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William E. Brown. AMINO ACID OXIDATION IN SKELETAL MUSCLE: ADAPTATION TO ENDURANCE TRAINING. (Under the direction of Dr. Lynis Dohm) Department of Biology, October, 1976.

The objectives of this study were as follows: (a) establish optimal assay conditions for leucine oxidation in muscle homogenates; (b) determine the relative importance of muscle in branched chain amino acid oxidation; (c) investigate the rate limiting step in leucine oxidation in muscle, heart, and liver; (d) and study the effect of endurance training on leucine oxidation in muscle homogenates. An in vitro assay for leucine oxidation and transamination was investigated and conditions that give near maximum rates of CO_2 production were established. Oxidation rates in heart and liver homogenates were 20-50 times greater than skeletal muscle. By assuming that muscle constitutes 45%, liver 4%, and heart 0.5% of total body weight, the total capacity of leucine oxidation was by order of tissue-liver, muscle, and heart. The rate limiting step in the oxidation of leucine by muscle, heart, and liver was investigated. The rate of transamination in muscle was 100-150 times higher than the rate of oxidation of L-(1-^{14}C)-leucine and 20-35 times greater in heart. In liver, transamination and the rate of oxidation were nearly the same suggesting that transamination is the rate limiting step. Comparing leucine oxidation by gastrocnemius muscle of trained and untrained rats demonstrated that trained animals tend to have a higher amino acid oxidation rate. Thus, it appears that muscle may play a significant part in the oxidation of branched chain amino acids in the body and during times of physiological stress (such as starvation or chronic exercise) muscle may adapt to give greater capacity for amino acid oxidation.

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AMINO ACID OXIDATION IN SKELETAL MUSCLE: ADAPTATION TO ENDURANCE TRAINING

A THESIS

Presented to

the Faculty of the Department of Biology

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by

William Edward Brown

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AMINO ACID OXIDATION IN SKELETAL MUSCLE: ADAPTATION TO ENDURANCE TRAINING

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Joseph G. Boyette

Dr. Joseph G. Boyette

Dedicated

to

My Mother and Father

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Until recently it was generally believed that nearly all branched chain amino acid oxidation occurred in liver. However, branched chain amino acid transaminase activity was found to be high in muscle and later work demonstrated that muscle could completely oxidize branched chain amino acids (1,2,3,4,5,6,7). Although it has been demonstrated that amino acid oxidation occurs in muscle, there has been a limited amount of research to show how important amino acids may be as energy sources in muscle.

Considering the importance of amino acid oxidation, Adibi *et al.* (8) reported that leucine was oxidized at a more rapid rate than palmitate for muscle tissue slices that were incubated in a medium in which the concentration of leucine and palmitate were equal in terms of carbon content. It is difficult to quantitate these results from tissue slices and intact muscle fibers because the resultant CO_2 production can be affected by the rate of transport of the radioactive substrate and by dilution of the labeled substrate with nonlabeled substrate inside the cell.

The regulation of leucine oxidation is thought to occur at one of the first two reactions, transamination or the oxidative decarboxylation of the resulting branched chain α-ketoacids. Shinnick and Harper (9) have reported that in liver the activity of transaminase and α-ketoisocaproic acid dehydrogenase were very similar, suggesting that transamination was probably the rate limiting step in leucine oxidation. Shinnick also reported that heart and skeletal muscle were high in

transaminase activity but low in α-keto acid dehydrogenase activity. This may tend to indicate that the α-ketoisocapraic acid dehydrogenase is rate limiting in heart or skeletal muscle.

The major portion of research concerning amino acid oxidation in skeletal muscle has dealt with the effects of dietary alterations; ie. low protein and high protein diets (10,11) and fasting and starvation (5,8). A preliminary study with amino acid oxidation in muscle slices (gastrocnemius) suggested that a strenuous program of running may result in an adaptation in the capacity of mixed muscles to oxidize leucine (12).

Because of the need for further information concerning amino acid oxidation the present study was undertaken. The goals of this research were: (1) to optimize assays for leucine oxidation in vitro for rat muscle homogenates; (2) to compare oxidation rates of red and white muscle tissue; (3) to investigate possible rate limiting enzymes in the oxidation of leucine; (4) to compare amino acid oxidation in muscle, heart, and liver to ascertain the importance of muscle in comparison with some other tissues in oxidation of leucine; (5) to compare the rate of leucine oxidation in muscle to previously published rates of palmitate oxidation to establish the relative importance of amino acids as energy sources in the muscle; and (6) to compare oxidation rates in muscle homogenates of trained and untrained rats.

LITERATURE REVIEW

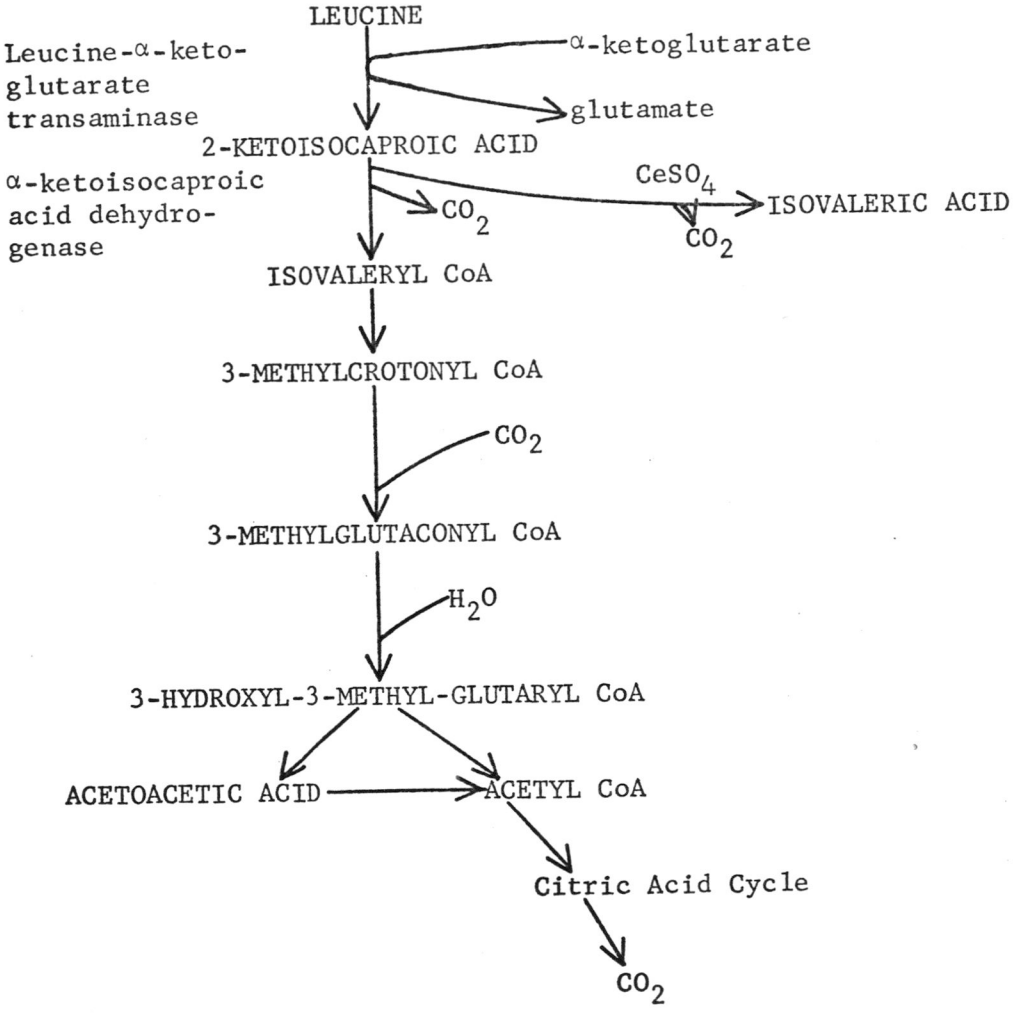
Figure 1 shows the metabolic pathway for the conversion of leucine to acetyl Co-A and acetoacetic acid. Acetoacetyl Co-A can be cleaved to form two molecules of acetyl Co-A, and acetyl Co-A may enter the tricarboxylic acid cycle enabling further degradation of the six carbon atoms from leucine (13). Although amino acids function primarily in formation of proteins and other biomolecules, this figure (Figure 1) shows a pathway by which leucine may be oxidized and thus become a source of energy for the body.

The actual rate of utilization of amino acids and the proportion of the different amino acids utilized in a given organism depends on many factors: the inherited ability to catabolize amino acids, the availability of amino acids, the dependence of the animal on the essential amino acids, the presence of other energy containing substances, the needs for protein synthesis, and the need for specific amino acids as precursors of other important biomolecules such as purines, pyrimidines, cell-wall components, hormones, and other specialized molecules (13).

The liver is generally believed to be the major site of amino acid catabolism in mammals (14). In recent years transaminases, including the branched-chain amino acid transaminases, have been found in high concentration in skeletal muscle (1,2) and it has been reported that amino acids may be oxidized by skeletal muscle (3,4,5,6,7). This has raised a question about the role of amino acids as a possible energy source for the muscle (5,15).

Very little work has been done concerning control of leucine oxidation. It has been shown that α-ketoglutarate stimulates CO_2

Figure 1. OXIDATION OF LEUCINE: Conversion of Leucine to Acetyl Co-A and Acetoacetic acid


LEUCINE
Leucine-α-keto-glutarate transaminase
α-ketoglutarate
glutamate
2-KETOISOCAPROIC ACID
α-ketoisocaproic acid dehydro-genase
CO_2
$CeSO_4$
CO_2
ISOVALERIC ACID
ISOVALERYL CoA
3-METHYLCROTONYL CoA
CO_2
3-METHYLGLUTACONYL CoA
H_2O
3-HYDROXYL-3-METHYL-GLUTARYL CoA
ACETOACETIC ACID
ACETYL CoA
Citric Acid Cycle
CO_2

production from leucine. It is thought that α-ketoglutarate regulates branched chain amino acid oxidation at two points: as an amino-group acceptor by accelerating transamination, and by accelerating the oxidation of the resulting branched chain α-keto acid (16).

A number of studies have been conducted to investigate the effect of dietary changes on amino acid catabolism. McFarlane and Von Holt (11) reported a significant decrease in oxidative degradation of leucine and phenylalanine in rats fed on a low protein diet, whereas, that of two nonessential amino acids (glutamate and alanine) were virtually unaffected. Sketcher et al. also found (in vivo) that leucine oxidation with DL-(1-^{14}C)-leucine decreased when adult rats were given a low protein diet. Researchers (10,11) have suggested that during periods of a low protein diet, there may be a block in the decarboxylation of α-ketoisocaproic acid as a result of reduced dehydrogenase activity as shown in liver mitochondrial preparations.

It has been reported that in diaphragms of rats that were fasted for three days, protein synthesis was fifty percent lower than normal values, whereas oxidation of leucine, isoleucine, and valine increased three to fivefold. Upon refeeding, protein synthesis in the diaphragm increased and leucine oxidation decreased to normal values (5).

After an overnight fast, alanine and glutamine represented over fifty percent of the amino acids released by skeletal muscle in vivo (17,18,19,20). An alanine cycle has been proposed in which synthesis of alanine from pyruvate by skeletal muscle provides a vehicle for transporting carbon and nitrogen to the liver for gluconeogenesis and urea formation (17,21). Odessey et al. (22) have shown the incorporation

of glucose into alanine in the muscle, and addition of branched chain amino acids increased the production of alanine from glucose. Thus, the transamination of branched chain amino acids may be important in control of the alanine cycle.

Hecker et al. (12) has reported that oxidation of (^{14}C) leucine was higher in trained than in untrained rats in vivo and the capacity for leucine oxidation of muscle slices from trained rats was higher than untrained rats. Several studies have shown that a program of running can result in a two-fold increase in the level of activity of a number of mitochondrial enzymes in skeletal muscle of rats (23,24,25,26). Baldwin et al. (27) has reported that endurance exercise produces an increase in the respiratory capacity of white, red, and intermediate muscle fiber types and red muscle fiber was found to have a significantly higher respiratory capacity than white muscle fibers (27,28). Although these studies suggest amino acid oxidation may be increased in muscle of trained rats, further studies measuring individual enzymes of the pathway in muscle homogenates are necessary.

MATERIALS AND METHODS

Animals and training: Male rats, from the Holtzman Company (Madison, Wisconsin) were housed one per cage and received a diet of Wayne Lab Blox ad libitum. At the time of sacrifice the trained rats weighed an average of 325 g and the untrained rats 375 g. Half of the rats were trained for six weeks as outlined in Table I and the other half served as untrained controls.

Tissue preparations: Animals were killed by a quick blow to the head followed by decapitation. Trained animals were sacrificed 18-24 hours after the last bout of exercise. The gastrocnemius and quadricep muscles were removed from the animals and placed in chilled homogenization media (10 mM potassium phosphate plus 1 mM EDTA, pH 7.5). Each muscle was pressed through a tissue press (Harvard Apparatus Co.) at 5,000 pounds of pressure. Muscles were homogenized by hand in a dual glass homogenizer (Ten Broeck from A. H. Thomas Co.). As indicated by Sketcher (10) hand homogenization was necessary because of destruction of α-ketoisocaproic acid degydrogenase activity if more vigorous techniques were used. A 1:6 homogenate (one gram of muscle to 5 ml of homogenization media) was filtered through four layers of cheese cloth and a portion of the homogenate from each muscle was boiled for 3-5 minutes to serve as a blank.

Radioactive materials: All radioactive materials were obtained from Amersham/Searle Corporation. L-(1-^{14}C)-leucine and L-(U-^{14}C)-leucine were purchased in 250 μCi packages and made up to 5 μCi/100 mM (pH 7.0) and stored at -20°C until use. Radioactivity was determined in a Beckman

Table I. 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.

liquid scintillation system (LS-233). The scintillation fluid contained 4 l toluene, 33 g PPO, 1 g POPOP, and 2 l triton.

Chemicals: Ceric sulfate (anhydrous) was purchased from Fisher Scientific Company. A saturated solution of ceric sulfate was prepared in 4 N sulfuric acid and placed in a two liter polyethylene bottle which was stored at room temperature.

Ethanolamine and 2-methoxyethanol were obtained from the Eastman Company. One part ethanolamine was mixed with 2 parts 2-methoxyethanol and stored at room temperature in a polyethylene bottle.

4 N sulfuric acid was made up to stop the enzymatic reaction. It was stored at room temperature in a glass bottle.

A 200 mM solution of α-ketoglutaric acid (Eastman Company) was prepared, adjusted to pH 7.0, and stored at -20°C in a polyethylene bottle.

Adenosine 5' triphosphate was purchased from Sigma and a 20 mM solution (pH 7.0) was prepared. It was placed in the freezer at -20°C in a polyethylene bottle where it remained until the day of the experiment.

L-malic acid was obtained from Sigma and made up to a concentration of 2 mM at pH 7.0. The malate solution was stored in a polyethylene bottle at -20°C until needed.

Coenzyme-A was prepared fresh each day of the experiment. It was made up to a concentration of 2 mM at pH 7.0. Purchases of coenzyme-A were from Sigma.

Magnesium chloride was obtained from Sigma and made up to a concentration of 40 mM at pH 7.0. The solution was placed in a polyethylene bottle and stored at -20°C.

On each day of the experiment dithiothreitol (Sigma Co.) was made up to 20 mM and pH 7.0.

Nicotinamide adenine dinucleotide was purchased from P-L Biochemicals, Inc. and was prepared fresh daily at a concentration of 2 mM and at pH 7.0. The solution was stored in a polyethylene bottle.

Sorrenson's phosphate buffer was made up to a concentration of 250 mM disodium hydrogen phosphate and 250 mM potassium dihydrogen phosphate at pH 7.5. This concentration was ten times the concentration actually used in the incubation chamber. The buffer was placed in a polyethylene bottle and stored at -20°C. Disodium hydrogen phosphate and potassium dihydrogen phosphate were obtained from Fisher.

Phosphate homogenization media was prepared to contain 10 mM potassium phosphate (pH 7.5) and 1 mM ethylenediamine tetraacetic acid. The homogenization media was stored at -20°C in a polyethylene bottle. Potassium phosphate was obtained from Fisher and ethylenediamine tetraacetic acid was obtained from Sigma.

The substrate, buffer, homogenization media (which was used to bring the concentration of the reagents to their desired final concentration), and cofactors were combined in a stock solution on the day of the experiment. The final concentrations were as follows: 10 mM α-ketoglutarate, 0.2 mM thiamine pyrophosphate chloride, 1 mM adenosine 5' triphosphate, 0.1 mM L-malic acid, 0.1 mM coenzyme-A, 2 mM magnesium chloride, 1 mM dithiothreitol, 50 mM Sorrenson's phosphate buffer (ph 7.5), and homogenization media (2.3 mM potassium phosphate and 0.231 mM ethylenediamine tetraacetic acid. The mixture of substrate, buffer, homogenization media and cofactors brought the total incubation volume to 1.3 ml.

Assay of enzyme activities: A 25 ml Erlenmeyer flask with a polyethylene center well which protruded down into the flask from a rubber stopper served as the reaction vessel. Radioactive leucine (0.2 ml) and enzyme homogenate (0.5 ml) were added to each flask to initiate the reaction. This brought the total incubation volume to 2.0 ml. Immediately before capping the vessel, the flask was flushed with oxygen.

The reaction was incubated for 1 hour at 36°C. At the end of that period the enzymatic reaction was stopped by adding 0.2 ml of 4 N sulfuric acid. Also, at this time 0.2 ml ethanolamine methyl cellosolve (1:2) was injected into the polyethylene centerwell for the purpose of trapping CO_2. Collection of CO_2 was continued for the next hour. A 0.15 ml aliquot of ethanolamine methylcellosolve was pipetted into counting vials containing 10 ml of scintillation fluid and the radioactive CO_2 was counted in the liquid scintillation counter.

Fresh caps and polyethylene center wells were then placed on the Erlenmeyer flasks and 6.0 ml of saturated $Ce(SO_4)_2$ in 4 N sulfuric acid was pipetted into the chamber for quantitative decarboxylation of the labeled α-ketoisocaproic acid. Ethanolamine methyl cellosolve (0.2 ml) was added to the centerwell for collection of CO_2. Again, collection of CO_2 was allowed for the next hour and 0.15 ml of ethanolamine methyl cellosolve was removed for counting.

Protein determination: The Biuret method was utilized for determining the amount of protein in the homogenate (29). Advantages of the Biuret test were simplicity, rapidity, and reproducibility of results.

Statistical tests: The data were subjected to a one-way analysis of variance and group comparisons (untrained, trained, white quadriceps, and red quadriceps) were made by the Neuman-Keul Test (30).

RESULTS

Biuret Protein Assay: Figure II shows a graph of absorbance versus protein for the Biuret protein determination. This standard curve was a means by which the analysis of protein (mg) per sample of homogenate was determined. The curve is linear to approximately 10 mg and then, no longer follows Beer's law.

Assay of ^{14}C-leucine oxidation: The method of determining the oxidation of labeled leucine was an *in vitro* assay involving the addition of one microcurie of leucine per assay (2.2×10^6 dpm or 1.32×10^6 cpm at 60% counting efficiency). The reaction was allowed to incubate for one hour and collection of $^{14}CO_2$ in 0.2 ml of ethanolamine methylcellosolve followed for one hour. During the preliminary study, the average counts per minute oxidized for L-(1-^{14}C)-leucine and L-(U-^{14}C)-leucine were 265 cpm and 313 cpm respectively. This is 0.02% of the amount of radioactively labeled leucine which is available for oxidation. The high variability observed in blanks and assay reactions may be explained by the large amount of labeled leucine (1μci/assay) which was used in each reaction flask and the low oxidative activity (0.02% oxidized) which existed in untrained skeletal muscle.

A problem in determining actual oxidative capacity of the muscle arose when it was found (in some instances) that a blank reaction flask (no enzyme) had more counts per minute than a reaction flask (with enzyme). It was found that adding various amounts of boiled enzyme resulted in variable amounts of $^{14}CO_2$ collected as shown in Figure III. Thus, a routine blank was run with boiled enzyme equivalent to the protein in the reaction flask.

Figure 2. Standard curve for protein determination

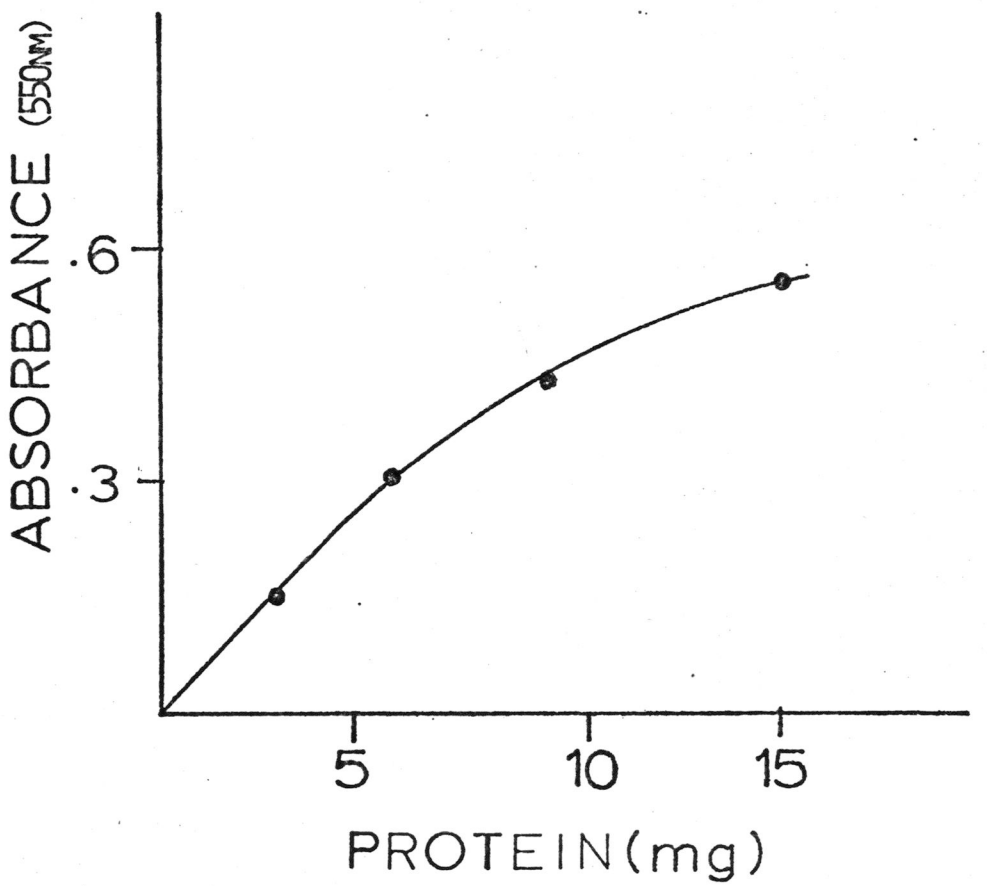
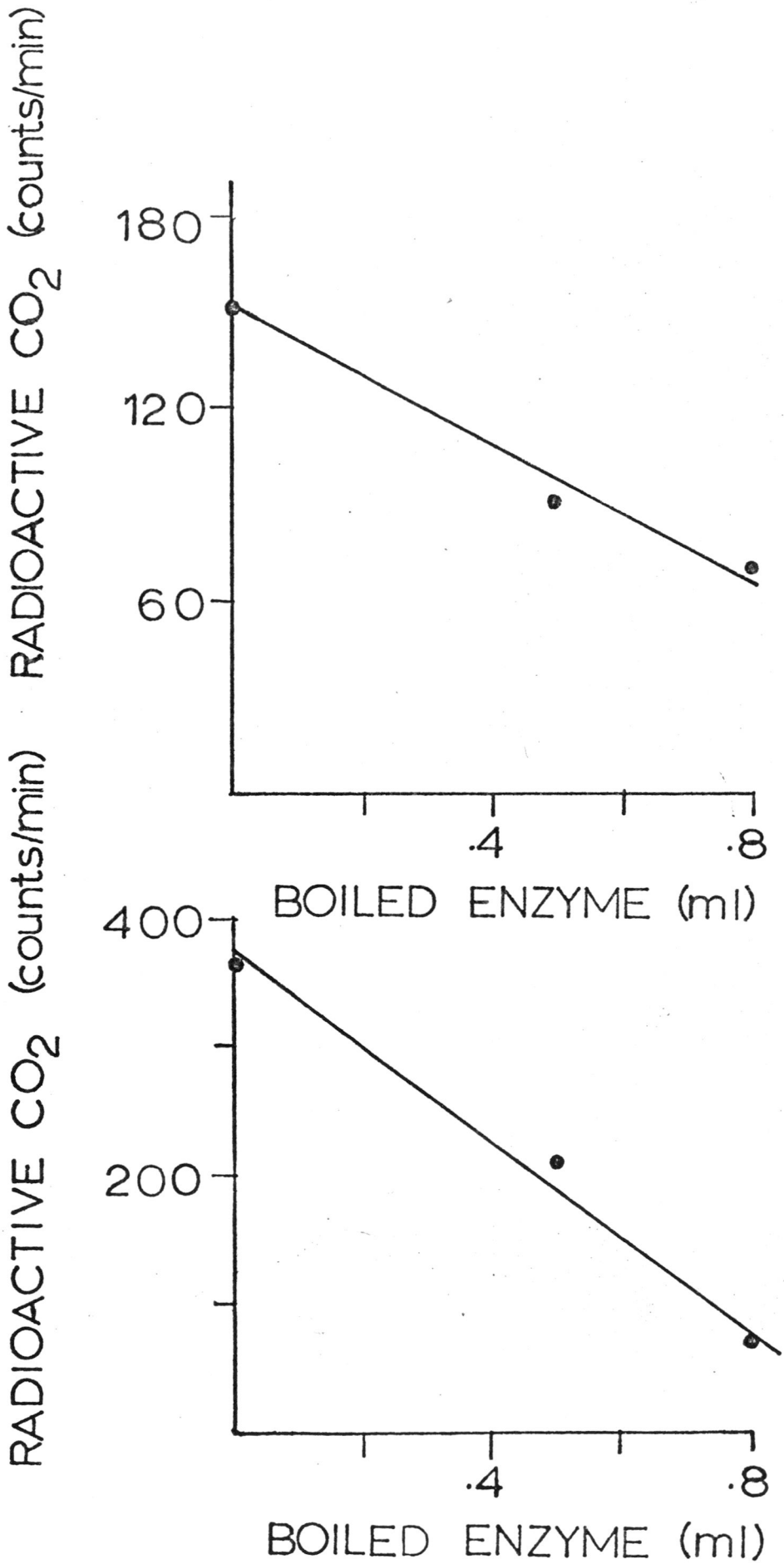

ABSORBANCE (550nm)
.6
.3
5
10
15
PROTEIN(mg)

Figure 3a. Dependence of the L-(U-^{14}C)-leucine blank on the amount of boiled homogenate

Figure 3b. Dependence of the L-(1-^{14}C)-leucine blank on the amount of boiled homogenate


RADIOACTIVE CO_2 (counts/min)
180
120
60
.4
.8
BOILED ENZYME (ml)
RADIOACTIVE CO_2 (counts/min)
400
200
.4
.8
BOILED ENZYME (ml)

Opitmal Buffer and Homogenization Media: Table II shows the results of a study of two different buffers (in the assay) and two homogenization medias. It became evident why Sketcher et al. had used a phosphate buffer when comparisons were made of a sucrose-Tris buffer (I) versus a phosphate buffer (II). The phosphate homogenization media (B) also gave better results compared to Chappell Perry homogenization media (A). The best results in oxidation and transamination were obtained when the phosphate buffer (II) was used with the phosphate homogenization media (B).

Optimal Concentrations of Substrates and Cofactors: One of the goals of this thesis was to establish assay conditions that would give maximal rates of leucine oxidation in skeletal muscle homogenates. Tables III, IV, and V are summaries of investigations into the concentrations of the cofactors and substrates that give the fastest rate of oxidation of L-(U-^{14}C)-leucine, L-(1-^{14}C)-leucine, and transamination of L-(1-^{14}C)-leucine respectively. The concentrations recommended by McFarlane and C. Von Holt (11) and Sketcher et al. (10) were 0.1 mM malate, 1 mM ATP, 1 mM DTT, 0.1 mM NAD, 0.1 mM Co-A, 0.2 mM TPP, 2 mM $MgCl_2$, 10 mM α-KG and 10 mM leucine. These concentrations were satisfactory.

Changes in $^{14}CO_2$ Production with Time and Amount of Enzyme: A very important aspect of the assay was to establish that transamination and oxidation were linear with time and amount of enzyme. Figures IV and V show the oxidation of L-(U-^{14}C)-leucine and L-(1-^{14}C)-leucine respectively. Although perfect linearity was not shown, oxidation increased with an increase in time and an increase in enzyme. Similar results were found in Transamination

Table II. Comparison of Homogenization Medias and Reaction Buffers

Activity (pmoles/min/mg)

Reaction	Homogenization Media	Buffer	Trial 1	Trial 2	Trial 3
Oxidation					
L-(U-^{14}C)-Leucine	A	I	27	23	3
	A	II	22		13
	B	I	40		7
	B	II		261	10
L-(1-^{14}C)-Leucine	A	I	35		
	A	II	3		
	B	I	29		
	B	II		161	
Transamination					
L-(U-^{14}C)-Leucine	A	I		1980	2460
	A	II			3110
	B	I			3580
	B	II		3480	3780
L-(1-^{14}C)-Leucine	A	I	802	1080	1810
	A	II	1020		2400
	B	I	1080		2940
	B	II		1430	4340

A Chappell-Perry (100 mM KCl, 50 mM Tris, 5 mM $MgSO_4$, 1 mM EDTA)
B KPi (10 mM KPi, 1 mM EDTA)
I Sucrose-Tris (100 mM Sucrose, 10 mM K_2HPO_4, 80 mM KCl, 0.1 mM EDTA)
II KPi (25 mM KH_2PO_4, 25 mM Na_2HPO_4)

Table III. Effects of Varying Cofactor and Substrate Concentrations on Oxidation of L-(U-^{14}C)-leucine

Variation	(mMol)	Activity (pmoles/min/mg) Exp. 10	Exp. 17	Exp. 18
Malate	0	4		
	0.05		3	3
	0.1	5	9	7
	0.2	4	10	4
	0.4	7		
ATP	0	5		
	0.4		6	10
	0.8	5	9	7
	1.7	3	6	7
	3.4	3		
DTT	0	1		
	0.5		8	9
	1	5	9	7
	2	4	8	8
	4	4		
NAD	0	6		
	0.05		8	7
	0.1	5	9	7
	0.2	5	5	3
	0.4	9		
Co-A	0	3		
	0.05		7	5
	0.1	5	9	7
	0.2	6	10	5
	0.4	7		
TPP	0	1		
	0.1	5	8	13
	0.2	9	9	7
	0.4		9	5
$MgCl_2$	0	6		
	0.5		7	10
	0.9	5	9	7
	1.9	6	5	11
	3.8	7		
α-Kg	0	2		
	2.5		5	2
	5		5	2
	7.5		5	6
	10	5	9	7
	20	3	6	3
Leucine	2.5		2	5
	5		4	4
	7.5		4	
	10		9	7
	20		6	

Table IV. Effects of Varying Cofactor and Substrate Concentrations on Oxidation of L-(1-^{14}C)-leucine

Variation	(mMol)	Activity (pmoles/min/mg) Exp. 12	Exp. 15	Exp. 16
Malate	0	17		
	0.05	16	6	19
	0.1	16	11	27
	0.2	13	13	38
ATP	0	17		
	0.4	13	14	19
	0.8	16	11	27
	1.7	18	13	16
DTT	0	12		
	0.5	5	9	20
	1	16	11	27
	2	7	8	28
NAD	0	4		
	0.05	7	6	20
	0.1	16	11	27
	0.2	10	22	23
Co-A	0	19		
	0.05	19	10	6
	0.1	16	11	27
	0.2	31	11	14
TPP	0	29		
	0.1	38	6	19
	0.2	16	11	27
	0.4	25	14	14
$MgCl_2$	0	42		
	0.5	31	10	17
	0.9	16	11	27
	1.9	22	12	27
α-Kg	0	20		
	2.5		13	25
	5	12	12	23
	7.5		11	25
	10	16	11	27
	20	22	12	19
Leucine	0			
	2.5		5	9
	5		6	17
	7.5		9	21
	10		11	27
	20		21	

Table V. Effects of Different Concentrations of Substrates and Cofactors on Transamination of L-(1^{14}C)-leucine

Variation	(mMol)	Activity (pmoles/min/mg) Exp. 12	Exp. 15	Exp. 16
Malate	0	577		
	0.05	442	725	942
	0.1	448	674	1530
	0.2	584	550	1850
ATP	0	509		
	0.4	507	773	1710
	0.8	448	674	1530
	1.7	583	807	1680
DTT	0	563		
	0.5	381	781	1720
	1	448	674	1530
	2	516	877	1610
NAD	0	472		
	0.5	489	630	1660
	0.1	448	674	1530
	0.2	487	580	1790
Co-A	0	490		
	0.05	705	582	1690
	0.1	448	674	1530
	0.2	564	346	1850
TPP	0	550		
	0.1	588	676	1850
	0.2	448	674	1530
	0.4	648	791	1520
$MgCl_2$	0	776		
	0.5	677	731	1850
	0.9	448	674	1530
	1.9	750	820	1520
α-Kg	0	11		
	2.5		776	1390
	5	676	710	
	7.5		941	1690
	10	448	674	1530
	20	231	674	1470
Leucine	2.5		399	540
	5		579	1470
	7.5		560	1260
	10		674	1530
	20		1320	

Figure IVa. Graph of L-(U-^{14}C)-leucine oxidation (nmoles/mg) versus time (min)

Figure IVb. Graph of L-(U-^{14}C)-leucine oxidation (nmoles/min) versus protein (mg)

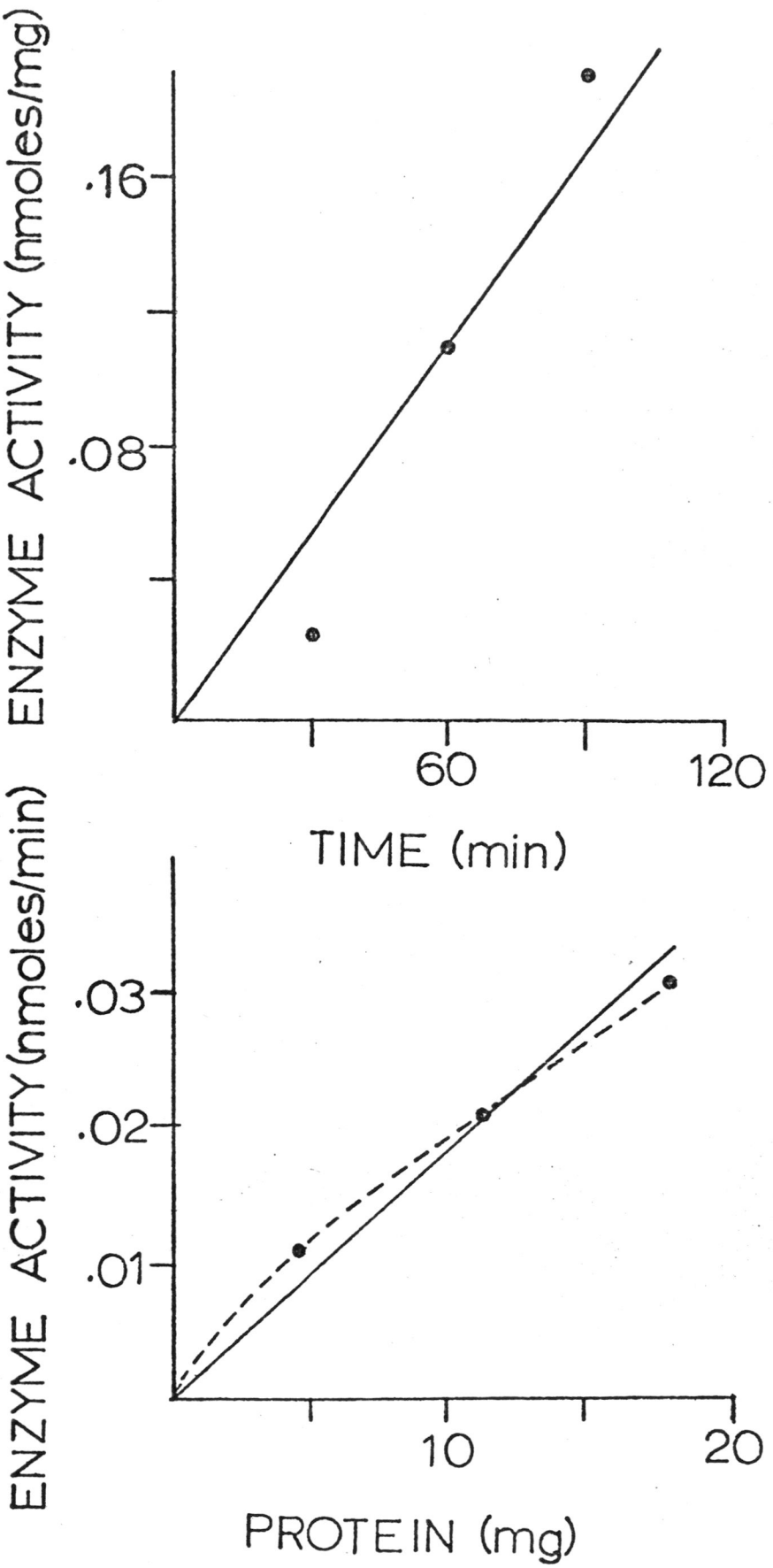

ENZYME ACTIVITY (nmoles/mg)
.16
.08
60
120
TIME (min)
ENZYME ACTIVITY (nmoles/min)
.03
.02
.01
10
20
PROTEIN (mg)

Figure Va. Graph of L-(1-^{14}C)-leucine oxidation (nmoles/mg) versus time (min)

Figure Vb. Graph of L-(1-^{14}C)-leucine oxidation (nmoles/min) versus protein (mg)

ENZYME ACTIVITY (nmoles/mg)
3
2
1
60
120
TIME (min)
ENZYME ACTIVITY (nmoles/min)
.4
.2
10
20
PROTEIN (mg)

(Figure VI). There was an increase in transamination when enzyme and time were increased. Again, there were problems in variability within the assay.

Leucine Oxidation in Muscle, Heart, and Liver of Rat, Hamster, and Guinea Pig: Table VI shows the results of a study to investigate the relative importance of amino acids as an energy source in muscle of three different animals. The oxidation of L-(U-^{14}C)-leucine was not greatly different in muscle, heart, and liver of all three species of animals; rat, hamster, and guinea pig. However, the oxidation rate in the heart and liver was 20-50 times greater than in muscle. The transaminase activity (Table VI) was 75-175 times higher than the rate of oxidation of L-(1-^{14}C)-leucine in muscle and 20-35 times greater in heart. In liver, however, the transaminase activity and the rate of oxidation of L-(1-^{14}C)-leucine were very similar. In the last column of Table VI, the percent of the total CO_2 oxidized from the number one carbon of L-(U-^{14}C)-leucine has been calculated. In the case of guinea pig muscle and liver it was clear that there was little or no oxidation past the α-ketoisocaproic acid dehydrogenase step as indicated by 93% and 99% respectively. In rat and hamster muscle, 40-50% of the L-(U-^{14}C)-leucine was the result of oxidation of the number one carbon atom. In heart 75% of the CO_2 came from the number one carbon atom.

Red Muscle Versus White Muscle: Table VII shows the transamination and oxidation rates of red quadriceps versus white quadriceps (activity/mg protein and activity/g tissue). There was a significant difference statistically between oxidation of red muscle versus white muscle with

Figure VIa. Graph of L-(1-^{14}C)-leucine transamination (nmoles/mg) versus time (min)

Figure VIb. Graph of L-(1-^{14}C)-leucine transamination (nmoles/min) versus protein (mg)

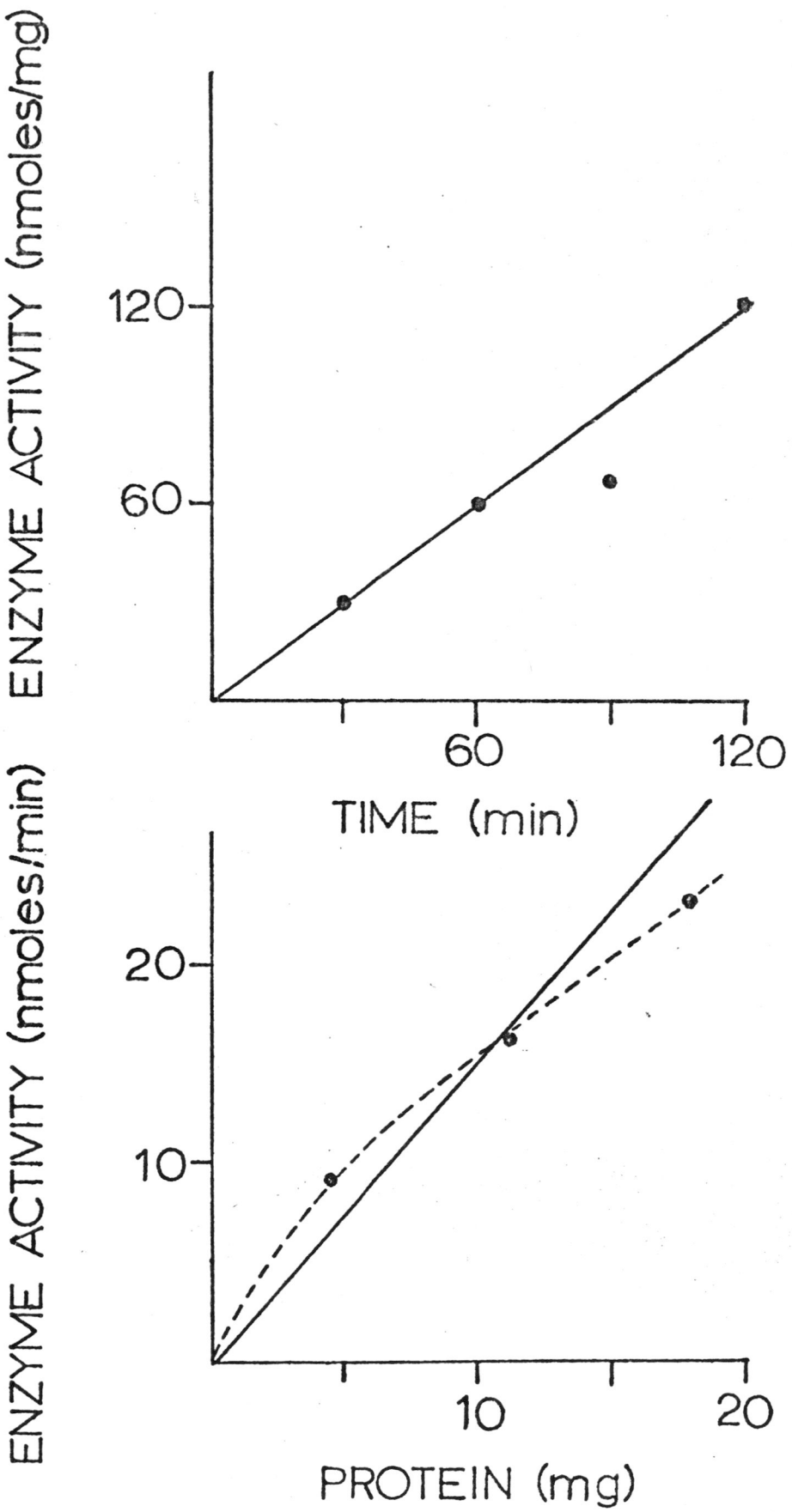

ENZYME ACTIVITY (nmoles/mg)
120
60
60
120
TIME (min)
ENZYME ACTIVITY (nmoles/min)
20
10
10
20
PROTEIN (mg)

Table VI. Oxidation of L-(1-^{14}C) and L-(U-^{14}C)-leucine and leucine transaminase activity of muscle, heart, and liver from rat, hamster, and guinea pig

Activity (nmoles/min/g tissue)

Species	Tissue	Leucine Transamination	1-^{14}C-Leucine Oxidation	U-^{14}C Leucine Oxidation	% of U-^{14}C-Leucine Oxidation from C-1
Rat	Gastrocnemius Muscle	79.5 ± 22.1	.456 ±.135	.192 ±.035	40
Hamster	Muscle	66.9 ± 3.9	.899 ±.186	.276 ±.045	54
Guinea Pig	Muscle	75.8 ± 5.5	.883 ±.082	.159 ±.025	93
Rat	Heart	909 ± 52	44.7 ± 5.7	10.2 ±.6	73
Hamster	Heart	764 ± 21	21.9 ±.8	4.87 ±.49	75
Guinea Pig	Heart	463 ± 74	15.3 ± 2.0	4.48 ±.46	57
Rat	Liver	42.6± 4.5	30.1 ± 6.4	7.70 ±.51	65
Hamster	Liver	68.6± 1.7	24.3 ± 1.9	5.23 ±.22	77
Guinea Pig	Liver	35.6± 3.6	17.2 ± 2.5	2.90 ±.34	99

Table VII. Oxidation and Transamination of Red Quadriceps muscle versus White Quadriceps muscle in Rat

Activity	(pmoles/min/mg protein or /g tissue)	(pmoles/min/mg protein or /g tissue)	(nmoles/min/mg protein or /g tissue)
Tissue	Uniform-^{14}C-leucine oxidation	1-^{14}C-leucine oxidation	Leucine α-ketoglutamate transaminase
	Activity/mg protein		
Red Quadriceps Muscle	7.18±1.41(8)	17.19±3.94(8)	2.09±0.13 (8)
White Quadriceps Muscle	3.71±0.41(7)*	7.1±1.64(8)*	1.65±0.28(8)
	Activity/g tissue		
Red Quadriceps Muscle	370.6±73.8(8)	898.9±207(8)	106.7±8(8)
White Quadriceps Muscle	182.7±20.5(7)	396±73(7)*	81±13.7(8)

*Red Quadriceps vs. White Quadriceps (P less than .05)

red muscle's oxidation rate being two times greater.

Effect of Training on Leucine Oxidation in Muscle: Table VIII shows the changes in the rate of oxidation of L-(U-^{14}C) and L-(1-^{14}C)-leucine and α-ketoglutarate transaminase activity in gastrocnemius muscle homogenates in response to exercise. In oxidation (activity/mg protein and activity/g tissue) there were trends of higher activity in trained rats versus untrained rats. This trend was so strong that it was decided to repeat the experiment with both the quadriceps and the gastrocnemius muscles. Similar results were found in Table IX (trained animals having higher oxidation activity than untrained animals).

Table VIII. Changes in the rate of oxidation of L-(U-^{14}C) and L-(1-^{14}C)-leucine and in leucine α-ketoglutarate transaminase activity in gastrocnemius muscle homogenates in response to exercise training (Experiment 1)

Activity	(pmoles/min/mg protein or /g tissue)	(pmoles/min/mg protein or /g tissue)	(nmoles/min/mg protein or /g tissue)
Group	Uniform-^{14}C-leucine oxidation	1-^{14}C-leucine oxidation	Leucine α-keto-glutamate transaminase
Gastrocnemius Muscle	Activity/mg Protein		
Untrained	3.73 ± 0.36 (15)	8.49 ± 1.33 (14)	1.62 ± 0.34 (8)
Trained	5.29 ± 0.82 (8)+	11.35± 2.88(8)	1.63 ± 0.14(8)
	Activity/g tissue		
Untrained	213 ± (15)	505 ± 91 (16)	94.1 ± 20.5 (8)
Trained	305 ± 47 (8)+	641 ± 155 (8)	92.5 ± 7.3 (8)

+Trained vs. Untrained (P less than .1)

Table IX. Changes in the rate of oxidation of L-(U-^{14}C)- and L-(1-^{14}C)-leucine and in leucine α-ketoglutarate transaminase activity in gastrocnemius and quadriceps muscle homogenates in response to exercise training (Experiment 2)

Activity	(pmoles/min/mg protein or /g tissue)	(pmoles/min/mg protein or /g tissue)	(nmoles/min/mg protein or /g tissue)
Group	Uniform-^{14}-C-leucine oxidation	1-^{14}-C-leucine oxidation	Leucine α-keto-glutamate transaminase
Gastrocnemius Muscle	Activity/mg protein		
Untrained	0.93 ± .10 (7)	2.02 ± .38 (7)	2.00 ± .11 (8)
Trained	1.36 ± .31 (7)	2.19 ± .44 (8)	1.68 ± .12 (8)
Quadriceps Muscle			
Untrained	0.73 ± .21 (7)	0.89 ± .19 (7)	2.02 ± .10 (8)
Trained	1.05 ± .22 (7)	1.56 ± .42 (8)	1.47 ± .10 (8)*
Gastrocnemius Muscle	Activity/g tissue		
Untrained	76 ± 10 (7)	172 ± 38 (7)	164 ± 8 (8)
Trained	126 ± 25 (7)+	208 ± 42 (8)	162 ± 9 (8)
Quadriceps Muscle			
Untrained	71 ± 20 (8)	87 ± 20 (8)	159 ± 10 (8)
Trained	102 ± 21 (7)	148 ± 40 (8)	141 ± 8 (8)

*Trained vs. Untrained (P less than .05)
+Trained vs. Untrained (P less than .1)

DISCUSSION

In working out the assay for leucine oxidation, it was very important for the incubation contents to be freshly prepared. This was a prominent factor in obtaining maximum rates of leucine oxidation. The optimum concentrations of cofactors and substrates have been indicated in the materials and methods and in the results of this paper. The assay conditions reported by McFarlane and Von Holt (11) and Sketcher et al. (10) appeared to give near maximal activity.

As was indicated earlier, the boiled homogenate which contained denatured protein worked well in providing a stable background activity. With this assay, it became evident that much variability existed in activity of skeletal muscle between rats. This was probably due to low oxidation rates in muscle. When examining the activity of heart and liver (Table VI), it was observed that in individual assays there was smaller variability than in skeletal muscle which may be the result of higher oxidation rates in liver and heart.

One of the major objectives of the experiments reported in Table VI was to try to quantitate the importance of skeletal muscle in amino acid oxidation. By observing columns four and five of Table VI, it may be seen that the oxidation rates of heart and liver (oxidation of 1-^{14}C and U-^{14}C leucine) were 20-50 times greater than skeletal muscle. By assuming that muscle constitutes 45%, liver 4%, and heart 0.5% of total body weight, the total capacity of leucine oxidation was by order of tissue-liver, muscle, and heart. This does not say that alterations in this order may not occur due to stress or diet.

Another objective of these studies (Table VI) was to investigate the importance of α-ketoisocaproic dehydrogenase on the total oxidation of leucine. As may be seen in Table VIII, IX, and column 3 of Table VI, transaminase was in high concentration (relative to oxidation) in skeletal muscle and heart. In muscle, transamination was 100-150 times higher than the rate of oxidation of L-(1-^{14}C)-leucine and 20-35 times greater in heart. With such high activity it would seem unlikely that transamination was rate limiting in oxidation of leucine in heart or muscle. In liver, however, transamination and the rate of oxidation were very close, suggesting that it is possible that transamination is the rate limiting step. This is in agreement with past work observed by Shinnick and Harper (9).

Felig and Wahren (32) observed an efflux of alanine from muscle and proposed a glucose alanine cycle. Odessey et al. (21) and Ruderman and Berger (31) have observed an increase in alanine released from the muscle when leucine was provided in the medium bathing the muscle. Odessey et al. (21) suggested the existence of a branched chain amino acid alanine cycle in muscle. Thus, the high activity of transaminases in muscle may possibly be a significant factor in the alanine cycle by providing an amino group for the transamination of pyruvate formed as a result of glycolysis.

The amount of CO_2 from C-1 of L-(U-^{14}C)-leucine oxidation (last column in Table VI) was calculated by dividing the rate of CO_2 production from L-(1-^{14}C)-leucine oxidation by six. This calculated number was divided by the rate of CO_2 production from L-(U-^{14}C)-leucine oxidation. This gave the percentage of U-^{14}C-leucine oxidized from the number one

carbon of leucine. If the oxidation of leucine is rate limiting at the α-ketoisocaproic acid dehydrogenase step and the rest of the molecule of leucine is oxidized rapidly and completely, then the percentage of U-^{14}C-leucine oxidized from carbon one would be 17 percent theoretically. Odessey and Goldbert (5) have suggested that this ideal figure (17%) may not be obtained since degradation of L-(U-^{14}C)-leucine forms many radioactive intermediates such as acetate, acetoacetate, etc., which may be diluted by non-radioactive intermediates. If no oxidation occurs after the α-ketoisocaproic acid dehydrogenase step, the value would be 100 percent. Based on this presumptive theory it appears that no oxidation occurs in guinea pig muscle (93%) and liver (99%) past the α-ketoisocaproic acid dehydrogenase step. It thus seems evident that α-ketoisocaproic acid dehydrogenase and α-ketoglutarate transaminase were not rate limiting in oxidation of leucine in guinea pig muscle and liver. No definite conclusions could be reached with heart (60%-75% oxidized from carbon one) or rat and hamster muscle (40% and 54% respectively).

Oxidation of leucine (Table VII) was shown to be significantly greater (P less than .05) in red muscle fibers. This was probably related to the greater respiratory capacity of red muscle fibers (27).

Comparing leucine oxidation (Table VIII) by gastrocnemius muscle of trained and untrained rats demonstrated that trained animals have a higher amino acid oxidation rate. This was repeated in subsequent work (Table IX). Although this was significant only to the P less than 0.1 level, it is a reproducible effect and thus seems to represent an adaptation to training. The quadriceps muscle also showed trends indicating

that trained rats oxidize leucine at a greater rate than untrained rats (Table IX). Transaminase activity was not altered by training; however, since transaminase was several fold higher than the rate of oxidation, an increase in transamination rate was not required for an increase in oxidation.

Hecker et al. have shown that the oxidation of (^{14}C) leucine was higher in trained than in untrained rats in vivo and the capacity for leucine oxidation of muscle slices from trained rats was higher than untrained rats (12). This study (12) also indicates an adaptation to oxidation of leucine with endurance training.

It has been shown that under conditions of protein calorie deficient rats (10,11), fasted rats (5), and exercised rats [Table VIII and IX and (12)]that oxidation of branched chain amino acids may increase. The purpose of this increase in catabolism may be to supply additional energy during these periods of deprivation of the normal food source. While it has been indicated that the liver is the primary site of amino acid catabolism in the normal animal, it appears that muscle may play a prominent role in oxidation of amino acids during unusual circumstances.

SUMMARY

1. The assay conditions for oxidation and transamination of leucine were investigated and conditions that give maximum rates of CO_2 production were established.

2. It was found that boiled homogenate worked well in gaining a stable background activity.

3. The order of total capacity for leucine oxidation was liver, muscle, and heart.

4. Transaminase was in high concentration in skeletal muscle. It was suggested that the transaminase was not rate limiting in muscle or heart. In liver, however, it appeared that transaminase was the rate limiting step.

5. The percent of the total CO_2 evolved from L-(U-^{14}C)-leucine that was the result of the oxidation of the number one carbon atom has been calculated.

6. In rat and hamster muscle it has been suggested that α-ketoisocaproic acid dehydrogenase could be the rate limiting reaction.

7. In the case of guinea pig muscle and liver, it was clear that there was little or no oxidation past the α-ketoisocaproic acid dehydrogenase step.

8. Trained animals may have a higher amino acid oxidation rate than do untrained animals.

9. Transamination was not affected in the gastrocnemius muscle of untrained and trained rats.

10. Oxidation was shown to be greater in red muscle fibers than in white muscle fibers.

REFERENCES CITED

1. Ichihara, A., and Koyama, E. 1966. Transaminase of branched chain amino acids. J. Biochem. 59:160-196.

2. Young, V. R. 1970. The role of skeletal and cardiac muscle in the regulation of protein metabolism. Pages 586-674 in H.N. Munro, Mammalian protein metabolism. Academic Press, New York.

3. Adibi, S. A., Morse, E., and Amin, P. 1971. Adaptive changes in leucine metabolism; alteration in pool size and oxidative degradation in tissue. Clin. Res. 19:470.

4. Buse, M. G., Biggers, F., Frederici, K., and Buse, J. F. 1972. Oxidation of branched chain amino acids by isolated hearts and disphragms of the rat. J. Biol. Chem. 247:8085-8096.

5. Goldberg, A. L., and Odessey, R. 1972. Oxidation of amino acids by diaphragms from fed and fasting rats. Am. J. Physiol. 223:1384-1391.

6. Manchester, K. L. 1965. Oxidation of amino acids by isolated rat diaphragm and the influence of insulin. Biochem. Biophys. Acta. 100:295-298.

7. Meikle, A. W., and Klain, G. J. 1972. Effect of fasting and fasting-refeeding on conversion of leucine into CO_2 and lipid in rats. Am. J. Physiol. 22:1246-1250.

8. Adibi, S. A., Krzysik, B. A., Morse, E. L., Amin, P. M., and Allen, E. R. 1974. Oxidative energy metabolism in the skeletal muscle: biochemical and ultrastructural evidence for adaptive changes. J. Lab. Clin. Med. 83:548-562.

9. Shinnick, F. L., and Harper, A. E. 1975. Branched chain amino acid catabolism in the rat. Fed. Proc. 34:880.

10. Sketcher, R. D., Fern, E. B., and James, W. P. T. 1974. The adaptation in muscle oxidation of leucine to dietary protein and energy intake. Br. J. Nutr. 31:333-342.

11. McFarlane, I. G., Von Holt, C. 1968. Metabolism of leucine in protein-calorie-deficient rats. Biochem. J. 11:565-571.

12. Hecker, A., Klain, G., Askew, E. W., and Wise, W. R. Jr. 1975. Adaptation to exercise: leucine metabolism in skeletal muscle. Physiologist. 18:242.

13. Lehninger, Albert L. 1972. Biochemistry. Worth Publishers, Inc., New York. 833pp.

14. Bollman, J. L., Mann, F. C., and Magath, T. B. 1926. Studies on the physiology of liver. Effect of total removal of the liver on deaminization. Am. J. Physiol. 78:258-269.

15. Lowenstein, J. M. 1972. Ammonia production in muscle and other tissues. Physiol. Rev. 52:382-414.

16. Buse, M. G., Jursinic, S., and Reid, S. S. 1975. Regulation of branched-chain amino acid oxidation in isolated muscles, nerves, and aortas of rats. Biochem. J. 148:363-374.

17. Felig, P., Pozefsky, T., Morliss, E., and Cahill, G. F. Jr. 1970. Alanine: Keyrole in gluconeogenesis. Science. 167:1003-1004.

18. Marlis, E. B., Aoki, T. T., Pozefsky, T., Most, A. S., and Cahill, G. F. Jr. 1971. Muscle and splanchnic glutamine and glutamate metabolism in postabsorptive and starved man. J. Clin. Invest. 50:814-817.

19. Pozefsky, T., Felig, P., Tobin, J. D., Soeldner, J. S., and Cahill, G. F. Jr. 1969. Amino acid balance across tissues of the forearm in postabsorptive man; Effects of insulin at two dose levels. J. Clin. Invest. 48:2273-2282.

20. Ruderman, N. B., and Tund, P. 1972. Amino acid metabolism in skeletal muscle; Regulation of glutamine and alanine release in the perfused rat hindquarter. Israel J. Med. Sci. 8: 295-302.

21. Mallette, L. E., Exton, J. H., and Park, C. R. 1969. Control of gluconeogenesis from amino acids in the perfused rat liver. J. Biol. Chem. 244:5713-5723.

22. Odessey, R., Khairallah, E. A., and Goldberg, A. L. 1974. Origin and possible significance of alanine production by skeletal muscle. J. Biol. Chem. 249:7623-7629.

23. Fuge, K. W., Crews, E. L. III, Patten Gale, P. K., Holloszy, J. O., and Shank, R. E. 1968. Effects of protein deficiency on certain adaptive responses to exercise. Am. J. Physiol. 215:660-663.

24. Holloszy, J. O. 1967. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. J. Biol. Chem. 242:2278-2282.

25. Holloszy, J. O., Oscai, L. B., Don, I. J., and Mole, P. A. 1971. Mitochondrial citric acid cycle and related enzymes: adaptive response to exercise. Biochem. Biophys. Res. Commun. 40: 1368-1373.

26. Mole, P. A., Oscai, L. B., and Holloszy, J. O. 1971. Adaptations of muscle to exercise: increase in levels of palmityl Co-A synthetase, carnitine palmityl transferase, and palmityl Co-A degydrogenase, and in the capacity to oxidize fatty acids. J. Clin. Invest. 50:2323-2330.

27. Baldwin, K. M. Klinkerfuss, G. H., Terjuny, R. L., Mole, P. A., and Holloszy, J. O. 1972. Respiratory capacity of white, red, and intermediate muscle: adaptative response to exercise. Am. J. Physiol. 222:373-378.

28. Beatty, C. H., Curtis, S., Young, M. K., and Borck, R. M. 1974. Oxidation of amino acids by red and white muscle fiber groups. Am. J. Physiol. 227:268-272.

29. Gornall, A. G. Barawill, C. J., and Maxima, D. M. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751-766.

30. Winer, B. J. 1971. Statistical principles in experimental design. Pages 184-221 in B. J. Winer, Design and analysis of single factor experiments. McGraw-Hill, New York.

31. Ruderman, Neil B., and Berger, Iichael. 1974. The formation of glutamine and alanine in skeletal muscle. J. Biol. Chem. 249:5500-5506.

32. Felig, P., and Wahren, J. 1971. Page 205 in Pernov, B. and Saltin, B. Muscle metabolism during exercise. Plenum Press, New York.

APPENDIX

ABBREVIATIONS

CO_2	Carbon dioxide
Co-A	Coenzyme-A
A	Absorbance
mM	Millimolar
mg	Milligram
CPM	Counts per Minute
PPO	2,5-Diphenyloxazole
PoPOP	1,4-bis- (2-(4-methyl-5-phenyloxazoyl))-Benzene
ATP	Adenosine Triphosphate
NAD	Nicotine Adenine Dinucleotide
DTT	Dithiothreitol
$MgCl_2$	Magnesium Chloride
TPP	Thiamine Pyrophosphate Chloride
α-Kg	α-Ketoglutarate
Ci	Curie
pmoles	Picomoles
nmoles	NaNomoles

INSTRUMENTS

Beckman Liquid Scintillation System LS-233

Perkin-Elmer Coleman Linear Absorbance Spectrophotometer Model 44

Tissue Press

Shaking Water Bath, American Optical Corporation Scientific Instruments Division Model 02156

Fisher Accumet Expanded Scale Research PH Meter Model 320

Mettler Balance P162N

Chemicals	Company	Lot No.
L-leucine	Sigma	34C-2770
L-Uniform Labled leucine	Amersham/Searle Corp.	CFB.67
L-[illegible]abeled leucine	Amersham/Searle Corp.	CFA.273
Toluene	Fisher	755405
PPO	Packard	3119
POPOP	Packard	0901
Triton X-100	Sigma	124C-0266
Sucrose	Mallinckrott	BNR
Potassium Dihydrogen Phosph.	Fisher	734634
Potassium Chloride	Fisher	714195
Ethylenediamine Tetraacetic Acid	Sigma	42C-3120
Sodium Phosphate Dibasic	Fisher	720358
Trizma	Sigma	43C-5440
Magnesium Sulfate	Fisher	730605
Dipotassium Hydrogen Phosph.	Fisher	712096
α-Ketoglutaric Acid	Eastman	A5A
Thiamine Pyrophosphate Chloride	Sigma	44C-1100
Magnesium Chloride	Sigma	1110-17216
Coenzyme-A	Sigma	84C-0138
Adenosine 5-Triphosphate	Sigma	52C-7240
Dithiothreitol	Sigma	84C-0138
L-Malic Acid	Sigma	510-0560
Nicotinamide Adenine Dinucleotide	P-L Biochemicals, Inc.	3208814
Ceric Sulfate	Fisher	746625
Ethanolamine	Eastman	C4AB
2-Methoxy ethanol	Eastman	B5A
Potassium Sodium Tartrate	Mullinckrodt	WITHL
Cupric Sulfate	Mullinckrodt	4744
Sodium Hydroxide	Fisher	74188