Leucine

A POSSIBLE REGULATOR OF PROTEIN TURNOVER IN MUSCLE

MARIA G. BUSE and S. SANDRA REID

From the Departments of Medicine and Biochemistry, Medical University of South Carolina, Charleston, South Carolina 29401

ABSTRACT Incorporation of radiolabeled precursors into muscle proteins was studied in isolated rat hemidiaphragms. A mixture of three branched-chain amino acids (0.3 mM each) added to media containing glucose stimulated the incorporation of [^{14}C]lysine into proteins. When tested separately, valine was ineffective, isoleucine was inhibitory, but 0.5 mM leucine increased the specific activity of muscle proteins during incubation with [^{14}C]lysine or [^{14}C]acetate in hemidiaphragms from fed or fasted rats incubated with or without insulin.

Preincubation with 0.5 mM leucine increased the specific activity of muscle proteins during a subsequent 30- or 60-min incubation with [^{14}C]lysine or [^{14}C]pyruvate without leucine. Preincubation with other amino acids (glutamate, histidine, methionine, phenylalanine, or tryptophan) did not exert this effect. When hemidiaphragms were incubated with a mixture of amino acids at concentrations found in rat serum and a [^{14}C]-lysine tracer, the specific activity of muscle proteins increased when leucine in the medium was raised from 0.1 to 0.5 mM.

Experiments with actinomycin D and cycloheximide suggested that neither RNA synthesis nor protein synthesis are required for the initiation of the leucine effect. Leucine was not effective when added after 1 h preincubation without leucine.

The concentration of lysine in the tissue water of diaphragms decreased during incubation with 0.5 mM leucine in the presence or absence of cycloheximide, suggesting that leucine inhibited protein degradation. During incubation with [^{3}H]tyrosine (0.35 mM) the addition of 0.5 mM leucine increased the specific activity of muscle proteins, while the specific activity of intracellular tyrosine remained constant and its concentration decreased, suggesting that leucine also promoted protein synthesis.

The concentration of leucine in muscle cells or a compartment thereof may play a role in regulating the turnover of muscle proteins and influence the transition to negative nitrogen balance during fasting, uncontrolled diabetes, and the posttraumatic state. Leucine may play a pivotal role in the protein-sparing effect of amino acids.

Parts of this paper have been presented at the 28th Annual Meeting of the Southern Society for Clinical Investigation (1974, *Clin. Res.* 22: 58A) and at the XIth International Cancer Congress (1974, Florence Italy. Abstracts, Vol. 2. 33).

Received for publication 27 December 1974 and in revised form 14 July 1975.

INTRODUCTION

The branched-chain amino acids leucine, isoleucine, and valine are the only essential amino acids that are oxidized mainly extrahepatically (1). Although a large number of tissues can oxidize them, skeletal muscle, because of its bulk, is thought to be the principal site of branched-chain amino acid catabolism (2-4). Recently, several laboratories reported that the rate of oxidation of the branched-chain amino acids by muscles is under metabolic and hormonal regulation (2, 3, 5–9). It is stimulated by fasting (2, 6, 7), severe stress (8), and uncontrolled diabetes (9), conditions that are associated with wasting of muscle protein and negative nitrogen balance.

The accelerated oxidation of branched-chain amino acids under conditions of stress and carbohydrate deprivation supplies energy to muscle as well as NH_2 groups for the synthesis of alanine and glutamine. The latter are released from muscle and used as substrates of gluconeogenesis by the liver and kidneys (10-13). In addition, it is conceivable that the accelerated oxidation of one or more of the branched-chain amino acids depletes a critical compartment of the muscle cells and thus serves as a signal to accelerate the catabolism of muscle proteins and/or inhibit their rate of synthesis. The data to be presented support this hypothesis and

suggest that leucine may act as a regulator of protein turnover in muscles, which is a major factor in the adaptive regulation of the body's fuel economy.

METHODS

Male rats of the Wistar strain weighing 120–180 g were used. In individual experiments the weight of the rats was within ±20 g. In one study, rats weighing 50–60 g were used. Unless otherwise indicated food was withheld for 20 h before the experiments, and water was available ad lib. Rats were decapitated with a guillotine.

The method for dissecting and incubating rat hemidiaphragms has been described elsewhere (14). One hemidiaphragm of each rat was incubated in the control, the other in the experimental medium, in a Dubnoff metabolic shaker (42 cycles/min). The medium was Gey and Gey's balanced salt solution (15) containing 5.5 mM or 10 mM glucose with the appropriate additions equilibrated with 95% O_2 and 5% CO_2 (pH 7.4) at 37.5°C. The hemidiaphragms were preincubated for 30–60 min, gently blotted, transferred to vessels containing 2–3 ml of fresh medium, and incubated for 30, 60, or 120 min. The volume and composition of preincubation and incubation media were usually identical except that the radioactive tracer and insulin (1 mU/ml) were added to the latter medium only. In some experiments, which are indicated in the text, the agents to be tested, e.g. amino acids and cycloheximide, were added to the media during preincubation only.

^{14}C incorporation into proteins. After incubation, the hemidiaphragms were briefly rinsed with balanced salt solution, blotted, and placed into tubes containing 5 ml of 10% trichloroacetic acid (TCA).[1] Tissue proteins were isolated by TCA precipitation, followed by extraction with hot TCA, performate digestion, and reprecipitation with TCA (16). The precipitate was washed three times with acetone and once with ether and dried under a heat lamp. Duplicate 3–4-mg samples of the dry protein powder were placed into scintillation counting vials and digested in 0.5 ml of 2 N NaOH at 60°C. When the solution was clear, 1.5 ml of Bio-Solv Solubilizer (formula BBS-2, Beckman Instruments, Inc., Fullerton, Calif.) and 10 ml of toluene scintillation mixture (14) were added, and the samples were counted in a β-liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) with 80% counting efficiency. Aliquots of media (50 μl) were dissolved in 1 ml of Hyamine (Packard Instrument Co.) and 14 ml of toluene scintillation mixture and counted. Counts were corrected to distintegrations per minute. The radioactivity incorporated into proteins was expressed in nanocuries per gram protein.

Determination of free amino acids in the tissue water. In some experiments the concentration of the amino acids and the specific activity of [^{14}C]lysine were measured. Hemidiaphragms were rapidly frozen in liquid N_2 either immediately after dissection or after preincubation or incubation. The tissues and aliquots of media were stored at −76°C. In the initial studies a small portion of the tissues was set aside for determination of water content. In later experiments tissue water was assumed to be 76% of tissue weight.

In initial studies the frozen tissues were pulverized in a mortar chilled in liquid N_2, rapidly weighed, placed into tubes containing 4 ml of hot 5% sulfosalicylic acid, and boiled for 5 min (17). In later experiments the tissues were weighed before incubation. The frozen tissues were homogenized in 4 ml of 5% sulfosalicylic acid at 4°C with a Polytron tissue homogenizer (Brinkmann Instruments, Inc., Westbury, N. Y.). Since the two methods yielded identical results, the latter procedure was adopted.

After centrifugation, the supernate was filtered through ultrafiltration membrane cones (Centriflo no. 2100, CF-50, Amicon Corp., Lexington, Mass.). The amino acids in the ultrafiltrate were measured by ion exchange chromatography (18) with a Beckman model 121 amino acid analyzer with an expanded sensitivity attachment. For each determination extracts prepared from 1–3 hemidiaphragms were used. The amino acid concentration was expressed in nanomoles per milliliter of tissue water.

In some experiments the effluent from the chromatography column was pumped first through an anthracene cell that was placed into the well of the β-liquid spectrometer (19); the effluent from the cell entered the reaction chamber of the amino acid analyzer. This allowed continuous monitoring of the specific activity of the different amino acids. Specific activity was expressed in microcuries of lysine per micromole.

Tyrosine incorporation into proteins. In one study (Table VIII) tared hemidiaphragms from young fasted rats (50–60 g) were incubated for 2 h in media containing 10 mM glucose, 0.35 mM [^3H]tyrosine (0.2 μCi/ml) with or without 0.5 mM leucine. The tissues were frozen in liquid N_2 at the end of incubation. The hemidiaphragms were homogenized in 1 ml of ice-cold 0.01 M potassium phosphate buffer (pH 7.4) with the Polytron homogenizer. The pestle and homogenizing tube were rinsed with an additional 1 ml of ice-cold buffer. The homogenate and wash were combined and precipitated with 0.5 ml of 50% cold TCA, mixed, and centrifuged (20). Aliquots of media were deproteinized with TCA in the same proportion as the muscle extracts. The acid-soluble supernates were decanted and analyzed for tyrosine fluorometrically (21). Aliquots of the supernate (0.4 ml) were added to a scintillation mixture (Scinti-Verse, Fisher Scientific Co., Fair Lawn, N. J.) and counted in the β-liquid spectrometer. The precipitates were washed with cold 5% TCA, extracted with hot TCA, washed with absolute ethanol and then with diisopropyl ether, and dried. Protein was digested in NaOH, and samples were removed for protein determination according to the method of Lowry et al. (22) with crystalline bovine serum albumin standards. Another aliquot of protein was solubilized in Bio-Solv and counted. The intracellular specific activity of tyrosine (disintegrations per minute per nanomole) was calculated with the following formula (20):

$$\frac{(\text{Total TCA-soluble dpm/mg}) - (0.35 \times \text{dpm}/\mu\text{l medium})}{(\text{Total TCA-soluble nmol Tyr/mg}) - (0.35 \times \text{nmol Tyr}/\mu\text{l medium})}.$$

The extracellular space was assumed to be 35% of the wet weight of the tissue (20), and it was also assumed that the specific activity of tyrosine in the extracellular space of muscle was at equilibrium with that of the medum. Tyrosine incorporation into protein (nanomoles per milligram) was calculated by dividing the specific activity of the protein (disintegrations per minute per milligram) by the specific activity of the intracellular tyrosine (disintegrations per minute per nanomole), measured at the end of incubation.

[1] *Abbreviation used in this paper:* TCA, trichloroacetic acid.

Reagents. All chemicals used were of the highest purity obtainable commercially. L-[-^{14}C]Lysine, sodium [1-^{14}C]acetate, sodium [2-^{14}C]pyruvate, [^{3}H]tyrosine, and [^{3}H]mannitol were purchased from New England Nuclear, Boston, Mass. The latter was stored at −76°C. L-Amino acids, sodium pyruvate, cycloheximide, and actinomycin D were purchased from Sigma Chemical Co., St. Louis, Mo. Bovine insulin (crystallized five times) was a gift of Eli Lilly Research Laboratories, Indianapolis, Ind.

RESULTS

The effect of the branched-chain amino acids on the incorporation of ^{14}C into muscle proteins (Table I). Rat hemidiaphragms were preincubated for 1 h with or without a mixture of the three branched-chain amino acids, each 0.3 mM. The muscles were then transferred to media of identical composition as the preincubation media, except that 0.1 mM [U-^{14}C]lysine and insulin were also present. The specific activity of proteins was 20% greater in muscles that were incubated with the three branched-chain amino acids than in the controls ($P < 0.01$). When the branched-chain amino acids were tested separately, valine had no effect, isoleucine inhibited [^{14}C]lysine incorporation into proteins by 12.5% ($P < 0.01$), but leucine alone increased the specific activity of the proteins by 25% ($P < 0.01$). Thus, leucine was responsible for the enhanced labeling of muscle proteins during incubation with branched-chain amino acids.

In the above studies hemidiaphragms from fasted rats were used. The stimulatory effect of leucine on ^{14}C incorporation into proteins was also observed in hemidiaphragms of fed rats (exp. 3, Table I).

Goldberg and Odessey reported that incubated rat hemidiaphragms oxidized negligible amounts of lysine as measured by $^{14}CO_2$ production from [^{14}C]lysine (7). We have confirmed this observation. However, leucine

TABLE I

The Effect of the Branched-Chain Amino Acids on the Incorporation of ^{14}C from [U-^{14}C]Lysine or [1-^{14}C]Acetate into Proteins

Exp.	^{14}C-Substrate	Additions to medium	^{14}C incorporation into proteins	% change	P
			nCi/g per 2 h		
1	Lysine, 0.1 mM	Control	41.4±2.7	+20.4±8.4	<0.05
		Leucine + isoleucine + valine, 0.3 mM each	46.4±2.9 (15)		
2		Control	47.0±3.9	+25.3±7.1	<0.01
		Leucine, 0.5 mM	58.2±4.7 (12)		
		Control	45.2±3.3	−12.5±3.3	<0.01
		Isoleucine, 0.5 mM	39.4±3.0 (11)		
		Control	47.6±5.7	+2.9±4.8	NS
		Valine, 0.5 mM	46.4±5.9 (11)		
3*		Control	50.4±2.3	+18.5±5.6	<0.02
		Leucine, 0.5 mM	58.8±1.4 (18)		
4	Acetate, 0.5 mM	Control	6.2±0.5	+15.0±4.6	<0.01
		Leucine, 0.5 mM	7.1±0.6 (11)		
5‡	Lysine, 0.1 mM	Control	65.2±2.0	+11.5±4.0	<0.02
		Leucine, 0.5 mM	72.2±2.1 (19)		
6‡		Control	67.8±5.9	+25.0±10.0	<0.01
		Insulin, 1 mU/ml	82.0±8.0 (12)		

Rat hemidiaphragms were preincubated in balanced salt solution (15) containing 5.5 mM glucose with or without the branched-chain amino acids indicated in the table. After 1 h the tissues were transferred to fresh media having the same composition as the preincubation media, except for the addition of the ^{14}C-tracer (0.02 μCi/ml) and insulin during incubation. Incubation and preincubation were at 37.5°C and pH 7.4, and the gas phase was 95% O_2 + 5% CO_2. Incubation lasted 2 h. One hemidiaphragm of each rat was incubated in control, and the other was incubated in experimental media. Means±SEM are tabulated. The numbers of paired observations are indicated in parentheses. P values were calculated by paired Student's t test.

* The hemidiaphragms were obtained from rats after an 18-h fast, except for exp. 3, where fed rats were used.

‡ Insulin (1 mU/ml) was added to all incubation media used in exps. 1–4. Insulin was omitted in exp. 5 and added in the experimental medium only in exp. 6.

could have increased the specific activity of the extracted muscle proteins by stimulating the transport of [^{14}C]-lysine into muscle cells, or the outward transport of unlabeled lysine from the intracellular pool. As shown in exp. 4 (Table I), the stimulatory effect of leucine is not restricted to [^{14}C]lysine as a substrate for protein synthesis. Hemidiaphragms which were incubated with 0.1 mM [1-^{14}C]acetate incorporated 15% more ^{14}C into muscle proteins when 0.5 mM leucine was added to the medium than in the absence of leucine ($P < 0.01$). Acetyl-CoA is one of the products of leucine oxidation and thus could be expected to decrease incorporation of ^{14}C from acetate into proteins by diluting the intracellular acetyl-CoA pool. The fact that leucine stimulated the incorporation of ^{14}C from acetate into proteins supports the hypothesis that leucine exerted its action beyond amino acid transport.

In exps. 1-4 (Table I) insulin was added to the incubation media. Insulin, however, is not required for the enhanced labeling of proteins in the presence of leucine (exp. 5). Hemidiaphragms that were preincubated and incubated with 0.5 mM leucine in the absence of insulin incorporated 11.5±4% more [U-^{14}C]lysine into proteins in 2 h than controls ($P < 0.02$). In parallel experiments insulin (1 mU/ml) in the absence of leucine increased the incorporation of [^{14}C]lysine by 25% of the baseline value.

The rats used in exps. 5 and 6 were younger than those in exps. 1-4 (mean wt 125 vs. 160 g) which may explain the somewhat faster rate of ^{14}C incorporation into proteins observed in the later studies. However, the two series of experiments were conducted several weeks apart and are not directly comparable. In our experience, the system is subject to sufficient variables to require paired studies using rats that are matched for weight and studied in parallel experiments to yield interpretable results.

Since the stimulatory effect of leucine on ^{14}C incorporation into proteins appeared to be most reproducible when hemidiaphragms from fasted rats were used and protein synthesis was stimulated by insulin during incubation, all subsequent studies were carried out under these conditions.

Time-course and specificity of the stimulatory effect of leucine on ^{14}C incorporation into proteins. The stimulatory effect of leucine on ^{14}C incorporation into proteins persisted for at least 1 h after leucine was removed from the medium (Table II). Rat hemidiaphragms were preincubated with or without 0.5 mM leucine, for 1 h, then transferred to media containing 0.1 mM [U-^{14}C]lysine but not leucine, and incubated for 30, 60, or 120 min. ^{14}C incorporation into proteins proceeded in an essentially linear fashion during the 2-h incubation. In tissues which had been exposed to leucine during preincubation the specific activity of proteins was 20% greater than in controls after incubation for 30 to 60 min in leucine-free media containing [^{14}C]lysine. During the 2nd h of incubation without leucine, there was no significant difference in the specific activity of proteins between the two groups.

Preincubation with 0.5 mM leucine also stimulated the subsequent incorporation of ^{14}C from 0.1 mM [2-^{14}C]-

TABLE II

The Effect of Preincubation with Leucine on the Subsequent Incorporation into Proteins of [^{14}C]Lysine or ^{14}C from Pyruvate

Preincubation (60 min)	Incubation	^{14}C-Substrate	^{14}C incorporation into proteins	% change	P
	min		*nCi/g per incubation time*		
Control	30	Lysine, 0.1 mM	20.6±2.1	+20.6	<0.05
Leucine	30		24.6±2.6 (8)	±8.9	
Control	60		50.6±3.9	+20.1	<0.001
Leucine	60		60.6±4.5 (8)	±3.0	
Control	120		103.6±9.3	+15.5	<0.1
Leucine	120		116.0±7.7 (8)	±7.4	
Control	60	Pyruvate, 0.1 mM	44.1±4.8	+18.2	<0.025
Leucine	60		50.6±5.0 (12)	±6.8	

Hemidiaphragms from fasted rats were preincubated for 1 h in balanced salt solution (15) containing 5.5 mM glucose with or without 0.5 mM leucine. They were briefly rinsed, blotted, and transferred to incubation media containing glucose and insulin (1 mU/ml) and either [U-^{14}C]lysine (0.02 μCi/ml) or sodium [2-^{14}C]pyruvate (0.1 μCi/ml). Leucine was not added to the incubation media. In experiments where the incorporation of ^{14}C from pyruvate was studied 0.2 mM glutamate was included in the incubation media to facilitate the transamination of pyruvate. Means±SEM are tabulated. The numbers of paired observations are indicated in parentheses. P was calculated by paired Student's t test.

pyruvate into proteins during 1 h incubation without added leucine ($P < 0.05$).

Table III compares the effect of preincubation with leucine to that of preincubation with other amino acids on the subsequent incorporation of [^{14}C]lysine into proteins. Glutamate, histidine, phenylalanine, tryptophan, and methionine (each 0.5 mM) were tested. In each experiment the effect of leucine was also tested to ensure that the lack of stimulation observed with the other amino acids was not due to failure of the experimental model. Only preincubation with leucine caused a significant increase in the specific activity of proteins during incubation with [^{14}C]lysine. Preincubation with tryptophan caused a small but significant decrease ($P < 0.05$).

The effect of increasing leucine in the medium in the presence of a mixture of amino acids. The effect of glutamate. The data in Table IV demonstrate that isolated hemidiaphragms can discriminate between two leucine concentrations (0.1 and 0.5 mM) that are within the range of leucine concentrations found in rat serum (2, 4, 17) and furthermore that the enhancement of [^{14}C]lysine incorporation into proteins by leucine occurs in the presence of a mixture of 18 amino acids including all the essential amino acids at concentrations approximating those found in the serum of fed rats (10).

Hemidiaphragms incubated with glucose, insulin, and 18 amino acids, including 0.5 mM leucine, incorporated 25% more [^{14}C]lysine into proteins than their pairs which were incubated in identical media, except for containing only 0.1 mM leucine. A significant difference between the two leucine concentrations could be demonstrated only when incubations were carried out in media lacking glutamate. When 0.2 mM glutamate was included in the incubation mixture, the difference in the specific activity of proteins between hemidiaphragms incubated with 0.1 or 0.5 mM leucine was no longer significant ($P < 0.1$).

The transamination of leucine is fully reversible in muscles (23); the transfer of the NH_2 group from leucine to α-ketoglutarate yields glutamate and α-ketoisocaproate. The effect of glutamate in the experiments shown in Table IV may represent reversal of the transamination of leucine which could maintain intracellular

TABLE III

The Effect of Preincubation with Different Amino Acids on the Subsequent Incorporation of [^{14}C]Lysine into Proteins

Preincubation (60 min)	^{14}C incorporation into proteins	% change	P
	nCi/g per 60 min		
Exp. 1			
Control	39.4±5.1	+27.3±8.3	<0.01
Leucine, 0.5 mM	48.6±6.0 (11)		
Control	29.4±3.1	0	NS
Glutamate, 0.5 mM	29.4±4.0 (8)		
Control	43.0±3.1	+11.3±7.0	NS
Histidine, 0.5 mM	47.4±4.3 (11)		
Control	48.8±3.6	−1.2±7.3	NS
Phenyalanine, 0.5 mM	48.2±3.8 (11)		
Exp. 2			
Control	36.6±3.4	+22.2±7.1	<0.025
Leucine, 0.5 mM	44.2±4.3 (11)		
Control	46.4±4.0	−9.8±4.2	<0.05
Tryptophan, 0.5 mM	41.2±3.3 (11)		
Control	33.2±3.9	−6.1±5.3	NS
Methionine, 0.5 mM	48.4±4.0 (8)		

The design of the experiments was essentially as described in Table II. Hemidiaphragms were preincubated for 60 min in balanced salt solution containing 5.5 mM glucose with or without one of the amino acids indicated. They were briefly rinsed, blotted, and transferred to incubation media containing glucose, insulin, and 0.1 mM [U-^{14}C]lysine (0.02 μCi/ml) and incubated for 1 h. Means±SEM are tabulated. The numbers of paired observations are indicated in parentheses. P was calculated by paired Student's t test.

TABLE IV

The Effect of Increasing the Concentration of Leucine in a Medium Containing a Mixture of Amino Acids at Concentrations Found in Rat Serum on the Incorporation of [^{14}C]Lysine into Muscle Proteins In Vitro

Leucine	Glutamate	^{14}C incorporation into proteins	% change	*P*
mM		*nCi/g per 2 h*		
0.1	0	48.4±5.3	32.2±9.3	<0.01
0.5	0	62.5±6.6 (15)		
0.1	0.2	59.5±6.9	7.2±4.1	<0.1
0.5	0.2	63.7±7.0 (16)		

Rat hemidiaphragms were preincubated for 1 h in balanced salt solution containing 5.5 mM glucose and 18 or 19 amino acids at concentrations approximating those found in serum of fed rats (10). These were millimolar: aspartate, 0.035; arginine, 0.2; proline, 0.18; glycine, 0.4; alanine, 0.45; valine, 0.2; isoleucine, 0.1; phenylalanine, 0.08; lysine, 0.4; histidine, 0.08; tryptophan, 0.07; threonine, 0.3; asparagine, 0.07; glutamine, 0.35; cysteine, 0.07; methionine, 0.07; tryosine, 0.07. The concentrations of leucine and glutamate are indicated in the table. The composition of the incubation medium was identical to that used during preincubation except that [U-^{14}C]lysine (0.02 μCi/ml) and insulin (1 mU/ml) were added. Means±SEM are indicated. The numbers of hemidiaphragm pairs are indicated in parentheses. *P* was calculated by paired Student's *t* test.

leucine close to a critical concentration during incubation with 0.1 mM leucine. The α-ketoglutarate/glutamate ratio may play a role in regulating the concentration of leucine in muscle cells at the level of transamination, while other factors (5, 6, 24) may regulate at the irreversible step, the oxidative decarboxylation of α-ketoisocaproate. Indeed, α-ketoglutarate has been shown to stimulate branched-chain amino acid oxidation by muscles (24).

The effect of leucine and actinomycin D on the incorporation of [^{14}C]lysine into muscle proteins (Table V). RNA synthesis is apparently not required for the stimulatory effect of leucine on the labeling of muscle proteins. After preincubation and incubation in media containing 0.5 mM leucine and actinomycin D the specific activity of muscle proteins was 16% greater than after exposure to actinomycin D without leucine ($P < 0.05$). In the same experiment pairs of hemidiaphragms were incubated with or without 0.5 mM leucine, in the absence of actinomycin D. The leucine effect was of the same magnitude whether or not actinomycin D was added to the medium. The dose of actinomycin D used (2 μg/ml) inhibits RNA synthesis by isolated rat diaphragms completely (25). The continuing, although slower protein synthesis observed during incubation with actinomycin D is due to the relatively long half-life of most mammalian mRNAs (25).

The effect of leucine and cycloheximide on the incorporation of [^{14}C]lysine into muscle proteins (Table VI). Incubation with cycloheximide (1 μg/ml) for 1 h inhibited protein synthesis by hemidiaphragms nearly completely. When hemidiaphragms were preincubated for 1 h with cycloheximide, rinsed for 30 s in balanced salt solution, and then incubated in the presence of [^{14}C]lysine and insulin, ^{14}C incorporation into proteins was only 57% of that observed in control tissues, indicating that the inhibitory effect of cycloheximide on protein synthesis had persisted at least for part of the incubation period. While the addition of 0.5 mM leucine during preincubation significantly stimulated the subsequent incorporation of [^{14}C]lysine into proteins in control tissues, preincubation with leucine in the presence of cycloheximide caused no significant stimulation (exp. 1).

The lack of a leucine effect under these conditions may have been due to continued partial inhibition of protein synthesis by cycloheximide during incubation. Therefore, a 30-min wash was inserted between preincubation and incubation in later studies, which reversed the cycloheximide-induced inhibition of protein synthesis nearly completely (study 2). Hemidiaphragms that had been preincubated with cycloheximide and leucine, washed for 30 min in media containing neither substance and incubated for 1 h with [^{14}C]lysine, incorporated 23% more ^{14}C into proteins than their pairs, which were subjected to an identical procedure, except that leucine was not included during preincubation ($P < 0.01$). Thus, protein synthesis is apparently not required for the initiation of the leucine effect.

When hemidiaphragms were preincubated for 1 h without added amino acids, the subsequent addition of leucine no longer promoted incorporation of [^{14}C]lysine into proteins (study 3). The inability of leucine to stimulate after a 60-min lag period was observed in the presence or absence of cycloheximide during preincubation. The data seem compatible with an inhibitory effect of leucine on a system(s) that activates protein

TABLE V

The Effect of Leucine and Actinomycin D on the Incorporation of [^{14}C]Lysine into Muscle Proteins

Additions to media	^{14}C incorporation into proteins	% change	*P*
	nCi/g per 2 h		
Control	350±35	+15.9±5.9	<0.05
Leucine	405±39 (8)		
Control	396±53	−31.7±3.4	<0.001
Actinomycin D	241±21 (8)		
Actinomycin D	235±23	+16.0±6.5	<0.05
Actinomycin D + leucine	272±17 (7)		

Rat hemidiaphragms were preincubated for 1 h in balanced salt solution containing 5.5 mM glucose with or without 0.5 mM leucine and/or actinomycin D (2 μg/ml). The composition of the incubation media was identical to those used during preincubation except that 0.1 mM [U-^{14}C]lysine (0.02 μCi/ml) and insulin (1 mU/ml) were added. Means±SEM are indicated. The numbers of hemidiaphragm pairs are indicated in parentheses. *P* was calculated by paired Student's *t* test.

TABLE VI

The In Vitro Effects of Cycloheximide and Leucine on the Incorporation of [^{14}C]Lysine into Muscle Proteins

Preincubation	Wash	Incubation	^{14}C incorporation into proteins	P
			nCi/g protein per 60 min	
Exp. 1 60 min	0.5 min	120 min		
(*a*) Control			39.0±2.2 (17)	$b > a$, $P < 0.05$
(*b*) Leucine			45.3±1.8 (18)	$c < a$, $P < 0.001$
(*c*) Cycloheximide	Control	Control*	22.3±2.2 (16)	$d > c$, NS
(*d*) Cycloheximide + leucine			26.0±2.5 (15)	
		60 min		
(*e*)		Control	32.0±1.9 (4)	$e > f$, $P < 0.001$
(*f*)		Cycloheximide	6.4±0.8 (4)	
Exp. 2 60 min	30 min	60 min		
(*a*) Cycloheximide	Control	Control*	37.6±2.4 (12)	$b > a$, $P < 0.01$‡
(*b*) Cycloheximide + leucine			45.0±2.2 (12)	% change, 23±7.1
Exp. 3 60 min	30 min	60 min		
(*a*) Control	Control	Control*	27.2±1.9 (7)	
(*b*) Control	Leucine	Leucine	27.8±2.7 (7)	
(*c*) Cycloheximide	Control	Control*	21.6±1.5 (7)	Leucine effect, NS
(*d*) Cycloheximide	Leucine	Leucine	22.8±2.3 (7)	

Rat hemidiaphragms were preincubated for 1 h in balanced salt solution containing 5.5 mM glucose (control) with or without the addition of 0.5 mM leucine and/or 1 μg/ml cycloheximide as indicated in the table. After preincubation, the tissues were washed for 0.5 or 30 min as indicated. The addition of leucine (0.5 mM) or cycloheximide (1 μg/ml) during incubation is indicated. Means±SEM are tabulated. The numbers of hemidiaphragms are indicated in parentheses. P was estimated by Student's t test.

* The control incubation medium contained in addition to glucose, insulin (1 mU/ml) and 0.1 mM [U-^{14}C]lysine (0.02 μCi/ml).

‡ By the paired Student's t test.

degradation and/or interferes with the initiation of protein synthesis; thus, once the lack of leucine has allowed these factor(s) to become operational, the introduction of leucine into the system would be ineffectual.

The effect of leucine and cycloheximide on lysine release into the medium and the concentration of lysine in the tissue water of hemidiaphrams during incubation (*Table VII*). Hemidiaphragms released into the medium approximately 0.8 μmol of lysine/g tissue per h incubation. The amount of lysine released was not affected by adding leucine (0.5 mM) or cycloheximide (10 μg/ml) to the incubation medium. While a fraction of the lysine that appeared in the medium could be accounted for by a decrease in the concentration of lysine in the tissue water, most of it was derived from net proteolysis. Lysine metabolism is unique in that it is the only amino acid that cannot be reaminated from the cellular ammonia pool (26).

The concentration of lysine in the tissue water was lower in hemidiaphragms that were incubated with leucine than in tissues incubated in control media ($P < 0.001$). The difference between the two groups was approximately 30% after 1 h exposure to leucine and 45% after 150 min.

The lysine concentration in the tissue water was not affected by the addition of cycloheximide. Leucine decreased it to the same degree in the presence of cycloheximide as in its absence. The dose of cycloheximide used in the above experiments was 10 times greater than that used in the studies shown in Table VI. Since protein synthesis was blocked virtually completely, it appears likely that leucine inhibited proteolysis.

When hemidiaphragms were preincubated for 1 h with or without leucine and then incubated for 1 h in media containing 0.1 mM [^{14}C]lysine but no leucine, the concentration or specific activity of lysine in the tissue water was not significantly different between the two groups at the end of incubation. (exp. 1*b*, Table VII). However, since the tissues contained significantly less lysine immediately after preincubation with leucine (exp. 1*a*), it is possible that the specific activity of [^{14}C]lysine in the intracellular water was greater in tissues that had been preincubated with leucine than in controls at the beginning of the incubation period. This

TABLE VII

Lysine Concentration in Tissue Water and Release into the Medium during Incubation of Rat Hemidiaphragms. The Effect of Leucine and Cycloheximide

Study	Preincubation		Incubation		Lysine in tissue water		Lysine released into medium
	Time	Leucine	Time	Leucine	Concn	Sp act	
	min	*mM*	*min*	*mM*	*μM*	*nCi/μmol*	*nmol/g per 2 h*
1*a*			60	0	947±118 (14)		
				0.5	664±80 (14)		
					% change, 30±5.4		
					$P < 0.001$		
1*b*	60	0	60	0	846±92 (9)	558±40 (9)	
		0.5	[^{14}C]Lys*	0	726±50 (9)	619±43 (9)	
					NS	NS	
2	30	0	120	0	662±37 (8)		1,650±70 (8)
		0.5		0.5	362±82 (5)		1,640±76 (8)
					$P < 0.01$§		NS
3	30	0	120	0	766±48 (7)		1,530±129 (6)
		0.5		0.5	359±46 (7)		1,520±103 (6)
		Cycloheximide‡			% change, 48±4.8		NS
					$P < 0.001$		

Hemidiaphragms from fasted rats were preincubated and incubated in balanced salt solution (15) containing 5.5 mM glucose and the additions indicated in the table. After incubation, the tissues were frozen in liquid N_2, and the media was deproteinized. Both were stored at −76°C until later analysis (see Methods). In early studies tissue analyses were carried out on two or three pooled hemidiaphragms, and in later studies individual hemidiaphragms were analyzed. Means±SEM are indicated. The numbers of observations are indicated in parentheses. *P* was calculated by paired Student's *t* test.

* [^{14}C]Lysine, 0.1 mM, 0.2 μCi/ml, and insulin, 1 mU/ml, were added during incubation in study 1*b*.

‡ Cycloheximide, 10 μg/ml, was included in the preincubation and incubation media in study 3.

§ By the standard Student's *t* test.

could account for the observed increased specific activity of proteins in the leucine-treated group, assuming that the intracellular lysine pool is homogeneous and uniformly on the pathway of protein synthesis. While this has been proposed for some amino acids (20), evidence for compartmentalization of the intracellular amino acid pool has been published (27, 28). The fact that leucine did not affect the amount of lysine released into the medium in 2 h, while the concentration of lysine in the tissue water decreased significantly, suggests that the intracellular lysine pool may be compartmentalized.

No apparent effect of leucine on the rate of [^{14}C]-lysine release from hemidiaphragm. Rat hemidiaphragms were equilibrated for 30 min at 37.5°C in balanced salt solution containing glucose and 0.1 mM [U-^{14}C]lysine. They were then rinsed for 30 s in medium containing no radioactivity, blotted, and transferred into media containing glucose and insulin with or without 0.5 mM leucine at 37.5°C. One hemidiaphragm of each rat was exposed to media containing leucine, its pair to control media. The tissues were transferred every 2 or 5 min to flasks containing 1 ml of fresh medium. The radioactivity that was released into the medium during each time interval was measured and related to the weight of the tissue. [^{14}C]lysine was released from the hemidiaphragms at the same rate in the presence or absence of leucine in the medium. Release appeared to be essentially linear between 5 and 20 min after removal from the radioactive medium.

In a separate experiment [^{3}H]mannitol (0.1 mM, 0.1 μCi/ml) was included in the incubation medium to determine the contribution of extracellular [^{14}C]lysine to the above measurements. The washout of mannitol was virtually complete during the first 5-min wash. The concentration of TCA-soluble [^{14}C]lysine in the intracellular water was more than twice that in the original medium at the end of the initial 30-min equilibration, and the ratio of TCA-soluble to TCA-precipitable radioactivity in the tissue was in the order of 15 : 1. The TCA precipitable radioactivity did not change or increased slightly during the 20-min wash period. Thus, between 5 and 20 min ^{14}C release into the medium represented mainly the transfer of [^{14}C]lysine from the intracellular compartment into the medium; this process did not seem to be affected by leucine.

The effect of leucine on the incorporation of [^{3}H]-

tyrosine into proteins of rat hemidiaphragms (*Table VIII*). The data in Table VII suggested that inhibition of protein degradation may account for the increased labeling of proteins observed during and after incubation with leucine. We attempted to discover whether or not the increased specific activity of proteins reflected the decreased size and increased specific activity of the precursor pool. Hemidiaphragms of small rats were employed, and the concentration of the labeled precursor was increased well above physiological levels to facilitate the rapid diffusion and equilibration of amino acids between the intracellular pools and the incubation medium (20). Tyrosine has been reported to equilibrate within 20 min with this preparation (20). In addition to not being produced or metabolized by muscle cells (20) tyrosine offered the advantage of the availability of a fluorometric assay (21), which allowed precise quantitation of non-protein-bound tyrosine in muscles and media.

The addition of 0.5 mM leucine to the incubation media decreased the intracellular concentration of tyrosine by 11% ($P < 0.05$). The specific activity of proteins was 22% greater after incubation with leucine than after incubation in control media ($P < 0.01$). The leucine effect was of the same magnitude whether or not the data were analyzed on the basis of the specific activity of the proteins or as tyrosine incorporated into proteins based on the specific activity of tyrosine in the intracellular water at the end of incubation. The specific activity of TCA-soluble tyrosine in the cells was identical after incubation with or without leucine. The absence of an effect of leucine on the specific activity of intracellular tyrosine in this experiment is attributed to the high concentration of tyrosine (0.35 mM) used in the medium, which served to minimize the contribution of tyrosine resulting from proteolysis to the specific activity of the intracellular tyrosine pool. Although not conclusive, e.g. leucine may have increased the specific activity of a critical pool in the cell or inhibited the degradation of a rapidly labeled protein with very fast turnover time, the data in Table VIII strongly suggest that leucine promoted protein synthesis.

TABLE VIII

The Effect of Leucine on the Incorporation of [^3H]Tyrosine into Proteins of Isolated Rat Hemidiaphragms

	Control	Leucine	*P*
Tyrosine in tissue water, *nmol/g wet muscle*	479±15 (6)	428±12 (6)	<0.05
Tyrosine in intracellular water, *nmol/g wet muscle*	349±14 (6)	292±15 (6)	<0.05
Tyrosine specific activity in intracellular water, *dpm/nmol tyrosine*	712±16 (5)	717±17 (5)	NS
Specific activity of protein, *dpm/mg protein*	899±35 (5)	1,093±35 (5)	<0.01
Tyrosine incorporated into proteins, *nmol/g protein per 2 h*	1,270±70 (5)	1,520±40 (5)	<0.02

Hemidiaphragms of small (50–60-g) fasted rats were incubated for 2 h in 3 ml of balanced salt solution containing 10 mM glucose and 0.35 mM tyrosine (0.2 μCi/ml) with or without 0.5 mM leucine. The concentration and specific activity of tyrosine in the tissue water and the media were measured at the end of incubation. The specific activity of intracellular tyrosine was calculated as described in Methods. Tyrosine incorporation into proteins was calculated as: protein specific activity/intracellular tyrosine specific activity. Means±SEM are tabulated. The numbers of observations are indicated in parentheses. *P* was calculated by paired Student's *t* test.

DISCUSSION

The data presented indicate that leucine may act as a regulator of the turnover of protein in muscle cells. They are compatible with the hypothesis that leucine inhibits protein degradation and promotes protein synthesis in muscle.

Insulin is the only agent known to exert a profound anabolic effect on the metabolism of muscle proteins whose action at the cellular level has been studied in detail. Insulin is thought to stimulate the synthesis of muscle proteins at the level of translation by promoting the initiation of peptide chains while at the same time the hormone inhibits the degradation of muscle proteins by an unknown mechanism (12, 29-32).

Peptide chain initiation is accelerated in hearts perfused with a mixture of essential amino acids at five times the physiological concentration as compared to hearts perfused with the normal amino acid mixture (31, 33). Recently, Rannels et al. observed stimulation of protein synthesis of the same magnitude in perfused rat hearts when only the concentration of the three branched-chain amino acids was increased (34). The effect on protein turnover reported in the present study appears to be specific for leucine. Since the three branched-chain amino acids are oxidized at about the same rate by muscles and hearts (5, 6), it seems unlikely that their effect is due merely to the increased availability of an oxidizable substrate (34). Indeed, the stimulation of protein synthesis observed in isolated rat hearts when noncarbohydrate substrates such as palmitate or acetate were added to the perfusion medium (34) may have resulted from increasing the intracellular concentration of leucine. Hearts perfused with palmitate and glucose maintained the intracellular concentration of leucine at 0.21 mM, while it decreased significantly during perfusion with glucose alone (34). It is noteworthy that in the studies cited (31, 33, 34) palmitate, insulin, or increased amino acids appeared to

prevent a block in peptide chain initiation, which developed in hearts perfused with glucose alone. Although direct evidence is lacking, our experiments are compatible with such an interpretation of the effect of leucine in muscles, since leucine was not effective when added after incubating the tissues without leucine and since leucine was effective in muscles blocked with actinomycin D or cycloheximide.

While this manuscript was in preparation Fulks et al. (29) reported that five-times-normal plasma concentrations of the branched-chain amino acids promoted protein synthesis and inhibited protein degradation in isolated quarter diaphragms of young rats. A mixture of the remaining plasma amino acids was ineffective. Leucine by itself or isoleucine and valine together were able to promote the incorporation of tyrosine into protein and inhibit tyrosine release. Our data are generally in good agreement with those of Fulks et al. (29). The observation that isoleucine and valine together had identical effects to leucine (29) needs further study. It is possible that the intracellular leucine concentration increases during incubation with five times the plasma concentration of a mixture of isoleucine and valine due to increased oxidation of the latter amino acids, which would compete with leucine for the branched-chain α-keto acid dehydrogenase system.

In bacteria, protein synthesis and degradation appear to be regulated coordinately and the supply of amino acyl-tRNA influences both processes (for review of literature see reference 35). The classical work from Munro's laboratory indicated that the supply of amino acids affected the aggregation of ribosomes in liver cells and that the availability of tryptophan was the limiting factor under certain conditions in this tissue (36). Jefferson and Korner subsequently reported that 11 amino acids were necessary for the stability of the polysomal profile in liver (37). In cultured hepatoma cells the level of tyrosine transaminase was markedly elevated by increasing the concentration of leucine in the medium (38). The leucine effect was dual; it represented stimulation of enzyme synthesis as well as inhibition of enzyme degradation. Lee and Kenney suggested that the effect may be mediated by the levels of leucyl-tRNA (38). As a working hypothesis we would like to propose that the concentration of leucine, possibly in a critical cellular compartment, may play a similar role in regulating protein turnover in muscle cells.

In uncontrolled diabetes, severe stress, and during the first 10 days of fasting, hepatic gluconeogenesis is stimulated (12, 13). Skeletal muscle is the primary source of amino acids for gluconeogenesis, and the above-cited catabolic states are associated with muscle wasting. The signals that are responsible for the accelerated hydrolysis of muscle proteins are not well understood; the lack of insulin plays a pivotal role in this process during fasting and uncontrolled diabetes (12, 13). During severe stress, such as in the burned patient, muscle catabolism is associated with elevated levels of insulin, growth hormone, cortisol, and catecholamines in plasma as well as insulin resistance (12, 39). Leucine oxidation by skeletal muscles is stimulated after hemorrhagic shock or after treatment with cortisol (8). Epinephrine (6), fasting (2, 6, 7), and diabetes (9) stimulate the oxidation of leucine by skeletal muscles.

The branched-chain amino acids in plasma rise above control levels between the 3rd and 10th day of fasting in man; they decrease thereafter and as the fast continues fall below normal levels (40). They are elevated in the plasma of uncontrolled diabetic patients (41, 42) and in diabetic rats (43). There is little information concerning the time-course of changes in the leucine concentration in muscle cells as related to fasting and diabetes; in the instances in which it was measured, however, elevated plasma levels of leucine were accompanied by increased leucine in muscles (43–45). While the concentration of leucine in plasma and muscles increases gradually in fasting rats, becoming significant only between the 4th and 6th day (44), Goldberg and Odessey reported a threefold acceleration of the oxidation of branched-chain amino acids by isolated muscles obtained from rats after a 67-h fast (7). The branched-chain amino acids are the only amino acids that are not concentrated by muscle cells to a significant degree (45). Thus, it is possible that the initial acceleration of protein catabolism is triggered by a depletion of leucine in the muscle cells or a compartment thereof due to accelerated removal of the substrate by oxidation. The slowly rising levels of leucine in plasma and muscles may reflect the stimulation of protein catabolism. The apparent inability of leucine under these conditions to restrain protein catabolism remains unexplained; it may reflect compartmentalization of muscle cells (27, 28) or may be a result of the interplay of the many hormonal and metabolic alterations that characterize the catabolic state.

Recently Sapir et al. (46) presented evidence showing that the daily infusion of 6-8 mmol each of the ketoanalogues of the branched-chain amino acids methionine and phenylalanine plus 3-4 mmol each of the remaining essential amino acids facilitated nitrogen sparing during prolonged starvation in man, in part by conversion of the α-ketoacids to amino acids (23) and in part by affecting the mechanism of nitrogen conservation. The latter effect persisted long after the keto acids were metabolized (46). A preliminary report (47) further suggested that administration of the α-keto analogues of the branched-chain amino acids alone resulted in protein sparing in these subjects. Reversal

of protein catabolism in uremic man (48), as well as in fasting man during periods of starvation with sepsis or trauma, (49) has been reported after treatment with essential amino acids or their α-keto analogues. The salient feature of these studies is that the nitrogen-sparing effect of the amino acids or ketosacids was not accounted for by their caloric contributions, suggesting that they may have a regulatory effect on the turnover of muscle proteins.

While in the above studies (46-49) mixtures of the essential amino acids or their keto analogues were supplied, it is possible that one or more of them played a pivotal role in promoting protein conservation. However, studies of the potential protein anabolic effect of leucine or its keto analogue in the treatment of anephric or catabolic states would have to take into account the possible deleterious effects that amino acid inbalance or antagonism may produce in vivo (50, 51).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Miss Dorothy A. Weigand in some of the studies.

This investigation was supported in part by U. S. Public Health Service grant AM-02001 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

REFERENCES

1. Miller, L. L. 1962. The role of the liver and non hepatic tissues in the regulation of free amino acid levels in blood. *In* Amino Acid Pools. J. T. Holden, editor. Elsevier Publishing Co., Amsterdam. 708–721.
2. Meikle, A. W., and J. G. Klain. 1972. Effect of fasting and fasting-refeeding on conversion of leucine into CO_2 and lipids in rats. *Am. J. Physiol.* **222**: 1246–1250.
3. Odessey, R., and A. L. Goldberg. 1972. Oxidation of leucine by rat skeletal muscle. *Am. J. Physiol.* **223**: 1376–1383.
4. Yamamoto, H., T. Aikawa, H. Matsutaka, T. Okuda, and E. Ishikawa. 1974. Interorganal relationship of amino acid metabolism in fed rats. *Am. J. Physiol.* **226**: 1428–1433.
5. Buse, M. G., J. F. Biggers, K. H. Friderici, and J. F. Buse. 1972. Oxidation of branched chain amino acids by isolated hearts and diaphragms of the rat. The effect of fatty acids, glucose, and pyruvate respiration. *J. Biol. Chem.* **247**: 8085–8096.
6. Buse, M. G., J. F. Biggers, C. Drier, and J. F. Buse. 1973. The effect of epinephrine, glucagon, and the nutritional state on the oxidation of branched chain amino acids and pyruvate by isolated hearts and diaphragms of the rat. *J. Biol. Chem.* **248**: 697–706.
7. Goldberg, A. L., and R. Odessey. 1972. Oxidation of amino acids by diaphragms from fed and fasted rats. *Am. J. Physiol.* **223**: 1384–1391.
8. Ryan, N. T., B. C. George, R. Odessey, and R. H. Egdahl. 1974. Preliminary report: effect of hemorrhagic shock, fasting, and corticosterone administration on leucine oxidation and incorporation into protein by skeletal muscle. *Metab. Clin. Exp.* **23**: 901–904.
9. Buse, M. G., H. F. Herlong, and D. A. Weigand. 1974. Leucine oxidation by muscles and nerves of diabetic rats. The effect of insulin. *Diabetes.* **23**: 366.
10. Mallette, L. E., J. H. Exton, and C. R. Park. 1969. Control of gluconeogenesis from amino acids in the perfused rat liver. *J. Biol. Chem.* **244**: 5713–5723.
11. Felig, P., T. Pozefsky, E. Marliss, and G. F. Cahill, Jr. 1970. Alanine: key role in gluconeogenesis. *Science (Wash. D. C.).* **167**: 1003–1004.
12. Cahill, G. F., Jr., T. T. Aoki, and E. B. Marliss. 1972. Insulin and muscle protein. *Handb. Physiol.* **1** (*Sect. 7*): 563–577.
13. Felig, P., and J. Wahren. 1974. Protein turnover and amino acid metabolism in the regulation of gluconeogenesis. *Fed. Proc.* **33**: 1092–1097.
14. Buse, M. G., and J. Buse. 1967. Effect of free fatty acids and insulin on protein synthesis and amino acid metabolism of isolated rat diaphragms. *Diabetes.* **16**: 753–764.
15. Gey, G. O., and M. K. Gey. 1936. The maintenance of human normal cells and tumor cells in continuous culture. I. Preliminary report: cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation. *Am. J. Cancer.* **27**: 45–76.
16. Manchester, K. L., and M. E. Krahl. 1959. Effect of insulin on the incorporation of C^{14} and C^{14}-labeled carboxylic acids and bicarbonate into the protein of isolated rat diaphragm. *J. Biol. Chem.* **234**: 2938–2942.
17. Scharff, R., and I. G. Wool. 1965. Accumulation of amino acids in muscle of perfused rat heart. Effect of insulin. *Biochem. J.* **97**: 257–271.
18. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Biochem.* **30**: 1190–1206.
19. Piez, K. A. 1962. Continuous scintillation counting of carbon 14 and tritium in effluent of the automatic amino acid analyzer. *Anal. Chem.* **4**: 444–458.
20. Li, J. B., R. M. Fulks, and A. L. Goldberg. 1973. Evidence that the intracellular pool of tyrosine serves as precursor for protein synthesis in muscle. *J. Biol. Chem.* **248**: 7272–7275.
21. Waalkes, T. P., and S. Udenfriend. 1957. A fluorometric method for the estimation of tyrosine in plasma and tissues. *J. Lab. Clin. Med.* **50**: 733–736.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
23. Walser, M., P. Lund. N. B. Ruderman, and A. W. Coulter. 1973. Synthesis of essential amino acids from their α-keto-analogues by perfused rat liver and muscle. *J. Clin. Invest.* **52**: 2865–2877.
24. Buse, M. G., S. Jursinic, and S. S. Reid. 1975. Regulation of branched-chain amino acid oxidation in isolated muscles, nerves and aortas of rats. *Biochem. J.* **148**: 363–374.
25. Manchester, K. L. 1964. The rate of turnover of messenger ribonucleic acid in rat diaphragm muscles. *Biochem. J.* **90**: 5C.
26. Rosenberg, L. E., and C. R. Scriver. 1974. Disorders of amino acid metabolism. *In* Duncan's Diseases of Metabolism. P. K. Bondy and L. E. Rosenberg, editors. W. B. Saunders Co., Philadelphia. 465–654.
27. Kipnis, D. M., E. Reiss, and E. Helmreich. 1961. Functional heterogeneity of the intracellular amino acid pool in mammalian cells. *Biochim. Biophys. Acta.* **51**: 519–524.
28. Hider, R. C., E. B. Fern, and D. R. London. 1969. Relationship between intracellular amino acids and protein synthesis in the extensor digitorum longus muscle of rats. *Biochem. J.* **114**: 171–178.

29. Fulks, R. M., J. B. Li, and A. L. Goldberg. 1975. Effect of insulin, glucose, and amino acids on protein turnover in rat diaphragm. *J. Biol. Chem.* **250**: 290–298.
30. Wool, I. G., J. J. Castles, D. P. Leader, and A. Fox. 1972. Insulin and the function of muscle ribosomes. *Handb. Physiol.* **1**(*Sect. 7*): 385–394.
31. Morgan, H. E., L. S. Jefferson, E. B. Wolpert, and D. E. Rannels. 1971. Regulation of protein synthesis in heart muscle. II. Effect of amino acid levels and insulin on ribosomal aggregation. *J. Biol. Chem.* **246**: 2163–2170.
32. Jefferson, L. S., D. E. Rannels, B. L. Munger, and H. E. Morgan. 1974. Insulin in the regulation of protein turnover in heart and skeletal muscle. *Fed. Proc.* **33**: 1098–1104.
33. Morgan, H. E., D. C. N. Earl, A. Broadus, E. B. Wolpert, K. E. Giger, and L. S. Jefferson. 1971. Regulation of protein synthesis in heart muscle. I. Effect of amino acid levels on protein synthesis. *J. Biol. Chem.* **246**: 2152–2162.
34. Rannels, E. D., A. C. Hjalmarson, and H. E. Morgan. 1974. Effects of noncarbohydrate substrates on protein synthesis in muscle. *Am. J. Physiol.* **226**: 528–539.
35. Goldberg, A. L., E. M. Howell, J. B. Li, S. B. Martel, and W. F. Prouty. 1974. Physiological significance of protein degradation in animal and bacterial cells. *Fed. Proc.* **33**: 1112–1120.
36. Wunner, W. H., J. Bell, and H. N. Munro. 1966. The effect of feeding with a tryptophan free amino acid mixture on rat-liver polysomes and ribosomal nucleic acids. *Biol. Chem. J.* **101**: 417–428.
37. Jefferson, L. S., and A. Korner. 1969. Influence of amino acid supply on ribosomes and protein synthesis of perfused rat liver. *Biochem. J.* **111**: 703–712.
38. Lee, K-L., and F. T. Kenney. 1971. Regulation of tyrosine-α-ketoglutarate transaminase in rat liver. Regulation by L-leucine in cultured hepatoma cells. *J. Biol. Chem.* **246**: 7595–7601.
39. Hinton, P., S. P. Allison, S. Littlejohn, and J. Lloyd. 1971. Insulin and glucose to reduce catabolic response to injury in burned patients. *Lancet.* **1**: 767–769.
40. Felig, P., O. E. Owen, J. Wahren, and G. F. Cahill, Jr. 1969. Amino acid metabolism during prolonged starvation. *J. Clin. Invest.* **48**: 584–594.
41. Carlsten, A., B. Hallgren, R. Jagenburg, A. Svanborg, and L. Werko. 1966. Amino acids and free fatty acids in plasma in diabetes. I. The effect of insulin on arterial levels. *Acta Med. Scand.* **179**: 361–370.
42. Felig, P., E. Marliss, J. L. Ohman, and G. F. Cahill, Jr. 1970. Plasma amino acid levels in diabetic ketoacidosis. *Diabetes.* **19**: 727–729.
43. Scharff, R., and I. G. Wool. 1966. Effect of diabetes on the concentration of amino acids in plasma and heart muscle of rats. *Biochem. J.* **99**: 173–178.
44. Adibi, S. A. 1971. Interrelationships between level of amino acids in plasma and tissues during starvation. *Am. J. Physiol.* **221**: 829–838.
45. Bergström, J., P. Fürst, L-O. Norée, and E. Vinnars. 1974. Intracellular free amino acid concentration in human muscle tissue. *J. Appl. Physiol.* **36**: 693–697.
46. Sapir, D. G., O. E. Owen, T. Pozefsky, and M. Walser. 1974. Nitrogen sparing induced by a mixture of essential amino acids given chiefly as their keto-analogues during prolonged starvation in obese subjects. *J. Clin. Invest.* **54**: 974–980.
47. Sapir, D. G., O. E. Owen, T. Pozefsky, and M. Walser. 1974. Nitrogen-sparing induced by keto-analogues of essential amino acids. *J. Clin. Invest.* **53**: 70a.
48. Walser, M., A. W. Coulter, S. Dinge, and F. R. Crantz. 1973. The effect of keto-analogues of essential amino acids in severe chronic uremia. *J. Clin. Invest.* **52**: 678–690.
49. Blackburn, G. L., J. P. Flatt, G. H. A. Clowes, Jr., T. F. O'Donnell, and T. E. Hensle. 1973. Protein sparing therapy during periods of starvation with sepsis or trauma. *Ann. Surg.* **177**: 588–594.
50. Harper, A. E. 1964. Amino acid toxicities and inbalances. *In* Mammalian Protein Metabolism II. H. N. Monroe and J. B. Allison, editors. Academic Press, Inc., New York. 87–134.
51. Krishnaswamy, K., and C. Gopalan. 1971. Effect of isoleucine on skin and electroencephalogram in pellagra. *Lancet.* **2**: 1167–1169.