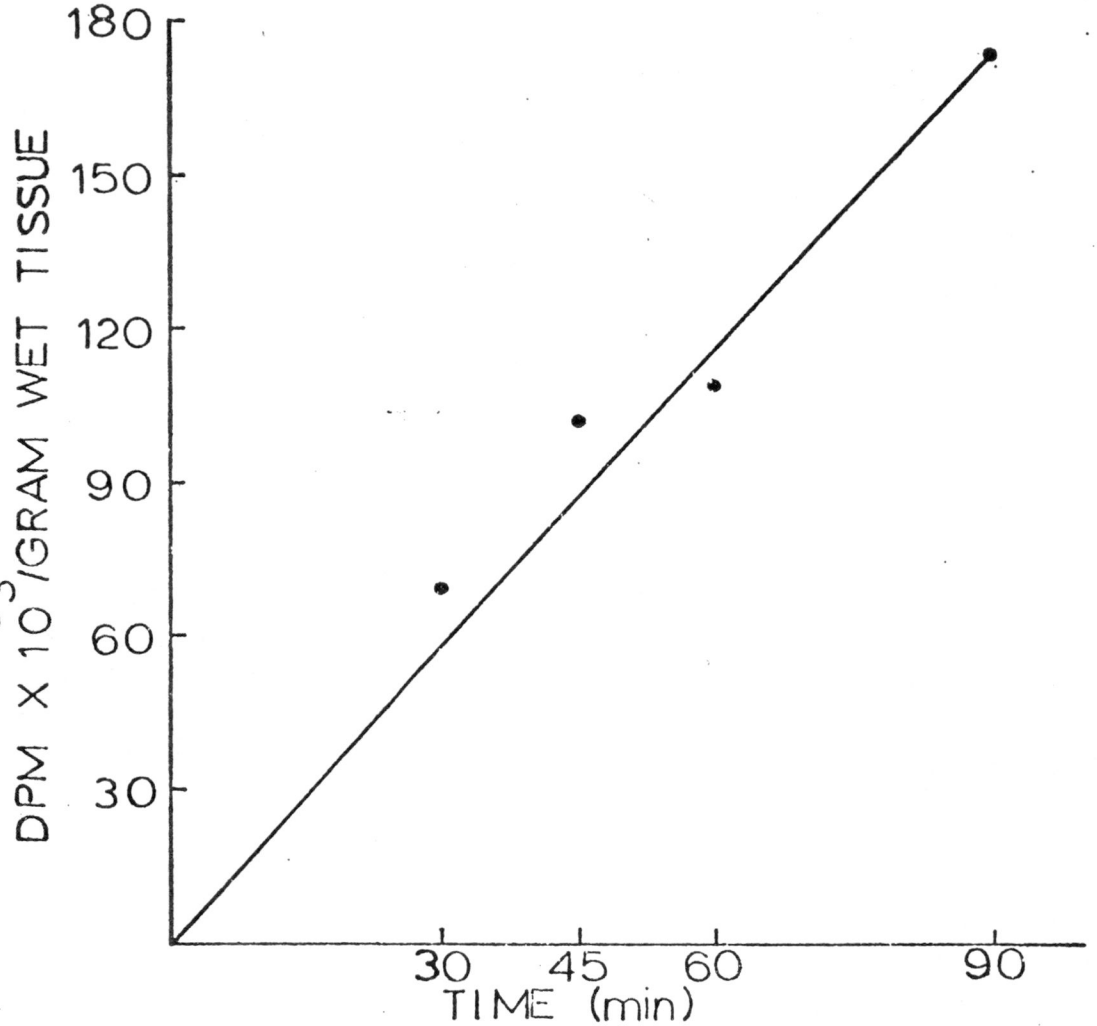

180
150
120
90
60
30
DPM X 10^-3/GRAM WET TISSUE
30
45
60
90
TIME (min)

Figure 9. Concentration of ^{14}C-alpha-aminoisobutyric acid versus time after injection in liver

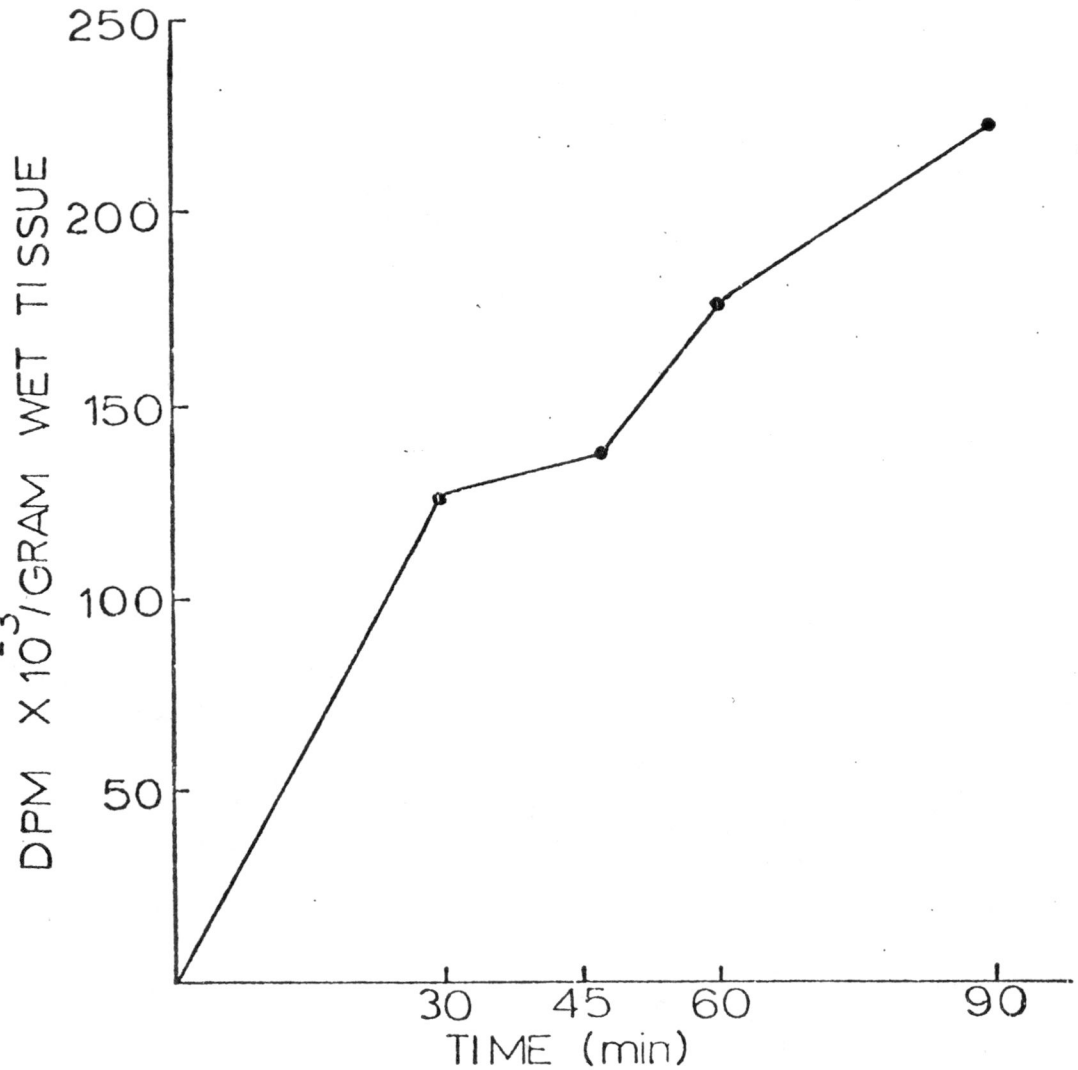

250
200
150
100
50
DPM X 10^{-3}/GRAM WET TISSUE
30
45
60
90
TIME (min)

Figure 10. Concentration of ^{14}C-alpha-aminoisobutyric acid versus time after injection in heart

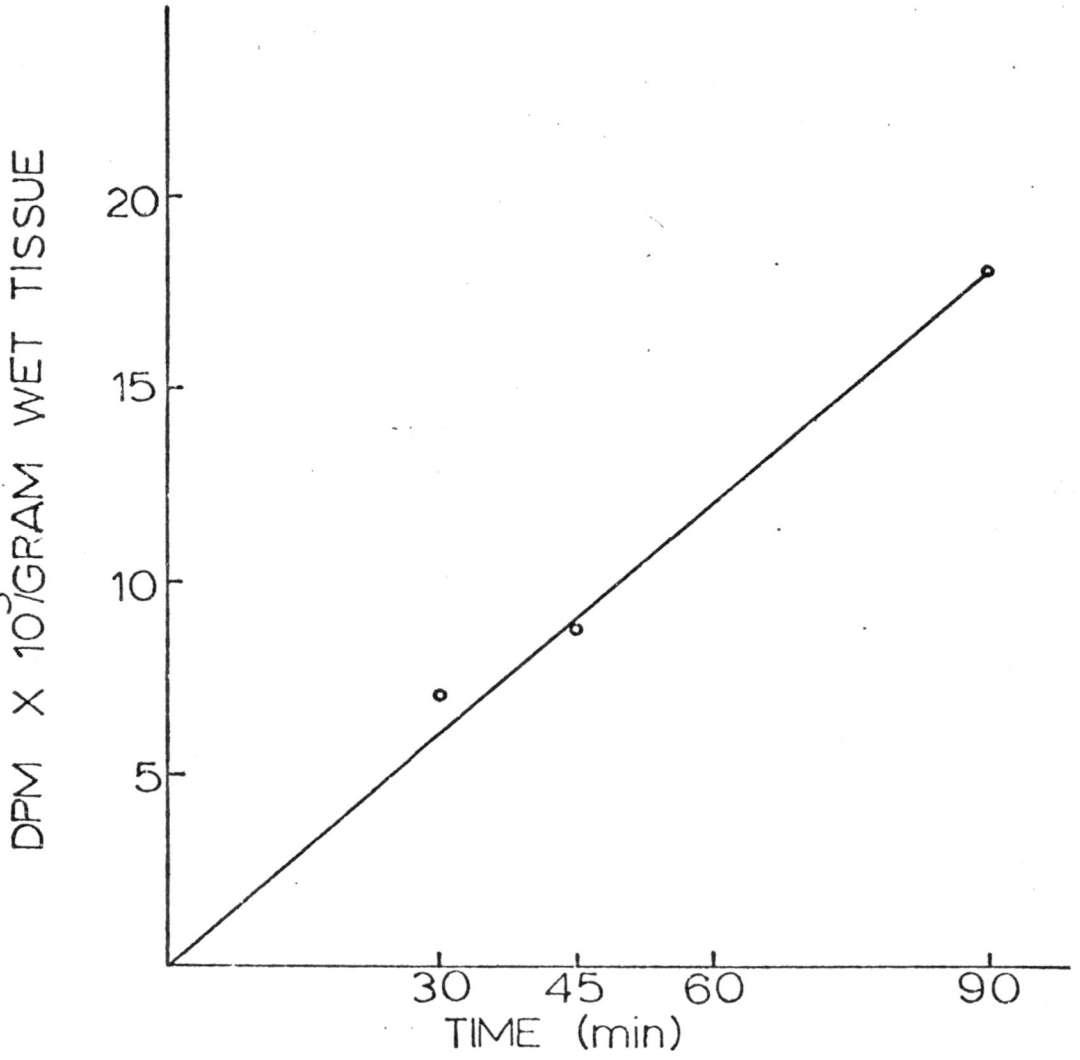

DPM X 10^{-3}/GRAM WET TISSUE
20
15
10
5
30
45
60
90
TIME (min)

Figure 11. Concentration of ^{14}C-alpha-aminoisobutyric acid versus time after injection in blood (●) and plasma (o)

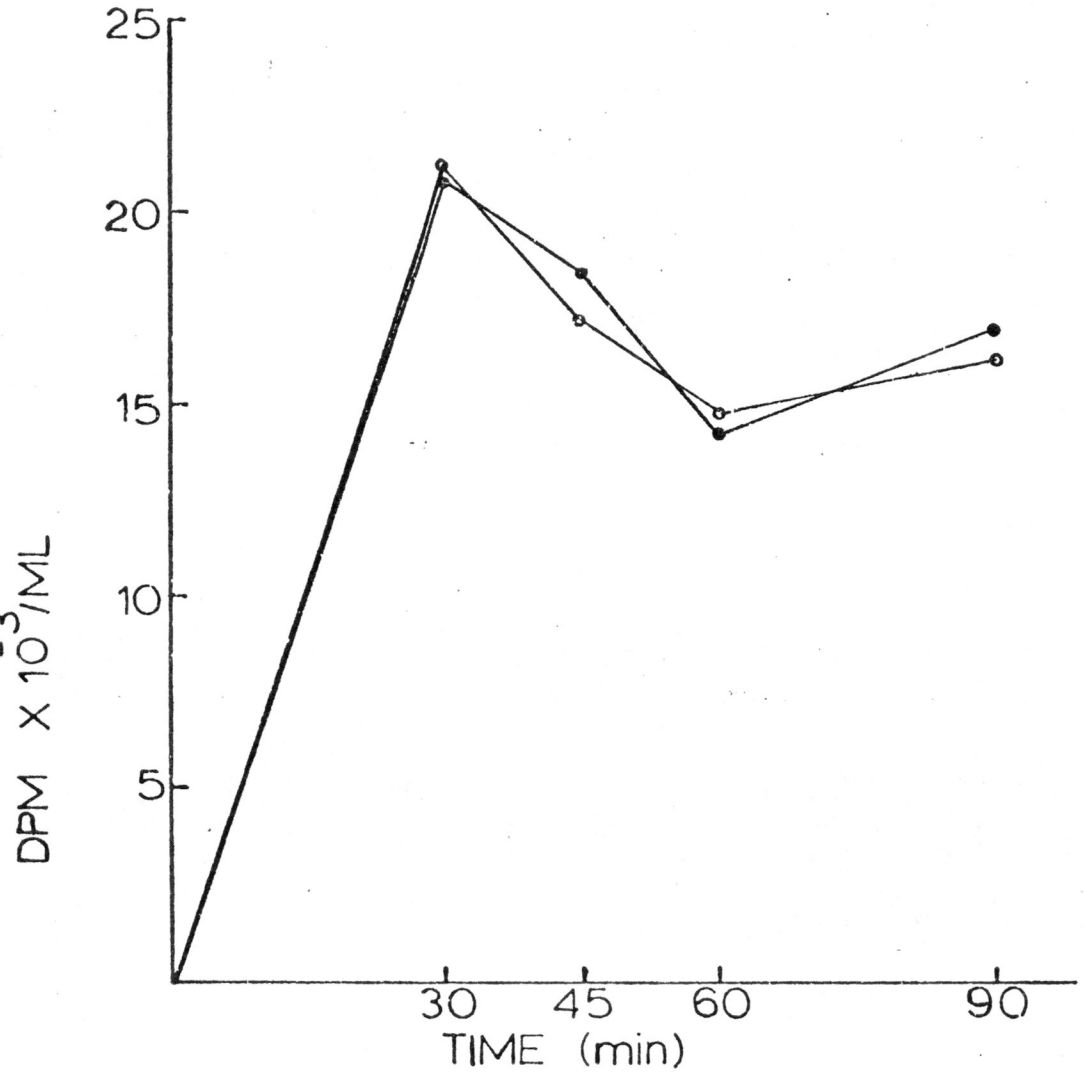

25
20
15
10
5
DPM X 10^-3/ML
30
45
60
90
TIME (min)

Table 4. Effect of Endurance Training on Transport of ^{14}C-Alpha-Aminoisobutyric Acid

Tissue	Net AIB Uptake (dpm/g)	
Gastrocnemius		
Untrained	11,802±1187	(8)
Trained	13,283±692	(9)
Red Quadriceps		
Untrained	17,020±1828[a]	(9)
Trained	13,456±487	(9)
White Quadriceps		
Untrained	7,201±375	(9)
Trained	8,078±379	(9)
Heart		
Untrained	17,543±758	(8)
Trained	13,362±814*	(8)
Liver		
Untrained	43,605±4440	(9)
Trained	43,529±2937	(9)

[a] Values are of the mean ± SEM
* Values are significantly different from Untrained ($P<.05$)

Table 5. Influence of Training on Leucine [^{14}C Carboxyl] Incorporation into Heart, Liver, Quadriceps, and Gastrocnemius Proteins

	Leucine Incorporation (DPM/mg Protein)	
	Untrained	Trained
Heart	1188+57[a] (8)	1040+37* (10)
Liver	2197.8+71 (10)	1936.2+61.3* (11)
Quadriceps	503.9+35.15 (10)	569.2+26.6 (11)
Gastrocnemius		
Total	439.8+23.1 (10)	491.8+23.8 (11)
Sarcoplasmic	501.7+31.2 (10)	563.5+25.2 (11)
Myofibrillar	455.0+30 (8)	498+26 (11)
Stromal	740.5+51 (10)	607.7+27* (10)

[a] Values are of the mean $\pm$ SEM
* Values are significantly different from Untrained ($P<.05$)

but there was a significant decrease in leucine incorporation into the stromal protein of gastrocnemius as a result of training. There were no differences in the specific radioactivities of the soluble fractions between untrained and trained except for heart (Table 6). This decrease in the soluble fraction of trained hearts may have been due to a decrease in amino acid absorption into the intracellular pool.

Although there was a decrease in the specific radioactivity of the soluble TCA fraction of trained hearts, the data nevertheless suggest that the decrease in amino acid incorporation reflects a decrease in protein synthesis rate. This conclusion was reached by the fact that in a previous study, Dohm (unpublished data) showed a decrease in [^{14}C]-leucine incorporation in heart of trained rats without any alterations in the specific radioactivity of leucine in the soluble fraction. Also, the specific radioactivity of leucine in plasma was not altered by training (Table 6), and Martin et al. (11) demonstrated that in the early time periods after the injection of a labeled amino acid, the extracellular amino acid pools serve as the precursors for protein synthesis.

Determination of protein levels: The effect of endurance training on muscle protein levels was investigated to determine if alterations in protein catabolism would exert an effect on muscle protein content. It appears that endurance training depressed the protein content of the sarcoplasmic and myofibrillar fractions of gastrocnemius (Table 7). A similar but not significant trend was evident in the protein levels of heart (Table 8). Thus, it seems that endurance training does decrease the protein content of muscle. This effect appears to be specific since only certain fractions were affected.

Table 6. Specific Radioactivities in Soluble Fractions of Plasma, Heart, Liver, Gastrocnemius, and Quadriceps

	Untrained	Trained
Plasma (DPM/ml)	23,784+1803 ($\bar{9}$)	20,290+942 ($1\bar{1}$)
Heart (DPM/gm)	26,306+774[a] ($\bar{9}$)	19,009+700* ($1\bar{1}$)
Liver (DPM/gm)	44,522+1217 ($1\bar{0}$)	40,636+2434 ($1\bar{0}$)
Quadriceps (DPM/gm)	20,436+512 ($1\bar{0}$)	19,936+815 ($1\bar{0}$)
Gastrocnemius (DPM/gm)	15,267+839 ($1\bar{0}$)	14,150+706 ($1\bar{1}$)

[a] Values are of the mean + SEM

* Values are significantly different from Untrained ($P<.05$)

Table 7. Effects of Endurance Training on Gastrocnemius Protein Levels

	Untrained	Trained
Gastrocnemius Protein (mg/g)		
Total	203.41±4.78[a] (15)	196.21±6.29 (14)
Sarcoplasmic	68.02±0.78 (18)	64.88±0.93* (17)
Myofibrillar	130.70±1.44 (18)	122.48±2.20* (17)
Stromal	46.16±3.02 (18)	50.14±4.57 (17)

[a] Values are of the mean ± SEM
* Values are significantly different from Untrained ($P<.05$)

Table 8. Effects of Endurance Training on Heart Protein Levels

	Untrained	Trained
Heart Protein (mg/g)		
Total	199.84±4.21[a] (18)	193.41±4.63 (17)
Sarcoplasmic	74.97±1.63 (9)	76.97±1.41 (9)
Myofibrillar	79.12±1.37 (9)	77.89±2.10 (9)
Stromal	72.93±3.35 (18)	67.37±3.17 (17)

[a] Values are of the mean ± SEM

Determination of nitrogen balance: This investigation was undertaken on two different occasions using two groups of animals. Both studies involved the use of trained rats and untrained-control rats. In the second experiment, an additional group of rats designated as pair fed was analyzed. Food consumption and body weights were compared among the different groups and it was consistently found that the untrained-control rats ate and weighed significantly more than the trained group (Fig. 12). The pair fed group was found to be intermediate in body weight between the trained and untrained groups (Table 9).

The food consumed by the rats (Wayne Lab Blox) was found to consist of 4.2% nitrogen in dry food analysis which is the equivalent of 26.2% protein. The water content of the food was determined to be 7.74%.

Nitrogen balance was determined by measuring the amount of nitrogen absorbed and excreted (expressed in g/24 hr/Kg metabolic body mass). The nitrogen balance was calculated by subtracting the amount excreted from the amount absorbed as such:

$$\text{Nitrogen balance} = \frac{\dfrac{\text{Nitrogen absorbed - Nitrogen excreted (g)}}{\text{24 hours}}}{\text{Kg metabolic body size}}$$

In the first absorption experiment, no significant differences were found in the amount of nitrogen absorbed by the rats. The trained rats excreted more nitrogen than the untrained rats ($P<.05$). The trained rats also had a significantly lower nitrogen balance than the untrained rats as shown in Table 10.

In the second experiment, there was a significant difference in absorption between the untrained and pair fed groups and also between

Figure 12. Body weights of trained (▭) and untrained (●) rats versus elapsed training time

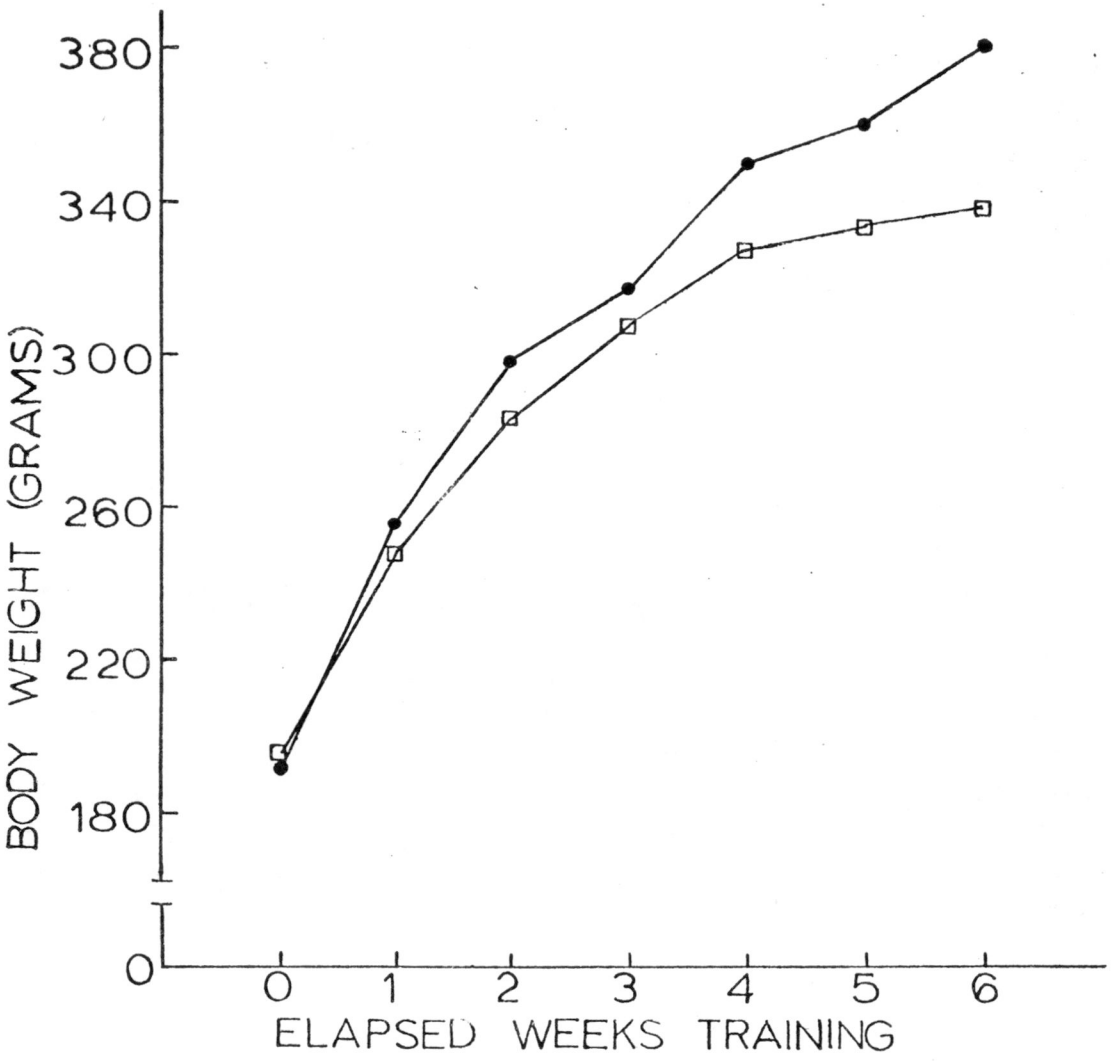

380
340
300
260
220
180
0
BODY WEIGHT (GRAMS)
0
1
2
3
4
5
6
ELAPSED WEEKS TRAINING

Table 9. Effects of Endurance Training on Food Consumption and Body Weights

First Experiment

	Untrained	Trained
Food Consumption (g/24 hr)	23.39±.43[a] (24)	21.02±.41* (28)
Body Weight (Kg)	.384±.006 (10)	.338±.008* (10)

Second Experiment

	Untrained	Pair Fed	Trained
Food Consumption (g/24 hr)	21.56±.55† (11)	19.8±.28 (12)	19.08±.50* (10)
Body Weight (Kg)	.357±.007† (11)	.337±.006 (12)	.327±.007* (10)

[a] Values are of the mean ± SEM
* Values are significantly different from Untrained ($P<.05$)
† Values are significantly different from Pair Fed ($P<.05$)

Table 10. Effects of Endurance Training on Nitrogen Balance

First Experiment

	Untrained	Trained
N Absorbed (g/24hr/Kg MBS)	1.38±.04[a] (10)	1.33±.05 (10)
% N Absorbed	76.03±1.3 (10)	75.86±1.4 (10)
N Excreted (g/24hr/Kg MBS)	.893±.03 (6)	.976±.03* (6)
Nitrogen Balance	.510±.03 (10)	.350±.07* (10)

Second Experiment

	Untrained	Pair Fed	Trained
N Absorbed (g/24hr/Kg MBS)	1.62±.04[a†] (10)	1.48±.03 (12)	1.39±.04* (10)
% N Absorbed	82.50±1.25 (10)	80.05±1.02(12)	76.39±.10*† (10)
N Excreted (g/24hr/Kg MBS)	.914±.05† (12)	1.11±.03 (12)	1.25±.05*† (9)
Nitrogen Balance	.731±.07† (10)	.507±.08 (12)	.275±.05*† (8)

[a] Values are of the mean ± SEM
* Values are significantly different from Untrained ($P<.05$)
† Values are significantly different from Pair Fed ($P<.05$)

the untrained and trained groups. The untrained absorbed the greatest amount of nitrogen followed by the pair fed and trained groups, respectively (Table 10). A significant difference in percent nitrogen absorbed was only found between the untrained and trained groups with the untrained being higher. In the first experiment, a trend was found in the same direction although it was not significantly different. The pair fed group also had a significantly higher percentage of nitrogen absorbed than the trained rats as shown in Table 10.

The results of nitrogen excretion were the opposite of that found for absorption; the trained animals excreted more nitrogen than both control groups. These data were reproduced in both experiments (Table 10).

In calculating the nitrogen balance, it was found that the trained animals had a lower value than the untrained groups. This result was significantly reproduced in both studies. In the second experiment, there was a significant difference between trained and pair fed groups and untrained groups. The pair fed group was also found to have a significantly lower nitrogen balance than the untrained rats.

DISCUSSION

The results of this study show that endurance training induces no changes in the amino acid transport to liver, red and white quadricep muscles and gastrocnemius muscle, but it caused a significant decrease in the transport of AIB into the heart of trained rats. The extracellular space was significantly increased in the gastrocnemius muscle of trained rats but no changes were observed in the rest of the tissues analyzed. Incorporation of [^{14}C]-leucine into protein was significantly decreased in liver, heart, and stromal fraction of gastrocnemius of trained animals. Protein levels were decreased in the sarcoplasmic and myofibrillar fractions of gastrocnemius muscle in trained rats. Nitrogen balance was significantly lower in trained rats due to a decrease in food consumption and nitrogen absorption, accompanied by an increase in urinary excretion of nitrogen.

The decrease in amino acid transport to heart as a result of endurance training shows that the effects are not local as in work-induced hypertrophy studies. The decrease in AIB transport demonstrates a decrease in absorption of the labeled amino acid into heart as a result of exercise. These results agree with the lowered specific radioactivity of [^{14}C]-leucine found in the TCA soluble fraction of trained hearts.

The extracellular space was greater in gastrocnemius of trained rats. This significant increase in extracellular space of muscle in trained rats suggests an increase in vascularity. This may be an adaptation of advantage since an enhanced circulation capacity in skeletal muscle of trained rats may result.

The decrease in [^{14}C]-leucine incorporation into protein of heart and liver of trained rats reflects a decrease in protein synthesis rate. This response to endurance training further demonstrates that the effects are not local as in Goldberg's hypertrophy studies.

A decrease in heart protein synthesis is paradoxical since training increases cardiac mass (3). Since protein synthesis is decreased but cardiac mass is increased, the rate of protein degradation must be depressed far more than protein synthesis. Thus, more attention should be given to the breakdown mechanisms of protein and its possible function as a regulatory mechanism.

The decrease in protein synthesis of trained heart may be due to a decrease in amino acid availability. This is shown by a decrease in [^{14}C]-AIB transport to heart and a decrease in the specific radioactivity of leucine in the soluble TCA fraction of heart. At this point, it cannot be determined whether the decrease in protein synthesis in heart results from a decrease in amino acid availability or if the two mechanisms (transport and synthesis) are independent of each other. This appears to be the case in liver since a decrease in protein synthesis was found to occur despite no changes in the amino acid transport.

There are several factors that are altered during exercise which have an effect on protein synthesis. Hartley et al. (32) showed that exercise alters plasma levels of several hormones which affect protein synthesis. Thus, any change in protein synthesis as a result of training may be responding directly to hormone levels; consequently, further investigations regarding changes in hormonal secretion as a result of endurance exercise should be pursued.

Our studies showed a decrease in protein synthesis in the stromal fraction of gastrocnemius muscle. These results are in the same direction as the *in vitro* studies of Beecher *et al.* (33) who showed a decrease in protein synthesis in muscle as a result of training. The opposite results were found in Goldberg's heavy work studies which may explain why no hypertrophy occurred in the muscle of trained rats.

This decrease in protein synthesis in the stromal fraction in muscle shows that training affects synthesis of specific proteins. However, the effect is not always in the same direction since Terjung (34) showed that synthesis of cytochrome c is increased as a result of training.

The significant decrease in protein levels of sarcoplasmic and myofibrillar fractions in gastrocnemius may be related to two factors; a decrease in protein synthesis or an increase in protein breakdown. Since no changes were found in protein synthesis, it seems that the protein degradation mechanisms may be increased in these two muscle proteins as a result of training. The opposite appears to occur in the stromal fraction of gastrocnemius muscle where a decrease in protein synthesis was found despite an increase in total stromal protein. Here again it seems that the rate of protein degradation must be depressed far more than synthesis in order to maintain an increased protein level. An increase in protein breakdown as a cause of lowered protein levels in trained rats is substantiated by the previous studies of Dohm *et al.* (4) who showed that training increased amino acid oxidation and urea excretion.

The decrease in nitrogen balance in trained rats was caused mainly by an increase in nitrogen excretion despite a lowered total nitrogen absorption. All nitrogen values were positive since growth was still taking place. These results indicate that catabolism of protein must be increased by training. The decrease in nitrogen balance as a result of training agrees with the increases in amino acid oxidation and urea excretion found in trained rats (4).

The results of this study agree with Haralambie's results (27). He showed that exercise increases the serum levels of α amino nitrogen and urea in men, and this demonstrates an increase in protein utilization. In addition, his studies also showed an increase in serum levels of tyrosine as a result of exercise which may be due to enhanced protein breakdown.

It appears that training increases protein catabolism thus suggesting that protein may be used as a fuel source during exercise. One possible way by which protein can be used as a fuel source is explained by Felig and Wahren's hypothesis of the glucose-alanine cycle (35). It appears that the release of alanine from peripheral tissue exceeds the release of the other amino acids. This difference is amplified even further during exercise, and the amount of alanine being released is proportionally greater than its content in constituent muscle proteins. In addition, the level of alanine at rest and during exercise is directly proportional to arterial pyruvate levels. It seems that oxidation of amino acids in muscle is preceded by transamination, adding an amino group to the carbon skeleton provided by pyruvate, thus releasing high levels of alanine in muscle. Alanine is deaminated in

the liver forming pyruvate and urea. In the liver, gluconeogenesis takes place from pyruvate, and glucose goes back to muscle where it serves as an energy source, thus completing the cycle.

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