Edward B. Tapscott, Jr. CHARACTERIZATION OF THE PERFUSED RAT HINDQUARTER AS A MODEL FOR THE STUDY OF MUSCLE PROTEIN METABOLISM. EFFECTS OF EXERCISE TRAINING AND ACUTE EXERCISE ON PROTEIN SYNTHESIS AND PROTEIN DEGRADATION IN PERFUSED MUSCLE. (Under the direction of Dr. G. Lynis Dohm) Department of Biology, June 1979.

The objectives of this investigation were to characterize the perfused rat hindquarter as a model for the study of protein synthesis and protein degradation and to determine the effects of both exercise training and acute exercise on both processes. It was determined that the perfused hindquarter would serve as an adequate model for the study of protein synthesis and degradation in resting muscle. Drastic decreases in high energy phosphate compounds in exercising perfused muscle might make the system unsuitable for the study of protein metabolism during exercise. Six weeks of exercise training did not result in a change in the rate of incorporation of [^{3}H] tyrosine into muscle protein although protein degradation was significantly increased in the trained rat. One hour of swimming resulted in both a depression of protein synthesis and an increase in protein degradation in the perfused muscle model.

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A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

Edward B. Tapscott, Jr.

June, 1979

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by

Edward B. Tapscott, Jr.

APPROVED BY:

SUPERVISOR OF THESIS

G. Lynis Dohm

Dr. G. Lynis Dohm

THESIS COMMITTEE

Wendall E. Allen

Dr. Wendall E. Allen

Takeru Ito

Dr. Takeru Ito

W. James Smith

Dr. W. James Smith

ACTING CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

Edward P. Ryan

Dr. Edward P. Ryan

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette

Dr. Joseph G. Boyette

ACKNOWLEDGEMENT

The author extends sincere thanks to Dr. G. Lynis Dohm for his guidance and untiring patience during the completion of this project. The expert technical assistance rendered by William Brown, Ave Edge, Alan Ayers, Tim Powell, Fernando Puente, and Ron Warren is gratefully acknowledged as are the time and talents given by my wife, Bonnie, in editing and typing the manuscript. Gratitude is also extended to Dr. Wendall Allen, Dr. Takeru Ito, and Dr. James Smith for their service on the thesis committee.

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INTRODUCTION

The importance of muscle protein as a source of metabolic fuel and the conditions which result in degradation of protein for this purpose have been areas of considerable debate. Internal stores of carbohydrate and fat have long been recognized as the principle sources of metabolic fuel during periods of dietary deficiency. Protein, having slower turnover, was not considered a primary source of metabolic energy. Muscle constitutes a large percentage of the body weight of most mammals however, and about eighty percent of the dry weight of muscle is protein. Therefore muscle protein is an important depot for metabolic energy for use during periods of stress. When supplies of carbohydrate are diminished, glucogenic amino acids released from protein provide glucose for maintenance of brain function (Felig 1975, Ruderman 1975). Muscle protein might also provide energy to sustain other metabolic functions even though carbohydrate stores were not depleted. Rates of protein synthesis and protein degradation might be altered during and subsequent to exercise to provide substrates for oxidation in muscle (Goldberg and Odessey 1972) and precursors for synthesis of new protein as well as substrates for gluconeogenesis.

Obviously synthesis and degradation of protein must be well regulated to prevent unnecessary muscle wasting. Considerable information is available regarding hormonal control of protein synthesis and degradation in muscle (Table I). The effects of exercise on levels of several hormones which have been shown to affect protein metabolism have also been measured (Table II), but mechanisms of regulation of these

TABLE I. Effect of Hormones on Protein Synthesis and Protein Degradation in Skeletal Muscle.

Hormone	Effect on Protein Synthesis	Effect on Protein Degradation	Reference
Insulin	+	-	Jefferson, et al. (1972)
	+	-	Jefferson, et al. (1974)
	+	-	Jefferson, et al. (1977)
	+	-	Rannels, et al. (1975)
	+	-	Fulks, et al. (1975)
Growth Hormone	+	n.d.	Florini and Breuer (1966)
	+	0	Flaim, et al. (1978b)
Glucocorticoids	n.d.	+	Munro, et al. (1978)
	n.d.	+	Thienhaus, et al. (1975)
	-	n.d.	Bullock, et al. (1968)
	-	n.d.	Peters and White (1970)
Thyroxine	n.d.	+	DeMartino, et al. (1977)
	-	0	Flaim and Jefferson (1978a)
Glucagon	n.d.	0	Fitzpatrick, et al. (1977)

The abbreviations are as follows: +, synthesis or degradation is increased; -, synthesis or degradation decreased; 0, no change; n.d., not determined.

TABLE II. The Effect of Exercise on Hormone Concentration.

Hormone	Effect of Exercise on Plasma Concentration	Reference
Norepinephrine	Increased	Hartley, et al. (1972b)
Epinephrine	No Change	Hartley, et al. (1972b)
Growth Hormone	Increased	Hartley, et al. (1972b)
Insulin	Decreased	Hartley, et al. (1972b)
Cortisol	Increased	Hartley, et al. (1972b)
Glucagon	Increased	Gyntelberg, et al. (1977)
Thyroxine	Increased	Terjung and Tipton (1971)
Testosterone	Decreased	Dohm and Louis (1978a)

factors and other factors controlling protein metabolism in exercising muscle are not well understood. Two of the major reasons for this have been the lack of an *in vitro* model which is suitable for the study of protein metabolism relative to the various physiological and biochemical parameters associated with exercise, and the piecemeal approach to the problem which this lack has precipitated.

Observations by Dohm *et al.* (1977) of decreased weight gain, increased urea excretion, and decreased nitrogen balance in run-trained male rats led to the proposal that exercise results in a more protein catabolic state, and that amino acids from degraded protein provides a substantial amount of energy. Reports of increased urinary nitrogen (Molè and Johnson 1971) and decreased nitrogen balance (Gontzea *et al.* 1974, 1975) in exercising men, and further observation by Dohm *et al.* (1978) of increased urine ammonia and loss of muscle protein in acutely exercised rats indicated that the effects of exercise on protein metabolism are more immediate than was suspected.

The objectives of this project were to develop an *in vitro* model in which protein synthesis and degradation could be measured simultaneously and to measure the effects of exercise training and acute exercise on protein metabolism.

A number of model systems have been used in the study of protein turnover. Studies of protein synthesis and degradation *in vivo* by pulse labeling techniques have been used. For example McManus *et al.* (1975) observed increased incorporation of [^{3}H] leucine into myofibrillar and sarcoplasmic protein in plantaris muscle from run-trained guinea pigs pulse labeled *in vivo*. However, they did not account for specific

activity of label in the precursor pool. Zimmer and Gerlach (1973), demonstrating the necessity of determining precursor specific activity, found that acute swimming exercise decreased absolute incorporation of labeled amino acid *in vivo*. Although apparent incorporation was increased, the specific activity of label was also higher in the exercised animal. When this was corrected the absolute protein synthesis was lower in the exercised tissue. In pulse labeled animals the specific radioactivity of the label decreases rapidly (Shimke 1970) due to catabolism, incorporation by extraneous tissues, and other factors, making estimation of absolute rates of synthesis difficult. Reutilization of label interferes with estimation of degradation rates.

In vivo measurement of protein synthesis has been improved with the constant infusion technique described by Garlick and Marshall (1972), in that specific activity of label may be maintained at an elevated level for longer periods. It is unlikely that this technique could be made workable to measure protein metabolism during normal exercise because of the delicate surgery involved, but it holds promise as a technique for *in situ* studies.

Recent work by Dohm *et al.* (1979) indicated that cell free protein synthesizing systems isolated from trained rats and acutely exercised rats incorporated less [^{14}C] leucine into protein than do those from untrained rested animals.

Studies on electrically stimulated isolated organs, especially extensor digitorum longus and diaphragm in the rat and sartorius in the frog, have led to varying results. Kendrick *et al.* (1967) observed that electrical stimulation of frog sartorius led to a gradual increase in

incorporation of label into muscle protein. Karpatkin and Samuels (1967) found, on the other hand, a decrease in incorporation of leucine in frog sartorius as did Pain and Manchester (1970) in electrically stimulated extensor digitorum longus. Goldberg (1972) found that electrical stimulation of rat diaphragm increased accumulation of [^{14}C] AIB, indicating increased transport of amino acids into the working muscle cell.

Isolated organs incubated *in vitro* obviously are of considerable value but these systems are not physiologically normal. The environment of the tissue is drastically altered. Delivery of oxygen and substrates no longer takes place in the normal fashion but rather these materials must diffuse through the tissue. Additionally, the sample tissue yield from these incubations is small necessitating micro-analytical techniques.

The use of isolated perfused rat hindlimbs has received increased attention as a model for the study of skeletal muscle metabolism (Ruderman *et al*. 1971), including the metabolism of protein (Jefferson 1972, 1977). There are several very positive features of perfused muscle preparations: (1) delivery of oxygen and substrates to the muscle cell occurs via the normal capillary bed, (2) endogenous humoral influences such as hormones may be eliminated from the system, and (3) hormones may be selectively added to the perfusate so that their effects may be accurately quantitated. Use of a fairly large perfusate pool permits constant evaluation of such parameters as circulating substrate concentration (this factor is the key to measurement of protein degradation simultaneously with protein synthesis). These preparations also yield a relatively large amount of tissue which could be extremely

important in studies involving RNA charging, for example, where concentrations of the materials to be measured are very low.

For these reasons, we chose to develop a perfused rat hindlimb preparation with which we could measure the effects of exercise on protein metabolism.

MATERIALS AND METHODS

Reagents. Bovine serum albumin, amino acids, enzymes, co-enzymes and selected metabolic intermediates, glucose and pyruvate were obtained from Sigma Chemical Company. Inorganic salts, organic solvents, fluors for scintillation counting and inorganic acids were obtained from Fisher Scientific Company. Sodium pentobarbital was obtained from Abbot Laboratories. Reagents for sample oxidation and for counting oxidized materials were from Packard Instrument Company. Outdated whole human blood was provided by Pitt Memorial Hospital. L-[ring-3,5-^{3}H] tyrosine and [methoxy-^{3}H]-methoxyinulin were obtained from New England Nuclear.

Experimental animals. Male Holtzman rats were housed singly and maintained on lab-blox (Wayne Feed Mills) and water ad libitum.

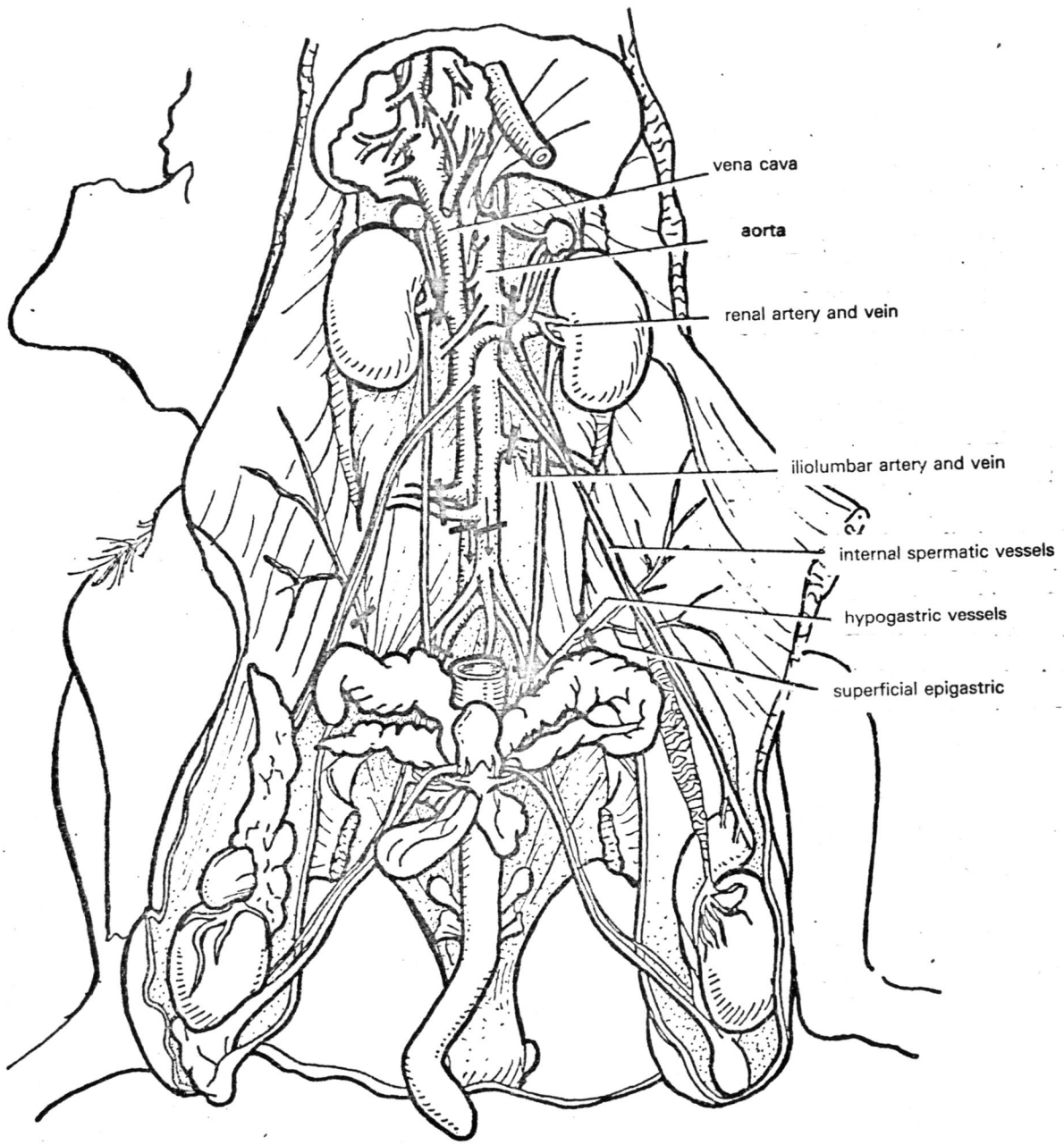
Animal treatments: Training. Animals initially weighing 180 - 200 grams were exercised six days weekly on a treadmill. Training began at a speed of 15 meters/minute for a duration of 15 minutes and gradually increased in speed, duration, and effective load until a speed of 35 meters/minute at a 7% grade for a duration of 60 minutes per day was reached at about the twenty-fifth day of training (beginning of the fifth week). Animals were maintained at this training level through the date of sacrifice in the seventh week. Control animals were age matched.

Animal treatments: Acute exercise. Acutely exercised animals were made to swim by placing them into a tank of lukewarm water with several other animals. It was found that animals placed in the tank singly soon learned to float, but if several animals were placed into the tank together they would keep each other moving. Control animals were age matched.

Surgical preparation of animals for perfusion. Two basic preparations were used in these experiments. The preparation of an intact animal for perfusion of the hindquarter (Figure 1) was similar to that described by Ruderman et al. (1971). Following anesthesia with sodium pentobarbital (5.0 mg/100g body weight), a midline abdominal incision was made and the skin reflected. The abdominal wall was incised from the pubic symphasis to the xyphoid process along the midline. In some experiments an electrocautery was used to make this incision in an effort to reduce bleeding. It was generally observed that clean incisions could be achieved with scissors by carefully following the midline. Following incision, the abdominal wall was reflected and the intestines were carefully removed to one side to permit access to the great vessels. Two ligatures were then placed about the lower colon (about 3cm apart) and the segment of colon between them was excised. The aorta and vena cava were carefully separated just below the insertion of the illiolumbar vessels. Ligatures which would later serve to anchor cannulae were placed loosely around each vessel. The hypogastric, illiolumbar, renal, and superficial epigastric vessels were then ligated.

After all of the ligatures were in place, the preparation was transferred to the perfusion enclosure. The arterial cannula (20 gauge needle) was inserted into the descending aorta so that the point came to lie approximately halfway between the illiolumbar and the bifurcation of the great vessels. Flow of perfusate was immediately initiated. It was found that by initiating flow immediately after insertion of the arterial cannula, the addition of heparin to the perfusate or treatment of the animal with anticoagulant could be avoided. As soon as the arterial

Figure 1. Placement of ligatures (--) and cannulae (↓) in preparation of hindquarter.

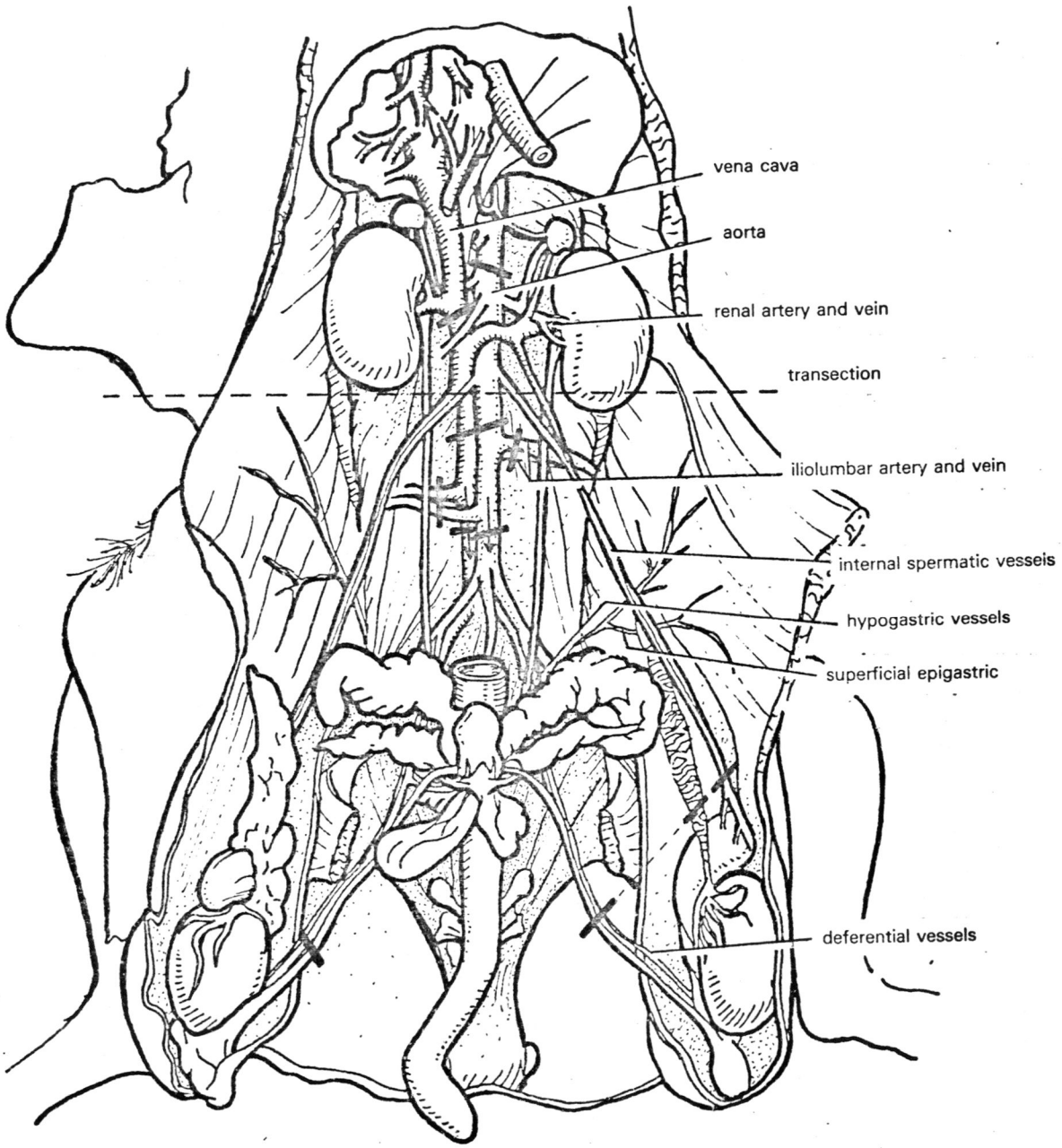

vena cava
aorta
renal artery and vein
iliolumbar artery and vein
internal spermatic vessels
hypogastric vessels
superficial epigastric

cannula was anchored, the venous cannula (20 gauge needle) was inserted and anchored. Perfusate pressure and flow were adjusted and the preparation was covered with parafilm.

The hemicorpus preparation was modeled after that described by Jefferson et al. (1972). Surgery (Figure 2) proceeded the same as for the hindquarter preparation up to and including separation of the aorta and vena cava and placement of the ligatures on these vessels. A single ligature was then placed about the internal spermatic vessel, the deferential vessels, and the vas defrens. Ligatures were placed posterior to the testes to prevent perfusate from collecting in the scrotum. The testes were excised. Loose ligatures were placed about the aorta just anterior to the left renal vessels and about the vena cava just posterior to the juncture with the right renal vein. Another ligature was placed around both great vessels just anteriorly to the juncture with the illiolumbar vessels. The illiolumbar vessels and the left renal vessels were ligated securely. The anteriormost ligature about the vena cava was then tightened. After allowing a few seconds for the vessels in the posterior of the animal to engorge, the anteriormost ligature on the aorta and the ligature just anterior to the illiolumbar were quickly secured. The animal was transected just anteriorly to the last secured ligature and the posterior portion (hemicorpus) was transferred to the perfusion chamber. Cannulae were inserted as described under preparation of the hindquarter.

With both preparations, care was taken to avoid interruption of blood flow to the posterior portion of the animal. Usually the interruption of

Figure 2. Placement of ligatures (-) and cannulae (↓) in preparation of hemicorpus.


vena cava
aorta
renal artery and vein
transection
iliolumbar artery and vein
internal spermatic vesseis
hypogastric vessels
superficial epigastric
deferential vessels

oxygenation of the hemicorpus following ligation of the aorta was less than two minutes until flow was reestablished.

Preparation of the perfusion medium. Krebs Henseleit buffer (Krebs and Henseleit 1932) was prepared by dissolving the salts listed in Table III in distilled deionized water.

A 30% (w/vol) solution of bovine serum albumin (BSA) in Krebs Henseleit buffer was dialysed twice for 10 hours against Krebs Henseleit buffer. The resulting solution was millipore filtered. Final BSA concentration in the solution was determined by the method of Gornall et al. (1949).

On the day on which perfusions were to be done, human erythrocytes were washed three to five times with four volumes of cold Krebs Henseleit buffer by alternate centrifugation, removal of supernatant and resuspension in fresh buffer. BSA was added to the washed red cells to bring the final BSA concentration in the perfusate to 4% (w/vol). Glucose was added to a final concentration of 5.5 mM, pyruvate to a final concentration of 0.15 mM, and millipore filtered Krebs Henseleit buffer was added to adjust the volume. For the protein synthesis-degradation experiments, a solution of the twenty common amino acids (containing 50 times the normal plasma level of amino acids other than tyrosine and 150 times the normal plasma level of tyrosine) (Beecher et al. 1979) in Krebs Henseleit buffer was prepared. The solution was millipore filtered. Ten ml of the amino acid solution was added along with 25 μCi [^{3}H] tyrosine per 100 ml perfusate to the erythrocyte-BSA mixture. The final volume was adjusted. The pH of the perfusion medium was measured and if necessary was adjusted by addition of dilute NaOH or HCl to pH 7.0 - 7.2. Because erythrocytes

TABLE III. Krebs Henseleit Bicarbonate Buffer.

Salts and Carbohydrates	Grams per Liter
NaCl	6.92
KCl	0.35
$MgSO_4 \cdot 7H_2O$	0.29
$CaCl_2$	0.28
KH_2PO_4	0.16
$NaHCO_3$	2.10
Glucose	0.991
Pyruvate	0.016

accumulate tyrosine, it was necessary to allow the perfusate containing amino acids to stand for an hour at 0 - 4°C before use to allow the erythrocyte amino acid pool and the free amino acid pool to equilibrate. Perfusion medium not containing amino acids and [^{3}H] tyrosine was used immediately. Perfusion medium was stored at 0 - 4°C until needed.

[^{3}H] tyrosine was chosen as labeled precursor because (1) it is not metabolized in muscle and (2) it may be measured fluorimetrically. Relatively higher concentrations of tyrosine were used so that the specific activity of the precursor amino acid pools would equilibrate as rapidly as possible.

Perfusion apparatus. The perfusion apparatus is described diagrammatically in Figure 3. Perfusate was drawn by a Buchler polystaltic pump from a reservoir through a multitube oxygenator past a manometer (or pressure gauge), through a bubble trap, and then into the aorta of the hemicorpus or hindquarter preparation. Perfusate removed from the rat returned to the reservoir by way of the pump and a glass wool filter. This apparatus was housed in a heated enclosure which was maintained at approximately 35°C. Because the stirrer which was used to mix the recycling perfusate in the reservoir tended to become quite warm during operation, it was helpful to keep the reservoir in a cool water bath to help prevent hemolysis in the medium. A unique feature of this apparatus was the oxygenator (Figure 4) which was designed and constructed by Dr. W.H. Waugh and Ted Bales of the Department of Medicine, School of Medicine, East Carolina University. Twelve parallel Silastic (trademark, Dow Corning) tubes (0.5 mm I.D. x 0.9 mm O.D.) carry the perfusion medium through an oxygen jacket made of Tygon (trademark, Norton). Gas exchange

Figure 3. Schematic of perfusion system.

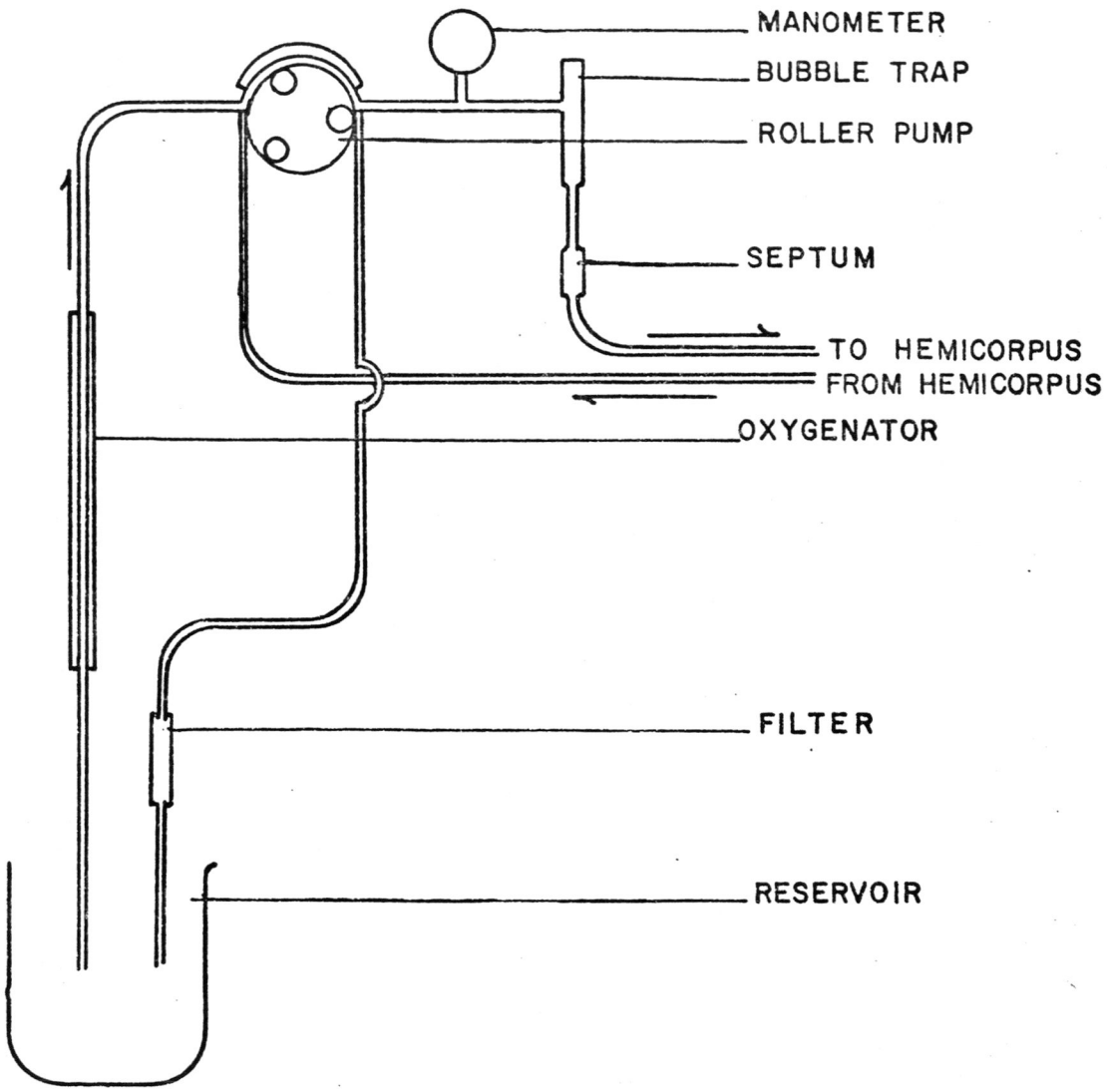
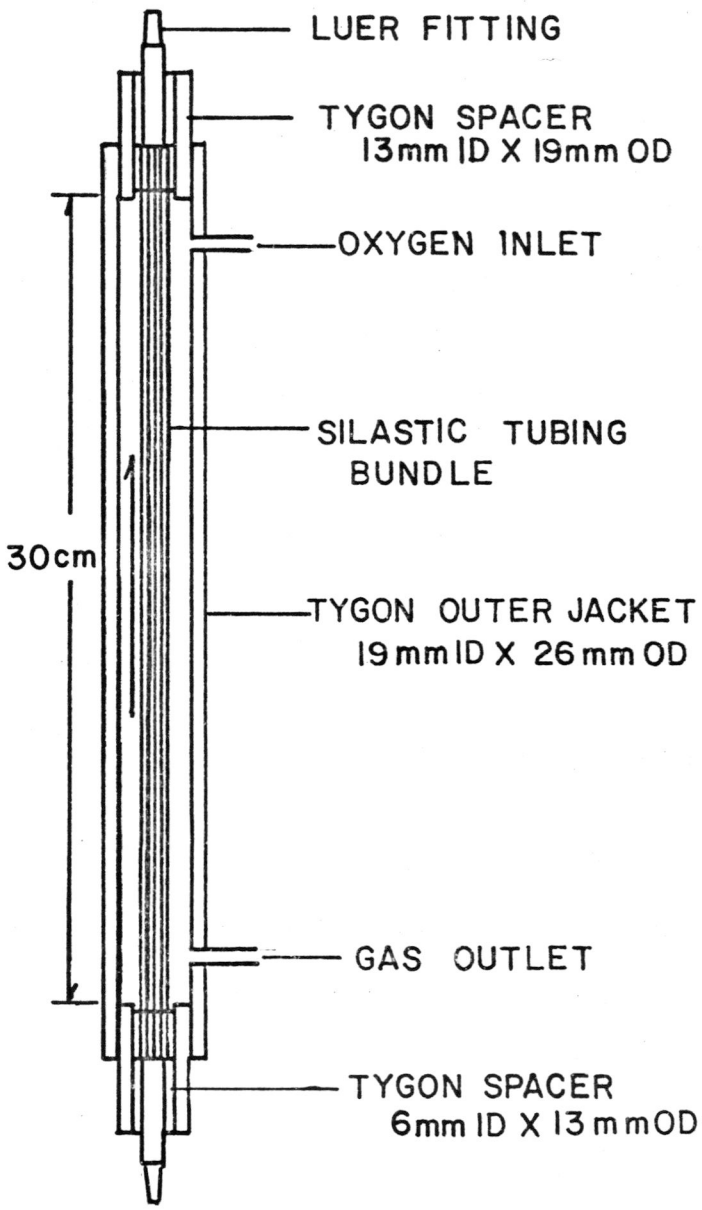

MANOMETER
BUBBLE TRAP
ROLLER PUMP
SEPTUM
TO HEMICORPUS
FROM HEMICORPUS
OXYGENATOR
FILTER
RESERVOIR

Figure 4. Oxygenator used in perfusion system.


LUER FITTING
TYGON SPACER
13mm ID X 19mm OD
OXYGEN INLET
SILASTIC TUBING
BUNDLE
30cm
TYGON OUTER JACKET
19mm ID X 26mm OD
GAS OUTLET
TYGON SPACER
6mm ID X 13mm OD

in this oxygenator takes place across the wall of the Silastic tubing. This minimizes liquid to air interface area thereby reducing hemolysis in the perfusate. The ends of the Silastic tubing bundle were molded together by casting the tubes in Silastic potting compound using a piece of 13 mm I.D. Tygon tubing as a mold.

Perfusion. Following cannulation and initiation of perfusate flow, some of the first effluent from the preparation was collected and discarded. In the early experiments where no labeled amino acid was added to the perfusate, neither the length nor the volume of this washout were critical, although generally washout volumes were maintained above 20 ml. In later experiments on protein synthesis and degradation, the washout period was limited to 5 minutes since it was hypothesized that the total amount of radioactivity which passed through the tissue in this initial washout period would not affect the apparent rate of synthesis as greatly as would the duration of exposure of the tissue to the label.

After the washout period, the effluent from the hemicorpus or hindquarter was returned to the reservoir and the perfusate was recirculated for the remainder of the perfusion. Insofar as possible, perfusate flow through the preparation was maintained at 10 - 12 ml/min. Typical arterial pressures varied between 100 and 140 mm Hg.

During the perfusions, samples of arterial and venous perfusate could be obtained through a septum in the arterial side of the system and at the venous return to the reservoir respectively. Samples could also be obtained directly from the reservoir. At the end of the alloted

perfusion time, the preparation was removed from the apparatus and desired experimental tissues were excised.

Measurements of metabolic intermediates in blood, perfusate, and muscle. Blood and perfusate samples were deproteinized by addition of 2.0 volumes of 8% $HClO_4$ in 40% ethanol. The pH of the resulting supernatant was adjusted with 0.5 M triethanolamine in 3 M potassium carbonate to pH 7.0 - 7.5. The supernatant volume was measured and the supernatant was stored at -20°C.

Muscle samples were excised as rapidly as possible, were quick frozen between liquid nitrogen cooled tongs, and were stored frozen. The frozen tissue was pulverized in a percussion mortar. Perchloric acid extracts from the muscle were prepared by homogenizing the powdered frozen muscle with two volumes of 8% $HClO_4$ in 40% ethanol for 60 seconds at full speed with an Omni Mixer (Dupont-Sorvall). The slurry was then centrifuged at 8000 x g for ten minutes. The supernatant was decanted and saved. The pellet was rehomogenized in two volumes of 6% $HClO_4$ (60 seconds at full speed) and centrifuged a second time. The resulting supernatant was pooled with the first and the pH of the combined extracts was adjusted to 7.0 - 7.5 with 0.5 M triethanolamine in 3 M K_2CO_3. The resulting suspension was centrifuged at 800 x g for 10 minutes. The supernatant volume was measured and the supernatant was stored at -20°C.

Selected glycolytic intermediates were measured enzymatically under conditions described by Maitra and Estabrook (1964) by monitering the change in absorbance due to coupled NAD (or NADP) reduction or NADH oxidation as the substrates were consumed. ATP, ADP, AMP, and creatine phosphate were also measured enzymatically (Lamprecht 1974a, 1974b,

and Jaworek 1974). Lactate was measured as described by Gutmann (1974). These assays were carried out in a 1.0 ml reaction volume according to the conditions shown in Table IV.

Muscle glycogen was determined by the method of Lo et al. (1970). A 0.10 to 0.15 g sample of frozen muscle powder was digested with 0.5 ml 30% KOH which was saturated with Na_2SO_4 in a boiling water bath for 30 minutes. The digestions were cooled on ice and 5.0 ml of 95% ethanol were added to precipitate the glycogen. This mixture was allowed to stand refrigerated overnight, then was centrifuged at 800 x g for 15 minutes. The supernatant was decanted and the pellet dissolved in 3.0 ml water. To 0.1 ml of this solution were added 0.4 ml water, 0.5 ml 5% phenol, and 2.5 ml 95% sulfuric acid. The absorbance of the resulting solution was measured at 490 nm and was compared to an absorbance curve prepared from a glycogen standard.

Analysis of blood and perfusate gasses was done with a Natelson microgasometer using the classical van Slyke technique. Oxygen and carbon dioxide were released from a 30µl sample of blood or perfusate by the addition of 10µl of a solution containing 0.16% (w/vol) potassium ferricyanide and 0.87% (w/vol) saponin. The pressure (P_1) of the gasses in the closed system was measured. The addition of 30µl of 3 N sodium hydroxide resulted in the absorption of the carbon dioxide present. The pressure (P_2) was again measured. The difference between P_1 and P_2 was proportional to the amount of carbon dioxide absorbed. Addition of 30µl of a solution of 20% sodium hydrosulfite in 1 N KOH resulted in the reabsorption of oxygen. The difference between the final pressure (P_3) measured and P_2 was proportional to the volume of oxygen present.

TABLE IV. Metabolite assay conditions.

Metabolite measured	Buffer salts and cofactors	Concentration	Extract volume	pH	Enzymes added
ATP	TEA	100 mM			Glucose-6-phosphate
Creatine	$MgCl_2$	25 mM			dehydrogenase
phosphate	KCl	100 mM	0.1 ml	7.5	Hexokinase
Glucose-6-	Glucose	0.5 mM			Creatine-
phosphate	NADP	0.5 mM			phosphokinase
ADP	TEA	100 mM			Lactate
AMP	$MgCl_2$	25 mM			dehydrogenase
	KCl	100 mM	0.1 ml	7.5	Pyruvate
	PEP	1.21 mM			kinase
	NADH	0.25 mM			Myokinase
	ATP	1.0 mM			
Fructose-6-	TEA	100 mM			Glucose-6-phosphate
phosphate	$MgCl_2$	25 mM	0.1 ml	7.5	dehydrogenase
Glucose-6-	KCl	100 mM			Hexokinase
phosphate	NADP	0.5 mM			Hexose phosphate
Glucose	ATP	1.0 mM			isomerase
PEP	TEA	100 mM			Lactate
Pyruvate	$MgCl_2$	25 mM	0.44 ml	7.5	dehydrogenase
	KCl	100 mM			Pyruvate
	NADH	0.1 mM			kinase
	ADP	1.0 mM			
Lactate	NAD	0.25 mM			Lactate
	Hydrazine	200 mM	0.1 ml	9.5	dehydrogenase
	Glycine	250 mM			

Measurement of protein degradation in perfused muscle. Perfusions were initiated with 100 ml of perfusate containing 25 μCi of [^{3}H] tyrosine. A 0.5 ml sample of the perfusate was taken at the outset for measurement of initial [^{3}H] tyrosine specific activity. At the end of the washout period, a 0.5 ml sample was taken from the collected effluent for measurement of the total radioactivity lost in the washout. Following the washout, 0.5 ml samples of perfusate were taken from the reservior at 10 minute intervals for the duration of the perfusion for determination of [^{3}H] specific activity. The perfusate samples were deproteinized by addition of 4.5 ml of 10% TCA. The resulting suspension was centrifuged and the clear supernatant was assayed for total free tyrosine by the method of Waalkes and Udenfriend (1957). To 0.5 ml of the supernatant in a culture tube were added 1.0 ml of a 0.1% solution of 1-nitroso-2-napthol in 95% ethanol, and 1.0 ml of a solution containing 0.05% sodium nitrite in 14% nitric acid. The tubes were capped and placed in a 55^{o}C water bath for 30 minutes. Upon removal from the bath, 10 ml of dichlorethane were added to extract the unreacted 1-nitroso-2-napthol. The tubes were shaken vigorously then centrifuged at approximately 1000 x g for five minutes to separate the phases. The fluorescense of the aqueous (top) layer was measured at an excitation wavelength of 460 nm and an emission wavelength of 570 nm.

[^{3}H] tyrosine in the supernatant was measured by counting 0.3 ml of the supernatant in 3.0 ml of a scintillation cocktail containing 0.825% (w/vol) 2,4-diphenyloxazole (PPO), 0.025% 1,4-bis-2-(4 methyl-5-phenyloxazolyl)-benzene(POPOP), and 30% Triton X-100 (Rohm and Haas) in toluene.

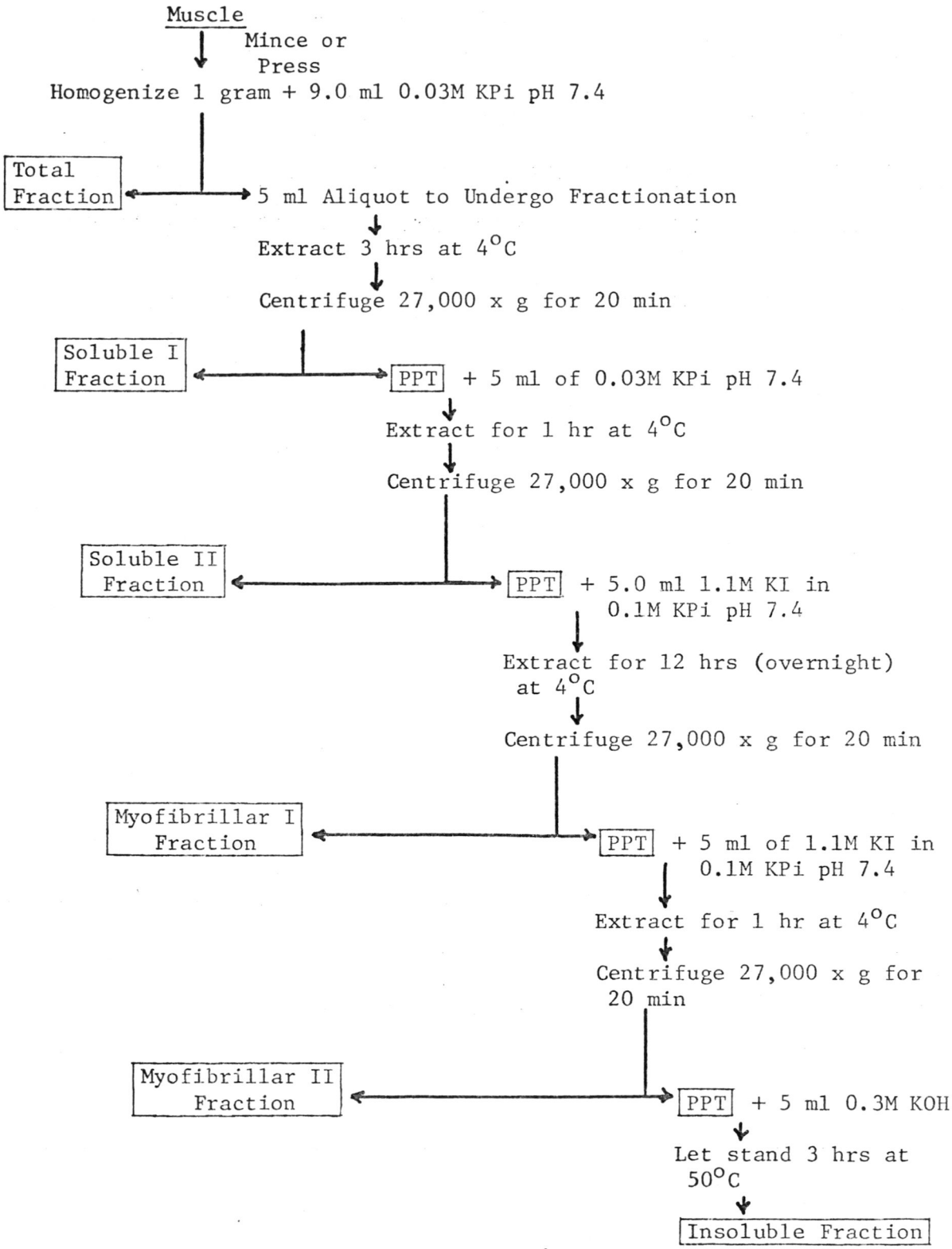
The total radioactivity in the system at the beginning of the recirculation (DPM_{t_1}) was determined by deducting the radioactivity (DPM) in the washout from the DPM in the original 100 ml of perfusate. By relating the DPM_{t_1} to the initial and final specific activity (SA_0 and SA_{60}) the nanomoles of tyrosine released by the preparation during the perfusion could be estimated (Jefferson et al. 1977).

$$\frac{DPM_{t_1}}{SA_{60}} - \frac{DPM_{t_1}}{SA_0} = \text{n moles released}$$

Measurement of protein synthesis in perfused muscle. The rate of protein synthesis was estimated by determining the rate of incorporation of [^{3}H] tyrosine into protein. At the end of perfusion, muscle samples were excised, minced using an Edco tissue press, and homogenized in nine volumes of 0.03 M potassium phosphate buffer (pH 7.4). Muscle protein classes were separated by buffer dissolution according to the flow diagram (Figure 5; Helander 1957).

Two ml aliquots of the protein solutions obtained were precipitated with 8.0 ml 10% TCA. In the early experiments these protein precipitates were washed three times by alternate centrifugation and resuspension in 5.0 ml portions of 10% TCA and finally in 5.0 ml ethanol saturated with sodium acetate. The protein pellet was dissolved in 5.0 ml 0.3 N KOH. Protein content in this solution was determined by the method of Gornall et al. (1949). Aliquots of the protein solution were air dried on combustable absorbent pads and were oxidized in a Packard Model 306 sample oxidizer. Radioactivity in the samples was determined using a Beckman LS233 liquid scintillation spectrometer. Recovery from the oxidizer and scintillation counting efficiencies were determined using internal standards.

Figure 5. Buffer dissolution of muscle protein classes.


Muscle
Mince or Press
Homogenize 1 gram + 9.0 ml 0.03M KPi pH 7.4
Total Fraction
5 ml Aliquot to Undergo Fractionation
Extract 3 hrs at 4°C
Centrifuge 27,000 x g for 20 min
Soluble I Fraction
PPT + 5 ml of 0.03M KPi pH 7.4
Extract for 1 hr at 4°C
Centrifuge 27,000 x g for 20 min
Soluble II Fraction
PPT + 5.0 ml 1.1M KI in 0.1M KPi pH 7.4
Extract for 12 hrs (overnight) 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
Extract for 1 hr at 4°C
Centrifuge 27,000 x g for 20 min
Myofibrillar II Fraction
PPT + 5 ml 0.3M KOH
Let stand 3 hrs at 50°C
Insoluble Fraction

It was observed that this technique of washing the precipitated protein did not remove free [^{3}H] tyrosine completely from the pellet. This resulted in an apparent high rate of incorporation during the washout period. To correct this error, several animals were perfused for the duration of the washout only. The apparent synthesis during this period was subtracted from the synthesis observed overall during the perfusion to give the net synthesis during the recirculation period.

In later experiments, 2.0 ml aliquots of the protein solutions obtained from the extraction procedure were precipitated by addition of 8.0 ml of 10% TCA. The precipitated protein was filtered onto Whatman #42 ashless filter paper using a ten place Hoefer filter holder. The retentate was washed with three 2.0 ml portions of 10% TCA. This procedure eliminated the apparent synthesis during the washout mentioned previously by effectively removing the free [^{3}H] tyrosine from the precipitated protein. The filters with the protein were oxidized in the sample oxizizer. Radioactivity was measured in the scintillation counter.

Characterization of the perfused hindquarter/hemicorpus. Before proceeding with the experiments on protein turnover it was necessary to demonstrate quantitatively that the perfused muscle model was metabolically sound. In order to do this, a number of parameters in perfused resting and perfused exercising muscle were compared with the same parameters in muscle rested and exercised *in situ*. Additionally, several parameters in the perfusate and blood from the *in situ* preparations were compared. It was also necessary to demonstrate that incorporation of labeled amino acid into muscle protein occurred at a constant rate over the duration of the perfusion, and that this incorporation rate was not

limited by compartmentalization between free amino acid pools in the tissue. Time studies were done to show that the specific activity of labeled amino acid in the muscle tissue was approximately the same as the specific activity in the perfusate over the duration of the perfusion, and to show that incorporation of label into protein was a function of the duration of the perfusion.

Determination of tissue integrity. Experimental animals weighing 450-520 grams were divided randomly into four groups and were treated as follows:

In situ rested animals were anesthesized with sodium pentobarbital. An abdominal incision was made and the abdominal wall was reflected. The femoral nerves were located. Electrodes were attached but the nerves were not stimulated. After 30 minutes, samples of venous and arterial blood were taken for blood gas analysis as described previously. Another venous blood sample was taken for glucose and lactate determination. The quadriceps muscles were then quickly excised and frozen with tongs which had been precooled in liquid nitrogen. The muscle samples were assayed for metabolites as described previously.

In situ exercised animals were treated like in situ rested animals with the addition of electrical stimulation of the femoral nerves at a rate of five per second at 1.2 millisecond duration with a Grass stimulator. Voltage was adjusted to be just high enough to elicit a muscular contraction (usually less than 3 volts). After 30 minutes, blood and muscle samples were taken as described above.

Perfused rested animals were prepared surgically as described previously for the perfused hindquarter. The femoral nerves were located and

electrodes were attached but the nerves were not stimulated. The preparation was perfused for 30 minutes. Samples of perfusate were taken initially and at the end of the perfusion for glucose and lactate analysis. Blood gas assays were done on arterial and venous samples taken at the end of perfusion. The quadriceps muscles were quick frozen for metabolites assays.

Perfused exercised preparations were similar with additional stimulation of the femoral nerves at a rate of five per second at 1.2 millisecond duration. Voltage necessary to induce muscular contraction increased during perfusion until no response was obtained.

In a preliminary experiment using a single perfused leg, [^{3}H] methoxyinulin was included in the perfusion medium so that extracellular space could be measured. The average extracellular space was estimated by comparing measured [^{3}H] methoxyinulin in a TCA soluble fraction from 24 perfused muscles from trained and untrained rats, with [^{3}H] methoxyinulin concentration in the final perfusate pool.

Time course of protein synthesis. Twenty-five hemicorpus preparations from animals weighing 335-370 grams were perfused with [^{3}H] tyrosine for either 15, 30, 50, 70, or 90 minutes such that five preparations were perfused for each duration given. Perfusate samples were taken initially and at ten minute intervals throughout the perfusion. Specific activity of [^{3}H] tyrosine in each sample was measured as described previously. At the end of the perfusion, the quadriceps muscles were excised and homogenized in potassium phosphate buffer. Two ml portions of the resulting homogenate were precipitated by the addition of 8.0 ml of 10% TCA. [^{3}H] tyrosine specific activity in the TCA

supernatant was determined as described previously. [^{3}H] tyrosine incorporation into the precipitated protein was determined.

Protein synthesis and degradation in trained animals. The effects of exercise training on protein synthesis and protein degradation in perfused muscle were measured in two very similar experiments. In both experiments animals initially weighing 180-200 grams were trained on a treadmill for six weeks. At the end of the training period hemicorpus preparations from the trained animals and from age matched controls were perfused with [^{3}H] tyrosine in the perfusate. In the first experiment, several preparations from both trained and untrained animals were perfused for the washout period only to serve as zero time controls. Improvement of technique (see discussion) later obviated this necessity and these blanks were not included in the second experiment or in the experiment on acute exercise.

Protein synthesis and degradation in acutely exercised animals. Animals weighing 290-330 grams were divided randomly into two groups. One group was made to swim for one hour, the other remained sedentary. Following the exercise bout, hemicorpus preparations were perfused. Determination of protein synthesis and degradation were done as described previously.

Statistical treatments. Statistical evaluation of the data obtained was performed using a one way analysis of variance.

RESULTS AND DISCUSSION

Characterization of the perfused muscle model. The tissue levels of metabolites which were measured were very similar in perfused resting muscle and muscle resting *in situ* (Table V). As might be expected, levels of high energy compounds decreased with exercise *in situ*, as did glycogen and glucose-6-phosphate. The increase in free glucose concentration in the exercising muscle, concomitant with the decrease in glucose-6-phosphate may indicate a decrease in hexokinase activity resultant from decreased ATP concentration. Lactate was observed to increase slightly in muscle exercised *in situ*. These same changes were observed in the perfused exercising muscle, but more drastic decreases in creatine phosphate and ATP and a doubling of the tissue lactate level suggested that the perfused exercising muscle operates under a more severe oxygen debt than muscle exercising *in situ*. Lactate/pyruvate ratios in the perfused exercising muscle indicate that the NAD^+/NADH ratio in the tissue is much decreased. This indicates a general lack of oxygen within the tissue.

Glucose and lactate levels (Table VI) measured in the perfusate and in the blood of the *in situ* preparations give some idea about why lactate accumulation in the perfused exercising muscles was so great. It was observed that glucose levels in the perfused preparations decreased slightly during the perfusion and that this was not increased by exercise. It has been proposed that the lack of insulin in the perfusate prevents removal of glucose from the medium. Although the final lactate level in perfusate from the resting preparations was only slightly higher

TABLE V. Tissue Levels of Selected Metabolites μmoles/g Dry Tissue *(mg/g dry tissue).

	In Situ Rested	Perfused Rested	In Situ Exercised	Perfused Exercised
ATP	20.10 ± 0.58	19.04 ± 0.27	15.94 ± 0.55	5.32 ± 1.01
ADP	3.81 ± 0.22	4.93 ± 0.13	4.88 ± 0.32	4.27 ± 0.08
AMP	0.37 ± 0.07	0.55 ± 0.06	0.76 ± 0.07	1.22 ± 0.01
Creatine Phosphate	25.75 ± 2.58	16.35 ± 2.92	19.38 ± 1.33	1.19 ± 0.18
Glucose	4.50 ± 0.40	3.59 ± 0.84	9.89 ± 1.51	9.34 ± 0.90
Glucose 6-phosphate	7.26 ± 0.76	7.73 ± 1.62	2.81 ± 0.48	3.13 ± 0.59
Pyruvate	0.40 ± 0.05	0.18 ± 0.05	0.16 ± 0.03	0.10 ± 0.02
*Glycogen	22.18 ± 1.30	22.69 ± 1.85	9.62 ± 1.58	6.83 ± 0.42
Lactate	18.78 ± 1.65	20.65 ± 1.28	23.13 ± 1.79	45.46 ± 2.67

TABLE VI. Glucose and Lactate Concentrations in Blood and Perfusate (μmoles/ml).

	Glucose		Lactate	
	Initial	Final	Initial	Final
In Situ Rested		4.31 ± 0.15		1.07 ± 0.10
In Situ Exercised		4.66 ± 0.33		1.28 ± 0.09
Perfused Rested	4.21 ± 0.14	3.28 ± 0.14	0.69 ± 0.03	1.93 ± 0.34
Perfused Exercised	4.96 ± 0.45	3.80 ± 0.38	1.56 ± 0.13	4.91 ± 0.53

than blood levels *in situ*, lactate was seen to increase markedly in the perfusate from the exercising preparations. This is partly due to the lack of a liver in the system to remove the lactate formed. As lactate levels in the perfusate increase, transport of lactate out of the cell should become increasingly difficult. This may also partly account for the high lactate levels within the exercising muscle.

Comparison of O_2 and CO_2 content in arterial and venous perfusate indicate that O_2 consumption within the perfused tissue occurs at a rate which approaches normal (Table VII). Oxygen consumption rate and CO_2 evolution rates were calculated from measured arterial/venous differences and measured flow rates of 11.6 ml/min for perfused resting, 10.0 ml/min for perfused exercising, and estimated flow of 10.0 ml/min through the hindquarter of the *in situ* preparations. Estimated oxygen consumption by perfused resting muscle appears to be higher than in perfused exercising muscle, but the oxygen consumption and carbon dioxide evolution rates for perfused exercising muscle compare favorably with the estimates for the *in situ* preparations. Even though the hematocrit in the perfusate was only one half of the normal (approx. 20%), the oxygen content on a volume percent basis in the perfusate was remarkably close to that of normal rat blood. This indicates that oxygenation of the perfusate was adequate, providing a higher percent oxygen saturation than normal, considering the reduced hematocrit.

Measurements of extracellular space in perfused muscle indicated that tissue swelling should not be a problem. Average extracellular space calculated for a group of 24 mixed trained and untrained animals was 15.2% of the muscle wet weight. There was no difference in

TABKE VII. Oxygen and Carbon dioxide Concentrations in Blood and Perfusate

	Venous		Arterial		O_2 Consumption ml/min/g muscle	CO_2 Evolution ml/min/g muscle
	Volume % CO_2	Volume % O_2	Volume % CO_2	Volume % O_2		
In Situ Rested	21.94 ± 2.61	12.63 ± 1.90	18.12 ± 2.13	16.66 ± 1.90	0.0050	0.0047
In Situ Exercised	20.14 ± 2.61	12.26 ± 1.12	18.33 ± 2.65	16.42 ± 1.78	0.0052	0.0022
Perfused Rested	21.10 ± 0.78	5.77 ± 1.47	9.12 ± 3.53	13.14 ± 1.53	0.0100	0.0162
Perfused Exercised	17.56 ± 2.93	8.46 ± 0.85	12.41 ± 0.35	12.96 ± 0.28	0.0054	0.0062

extracellular space between the trained and untrained groups. This value compares favorably with other estimates reported for normal rat quadriceps muscle (Puente 1978).

Incorporation of labeled amino acid ([^{3}H] tyrosine) by perfused muscle was observed to increase as a function of time (Figure 6). Extrapolation of the curve in Figure 6A to time "0" indicates an apparent synthesis of protein during the washout period. It was determined that this was due to incomplete removal of free label from the precipitated protein pellet. (Free radioactivity equivalent to the apparent 0 time incorporation in the soluble protein fraction from perfused muscle eluted from a sephadex G 100 column simultaneously with free tyrosine.) Improvement of our technique of washing the precipitated protein, as discussed in methods, eliminated this apparent synthesis (Figure 6B).

Specific activity of [^{3}H] tyrosine in the free amino acid pool in perfused muscle was found to increase rapidly to a level comparable to that of the perfusate. It was observed that this level was maintained after approximately 15 minutes for the duration of the perfusion (Figure 7), indicating that compartmentalization within the tissue does not limit apparent synthesis rates.

Rates of protein synthesis and degradation in the perfused hemicorpus of control animals compared very well with values in the literature for perfused controls (Jefferson et al. 1976). By assuming that a gram of wet muscle contains 200 mg protein and that the hemicorpus preparation Jefferson described weighs 100 g (probably conservative for a 250 gram animal) the synthesis rate which they observed in control animals (84 ± 8 nmole/hr/g muscle or 0.42 nmole/hr/mg protein) was comparable to our

Figure 6. Incorporation of [^{3}H] tyrosine into perfused muscle protein versus time.

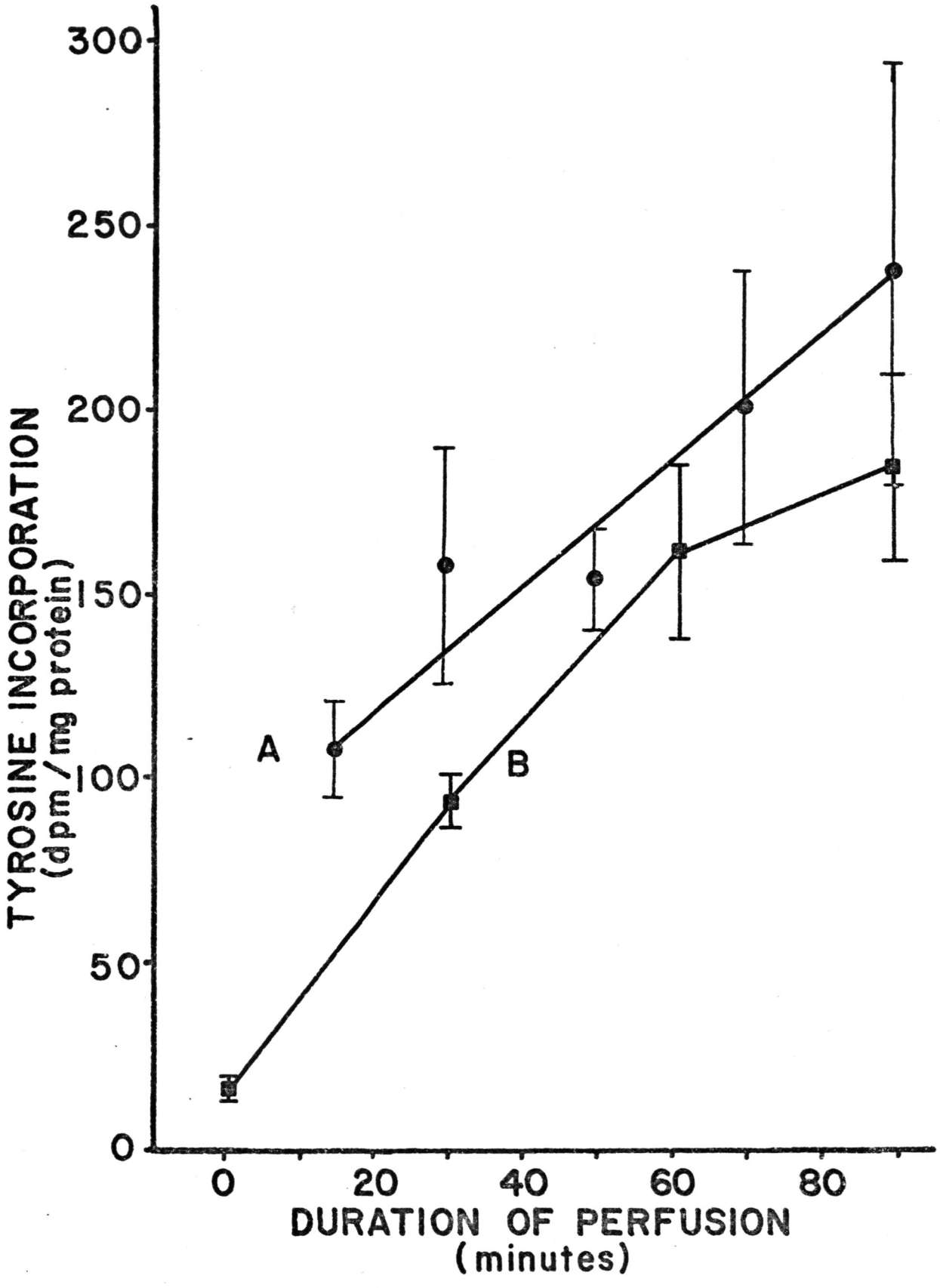
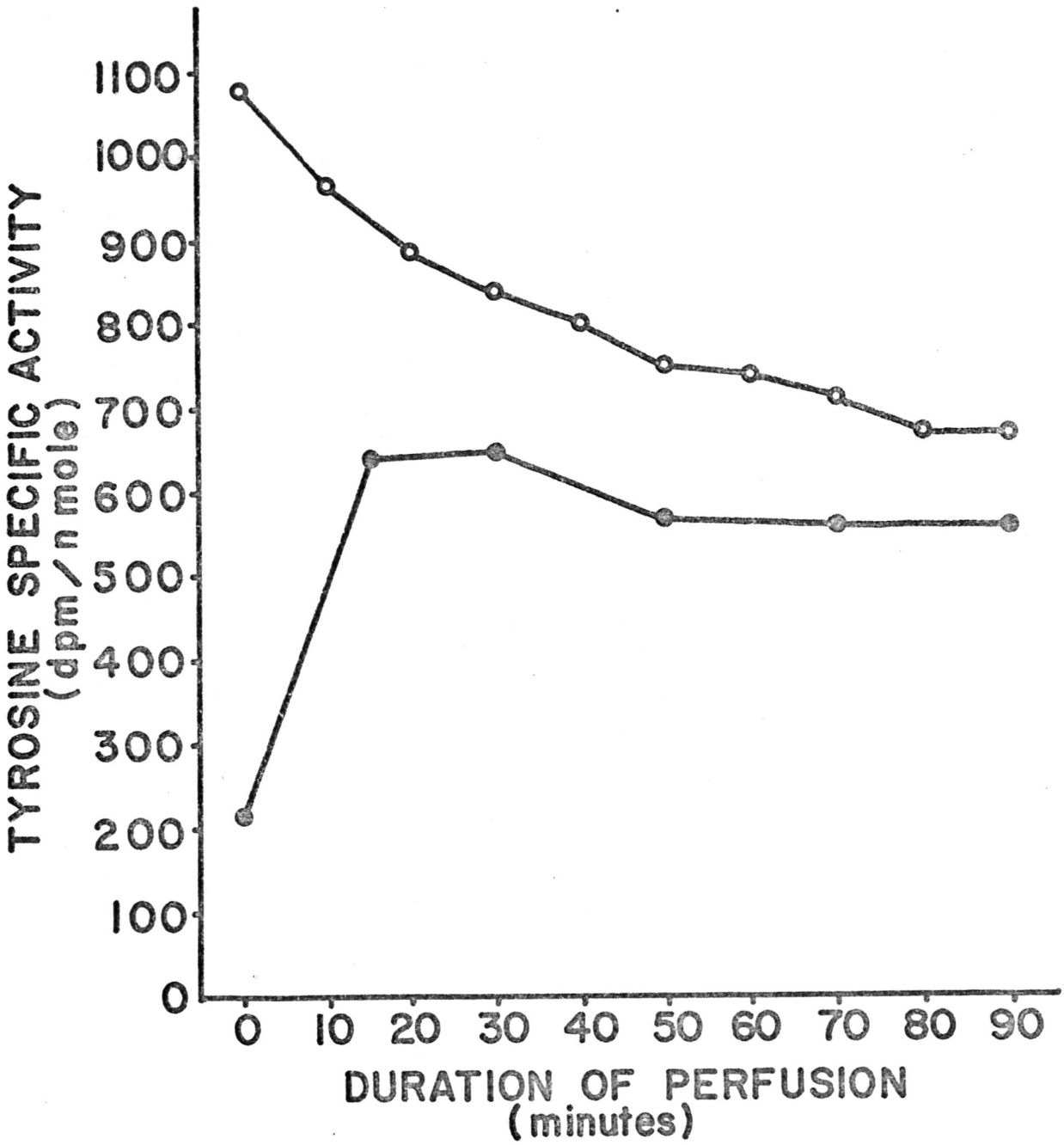

300
250
200
150
100
50
0
TYROSINE INCORPORATION
(dpm/mg protein)
A
B
0
20
40
60
80
DURATION OF PERFUSION
(minutes)

Figure 7. Specific activity of [^{3}H] tyrosine in perfusate (○–○) and muscle(●–●) versus time.


TYROSINE SPECIFIC ACTIVITY
(dpm/n mole)
1100
1000
900
800
700
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90
DURATION OF PERFUSION
(minutes)

measurements of 0.20 - 0.36 nmole/hr/mg protein. The degradation rate which they reported (88 $\pm$ 4 nmole/hr/g hemicorpus or 8.8 μmole/hr/hemicorpus) was comparable to our value of 8.9 μmole/hr/hemicorpus (≈ 160 nmole/hr/g muscle).

These experiments indicated to us that resting perfused muscle preparations are metabolically intact and that the perfused hemicorpus should provide an adequate model system for the study of protein metabolism in resting muscle. The indications of anoxia in the perfused exercising preparations led to some reservations regarding the use of perfused exercising muscle to estimate absolute rates of protein synthesis and degradation during exercise. It is proposed, however, that in properly controlled experiments perfused exercising perparations might also be used to some advantage. More specifically since the reduction of ATP and creatine phosphate and concomitant increases in AMP and lactate levels in the exercising perfused muscle resemble changes which occur during fatigue, this model could be useful in the study of various aspects of fatigue.

Effects of exercise training and acute exercise on protein synthesis. There was no difference observed between the rates of protein synthesis in trained and untrained animals in the perfused hemicorpus (Table VIII). There was however, a significant decrease in incorporation of radioisotope into all three protein classes in acutely exercised muscle (Table IX).

Several studies on the effects of exercise training have led to varying results. McManus et al. (1975) observed increased incorporation of [^{14}C] leucine into sarcoplasmic and myofibrillar protein fractions from

TABLE VIII. Effect of Training on Protein Synthesis in the Perfused Hemicorpus.

	[^{3}H] Tyrosine Incorporation (nmoles/hr/mg protein)			
	Soluble Protein	Myofibrillar Protein	Insoluble Protein	Whole Homogenate
Experiment #1				
Untrained	0.23 ± 0.05 (10)	0.16 ± 0.02 (10)	0.25 ± 0.03 (10)	0.20 ± 0.02 (10)
Trained	0.25 ± 0.05 (11)	0.14 ± 0.01 (11)	0.22 ± 0.02 (11)	0.19 ± 0.02 (11)
Experiment #2				
Untrained	0.20 ± 0.03 (10)†	0.12 ± 0.01 (10)	0.22 ± 0.03 (10)	0.17 ± 0.02 (10)
Trained	0.24 ± 0.04 (9)	0.12 ± 0.01 (9)	0.26 ± 0.02 (9)	0.20 ± 0.03 (9)

† Mean ± SEM with the number of observations in parenthesis.

TABLE IX. Effect of a Single Acute Bout of Exercise (1 hr swim) on Protein Synthesis in Perfused Muscle.

[^{3}H] tyrosine Incorporation (nmoles/hr/mg protein)

	Soluble Fraction	Myofibrillar Fraction	Insoluble Fraction	Whole Homogenate
Rested	0.36 ± 0.02 (9)	0.32 ± 0.01 (10)	0.31 ± 0.02 (8)	0.30 ± 0.10 (8)
Exercised	0.30 ± 0.02 (10)*	0.26 ± 0.02 (9)*	0.26 ± 0.01 (9)*	0.22 ± 0.15 (10)*

*Statistically significant difference ($P<0.05$) between acutely exercised and rested.

plantaris muscle in trained guinea pigs. The changes observed were small and since they did not measure precursor pool specific activity, it is possible that the changes in terms of absolute rate of synthesis were overestimated.

Dohm et al. (1977) observed a decrease in incorporation of [^{14}C] leucine into muscle protein (specifically insoluble protein) in vivo in trained rats. At the same time, a trend toward an increased incorporation of label into sarcoplasmic and myofibrillar protein was observed although these changes were not statistically significant. In that study, the specific activity of the [^{14}C] leucine in TCA soluble material from the muscle was observed to be similar in trained and untrained animals indicating that the decrease in incorporation observed reflected a real change in rate of synthesis.

Considering all of these studies, it appears that training results in no significant, reproducible change in protein synthesis. The variations in results from one report to another may be due to differences in techniques of measurement, and/or adaptive differences between muscle types and different species used.

Our findings that acute exercise decreases protein synthesis in the perfused hemicorpus corroborate the findings of Zimmer and Gerlach (1973). They observed a depression in protein synthesis rate following a bout of acute swimming exercise. The depression lasted approximately two hours following the exercise bout and the synthesis rates returned to normal later in the recovery period. Beecher et al. (1975) also observed that acute exercise resulted in a decrease in protein synthesis by isolated polyribosomes.

Measurements of protein synthesis in electrically stimulated isolated organs have led to varying results. Increases (Kendrick-Jones et al 1967) and decreases (Karpatkin and Samuels 9167) in incorporation of radio-label into electrically stimulated frog sartorius have been observed. Pain and Manchester (1970) found that electrically stimulated extensor digitorum longus from rat incorporated less label.

Again some variation in the effects of exercise due to species differences and tissue treatments might be expected but these results show consistantly that acute exercise results in a decrease in protein synthesis which might conserve amino acids for oxidation or gluconeogenesis. Reduced capacity for incorporation of label by isolated polyribosomes from acutely exercised animals indicates that some of the control over protein synthesis is exerted at the translational level. Other control points such as tRNA charging, chain initiation and elongation are yet to be investigated.

Effects of exercise training and acute exercise on protein degradation. Protein degradation rates were observed to be elevated in perfused preparations from both trained and acutely exercised animals (Tables X and XI). It is possible that the increased degradation observed in trained animals is a carryover from the last bout of exercise. These results are in accord with the findings of Kasperek et al. (1979). They found that training increased urinary 3-methylhistidine excretion and that acute exercise increased the concentrations of small peptides and tyrosine in muscle, and concentrations of 3-methylhistidine in plasma. These observations indicate that not only does exercise result in degradation of muscle protein, but that it results in degradation of

TABLE X. Effect of Training on Protein Degradation in the Perfused Hemicorpus.

	Tyrosine Released (nmoles/hr/g muscle)	
	Untrained	Trained
Experiment #1	166 ± 23 (10)†	208 ± 25 (11)
Experiment #2	167 ± 32 (8)	265 ± 86 (3)
Experiment #3	165 ± 11 (8)	238 ± 28 (8)*

†Mean ± SEM with the number of observations in parenthesis.

*Statistically significant difference ($P<0.05$) between trained and untrained.

TABLE XI. Effect of a Single Acute Bout of Exercise (1 hr swim) on Protein Degradation in Perfused Muscle.

	Protein Degradation	
	μmoles tyrosine released/hr/hemicorpus	nmoles tyrosine released/hr/g muscle
Rested	8.9 ± 0.6 (10)	163 ± 6 (10)
Exercised	13.8 ± 1.4 (8)	250 ± 38 (8)*

*Statistically significant difference ($P<0.05$) between acutely exercised and rested.

myofibrillar protein as evidenced by increased plasma levels of 3-methylhistidine.

The consequences of increased protein degradation in the exercising animal are bivalent. Protein which is degraded may provide amino acids for oxidation; on the other hand when myofibrillar elements are destroyed, the muscle becomes physically weaker. Excessive protein degradation would lead to a prolonged energy demand in the tissue following exercise as the proteins were resynthesized. Zimmer and Gerlach (1973) observed that the rate of protein synthesis increased to a level greater than control several hours into the recovery period after acute exercise. This may be an indication that protein turnover (both synthetic and degradative processes) is stepped up during the recovery period.

Kasperek et al. (1979) have investigated possible mechanisms responsible for the increase in protein degradation resultant from exercise. They found the activities of several proteases to be unaffected by training. In exhaustively exercised rats, the activity of free (unbound) cathepsin D in muscle was increased. This suggested that a release of lysosomal proteases might be responsible for the increased degradation observed following a bout of acute exercise.

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APPENDIX

ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
AIB	Aminoisobutyric acid
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
Ci	Curie
DPM	Disintigrations per minute
g	Gram, gravity
M	Molar
mg	Milligram
ml	Milliliter
mM	Millimolar
mm	Millimeter
µl	Microliter
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced
NADP	Nicotinamide adenine dinucleotide phosphate
nM	Nanometers
PEP	Phospho(enol) pyruvate
PPT	Precipitate
RNA	Ribonucleic acid
TCA	Trichloroacetic acid
TEA	Triethanolamine
w/vol	Weight per volume