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General Stimulation of Muscle Protein Synthesis by Branched Chain Amino Acids *in Vitro*[1] (40460)

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The branched chain amino acids leucine, isoleucine and valine are oxidized primarily by muscle (1). It has been suggested that these amino acids participate in the regulation of protein synthesis in heart and skeletal muscle. Perfusion with five times normal concentrations of amino acids prevents the deceleration of protein synthesis that occurs when hearts are perfused with normal concentrations of amino acids; the effect appears to involve initiation of translation (2) and is reproduced when the heart is perfused with five times normal concentrations of only the branched chain amino acids (3).

In isolated rat diaphragms, branched chain amino acids stimulate protein synthesis (4, 5), while five times normal plasma concentration of all other amino acids (4), or a series of individual amino acids (5) are ineffective. Incubation of diaphragm muscle with leucine alone increases protein synthesis to the same degree as a mixture of the three branched chain amino acids. The effect does not involve a by-product of leucine degradation (5, 6). Increasing the leucine concentration stimulates protein synthesis of isolated diaphragms in the presence or absence of all essential amino acids at physiological plasma concentrations (5).

Previous reports of the branched chain amino acid stimulus to protein synthesis were confined to total muscle protein. We examined the incorporation of tyrosine into the soluble and myofibrillar protein fractions of muscle to determine if either fraction is particularly responsive and whether or not, within each fraction, the branched chain amino acids stimulate the synthesis of particular proteins preferentially.

Methods. Tissue labeling. Male rats (70–100 g) of the Wistar strain were fasted for 24 hr, decapitated and the diaphragms cut into halves or quarters. Details of dissection and incubation procedures have been reported elsewhere (5, 6).

A double isotope technique was employed to compare protein labeling in the presence or absence of the branched chain amino acids. Hemidiaphragms were incubated at 37° for 2 hr in Gey and Gey's balanced salt solution (7) containing 10 mM glucose and either 0.35 m*M* [^{14}C]tyrosine (0.5–2.0 μCi/ml) or [^{3}H]tyrosine (1–5 μCi/ml). The branched chain amino acids (each 0.5 m*M*) were added to either the ^{14}C or ^{3}H-labeled medium, but not to both. Incubation was preceded by 30 min preincubation in media which were identical to those used for incubation, except that radio-labeled tyrosine was omitted.

To establish that the intracellular free tyrosine pool was labeled to the same degree under all experimental conditions each quarter diaphragm was incubated in one of four media: [^{3}H]tyrosine, [^{3}H]tyrosine plus branched chain amino acids, [^{14}C]tyrosine, and [^{14}C]tyrosine plus branched chain amino acids (Table I). The muscles were homogenized and the proteins precipitated with trichloroacetic acid, as previously described (6). Tyrosine was measured in the acid-soluble supernatant and in the incubation medium according to the method of Waalkes *et al.* (8). Aliquots of NaOH digested protein and radio-labeled incubation medium were counted in a liquid scintillation counter. Intracellular tyrosine specific activity was calculated by standard methods (4).

Muscle fractionation. In early studies (Table II), ^{14}C-labeled and ^{3}H-labeled hemidiaphragms from the same animal were com-

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bined and homogenized with a Polytron tissue homogenizer (Brinkman) in 0.24 *M* sucrose containing 1 mM EDTA and 1 m*M* B-mercaptoethanol, pH 7.4 (1 ml/100 mg tissue), and centrifuged at 25,000*g* for 40 min at 5°. The supernatant containing the sarcoplasmic proteins was collected.

The pellet was washed with water, resuspended in 2 ml of Weber's solution (0.6 *M* KCl, 0.04 *M* $NaHCO_3$, 0.01 *M* Na_2CO_3, 1 m*M* EDTA), shaken overnight at 5° and centrifuged at 4000*g* for 15 min. The supernatant, containing predominantly myosin and actomyosin, was diluted 1:20 with ice cold water (final KCl concentration 0.03 *M*). The pH was adjusted to 6.8 ± 0.05 with dilute acetic acid. The diluted solution was allowed to sit for at least one hour at 5° to permit complete precipitation of contractile proteins. Following centrifugation, the precipitate was redissolved in 1 ml of Weber's solution and reprecipitated by dilution.

TABLE I. SPECIFIC ACTIVITIES OF INTRACELLULAR AND EXTRACELLULAR POOLS OF MUSCLE FOLLOWING INCUBATION WITH LABELED TYROSINE.[a, b]

Medium	Intracellular sp act (dpm/nmole intracellular tyrosine)	Extracellular sp act (dpm/nmole tyrosine in media	Intracellular sp act/ extracellular sp act
^3H	4461 ± 175.1	6413 ± 108.1	0.70 ± 0.03
^3H + BC	4157 ± 395.7	6601 ± 115.3	0.63 ± 0.06
^{14}C	2473 ± 101.1	3272 ± 83.11	0.76 ± 0.04
^{14}C + BC	2125 ± 160.0	3315 ± 96.9	0.65 ± 0.05

[a] Quarter diaphrams were incubated in medium containing 1 μCi/ml [^3H]tyrosine, 0.3 m*M*, plus or minus 0.5 m*M* branched chain amino acids, or 0.5 μCi/ml [^{14}C]tyrosine, plus or minus branched chain amino acids for 2 hr.

[b] Means of nine observations ± SEM are tabulated. BC indicates presence of branched chain amino acids in the medium. No significant effects of branched chain amino acids were noted when analysed by standard or by paired *t* test.

To prepare soluble and myofibrillar muscle fractions for electrophoresis and isoelectric focusing, the following techniques were carried out at 5°. ^{14}C-labeled and ^3H-labeled hemidiaphragms from the same animal were combined and homogenized with a Polytron tissue homogenizer in 25 m*M* KCl containing 39 mM sodium borate and 0.5 m*M* dithiothreitol, pH 7.1. Myofibrils were isolated as described by John (9), a modification of the procedure of Perry and coworkers (10).

Soluble proteins were isolated from the supernatant of the first homogenization in KCl-borate buffer, which was dialyzed for 24 hr against distilled water and then concentrated 20-fold with a Minicon concentrator (Amicon).

Disc gel electrophoresis. Myofibrillar proteins were subjected to electrophoresis as described by John (9). Gels were stained with Coomassie Brilliant blue and destained in acetic acid: methanol: H_2O, 75 ml: 50 ml: 875 ml.

Absorption profiles of each gel were prepared by scanning in a densitometer (ISCO). Gels were sliced manually with a razor blade; 3–5 mm slices were placed into scintillation vials containing 200 μl of 30% H_2O_2 and dissolved at 70°.

TABLE II. THE EFFECT OF BRANCHED CHAIN AMINO ACIDS ON THE LABELING RATIOS OF MUSCLE HOMOGENATE AND PROTEIN FRACTIONS.[a]

	Tissue Protein		
Labeling ratio	Whole homogenate	Soluble fraction	Myofibrillar fraction
$^3H/^{14}C$	2.84 ± 0.30 (20)	3.29 ± 0.32 (14)	3.69 ± 0.41 (10)
^3H + BC/^{14}C	4.53 ± 0.64 (16)	5.51 ± 0.68* (10)	5.75 ± 0.70* (8)
$^3H/^{14}C$ + BC	1.63 ± 0.15* (12)	1.94 ± 0.09* (6)	2.04 ± 0.09* (2)
	Tissue/Medium**		
Labeling ratio	Whole homogenate	Soluble fraction	Myofibrillar fraction
$^3H/^{14}C$	1.07 ± 0.10 (6)	1.19 ± 0.14 (6)	1.27 ± 0.15 (5)
^3H + BC/^{14}C	1.57 ± 0.09† (6)	1.63 ± 0.02† (6)	1.90 ± 0.19† (6)

[a] Labeling ratios for whole muscle homogenate, the soluble protein fraction and the myofibrillar protein fraction was computed as [^3H]dpm/mg muscle ÷ [^{14}C]dpm/mg muscle. The presence of branched chain amino acids in either [^3H]medium or [^{14}C]medium is indicated by BC. Means ± SEM are tabulated. Number of observations are shown in parentheses.

* Different from control, without BC $P < 0.001$; $P < 0.01$ † $P < 0.02$.

** Labeling ratio in tissue fraction–labeling ratio in medium.

Samples were counted in a β-scintillation spectrometer with automatic quench compensation in 0.5 ml Hyamine hydroxide (New England Nuclear) and 10 ml of modified Bray's solution (12). ^3H efficiency was 30–36%, ^{14}C efficiency was 55–65%, and ^{14}C spill into tritium was 20–23%. $^3H/^{14}C$ ratios were calculated for all gel slices.

Isoelectric focusing. Soluble muscle proteins were separated by isoelectric focusing over a pH gradient of 3.5–8.0, according to the procedures described by Allen *et al.* (13), using 5% slab gels containing 3.5% bis acrylamide. Protein samples were applied on 10 mm × 20 mm paper tabs, 50 μl per tab, 150–200 μg protein per tab. Densitometric scans of the gels were prepared. Control and branched chain treated muscle preparations were focused beside each other on the same gel. Slab gels were sliced manually into 3–5 mm wide sections with a microtome blade. By careful slicing corresponding bands could be cut from control and branched chain treated gels simultaneously. Corresponding control and branched chain treated slices were placed into counting vials, dissolved in 200 μl H_2O_2 (30%) at 70° and counted in 0.3 ml Hyamine and 10 ml of Scintiverse in the liquid scintillation counter with a ^{14}C efficiency of 60–80%, ^3H efficiency 30–32%, and ^{14}C spill into ^3H of 30%.

Results. The presence of the branched chain amino acids with either [^{14}C] or [^3H] labeled tyrosine did not affect the intracellular or extracellular sp act. Further, when intracellular sp act was expressed as a percentage of the extracellular sp act, the degree of labeling of the intracellular pools was not different, irrespective of the isotope used, or of the presence or absence of branched chain amino acids (Table I).

Branched chain amino acids (0.5 m*M*) stimulated tyrosine incorporation into the proteins of total muscle, the soluble fraction and the myofibrillar fraction by 50–60%, as indicated by the change in the $^3H/^{14}C$ ratios (Table II). The small differences in the $^3H/^{14}C$ ratio seen between different fractions incubated with or without branched chain amino acids are not significant. Note that in early experiments the effect on branched chain amino acids was studied in homogenates alone to standardize the double isotope procedure. The ratio of $^3H/^{14}C$ in the media varied slightly from experiment to experiment. However, regardless of isotope ratio in media, an increase in amino acid incorporation was noted in all studies where branched chain amino acids were included.

This observation was significant when the labeling ratios of the whole homogenate, and that of the soluble and myofibrillar fraction were measured directly ($P < 0.01$) or when the isotope ratios observed in tissue proteins were divided by those in the medium ($P < 0.02$). The latter analysis was used in experiments involving electrophoresis and isoelectric focusing to minimize the effect of variations in the concentration of label used in different experiments. The apparent differences among means of the three protein fractions within either the control or the branched chain amino acid treated groups are not significant.

Figures 1a and 1b depict the electrophoretic pattern of myofibrillar proteins in the absence and presence of branched chain amino acids. The large protein bands at the top of the gel (A and B) represent myofibrils that were undissociated, and too large to move into the gel. B and C represent proteins with relatively rapid turnover with high incorporation of both ^3H and ^{14}C tyrosine. In contrast, protein E, which exhibited similar absorbance to C, incorporated very little tyrosine. The proteins designated as F, G, H, and I are rapidly moving small molecular weight proteins with low incorporation of label. According to published data (9) of the migration behavior of myofibrillar proteins under these electrophoretic conditions, protein C corresponds to actin, D and E to α and β tropomyosin, F to troponin and G, H and I to dissociated light chains of myosin.

Uptake of ^3H tyrosine was increased in all proteins when branched chain amino acids were included with the tritium labeled medium, as indicated by the increased $^3H/^{14}C$ in all myofibrillar protein bands. The branched chain amino acid effect in protein A seems particularly striking because of the high concentration of protein present. However, it was not significantly different from that seen in other protein bands.

Isoelectric focusing sharply resolved the soluble proteins of muscle (Fig. 2). The pres-

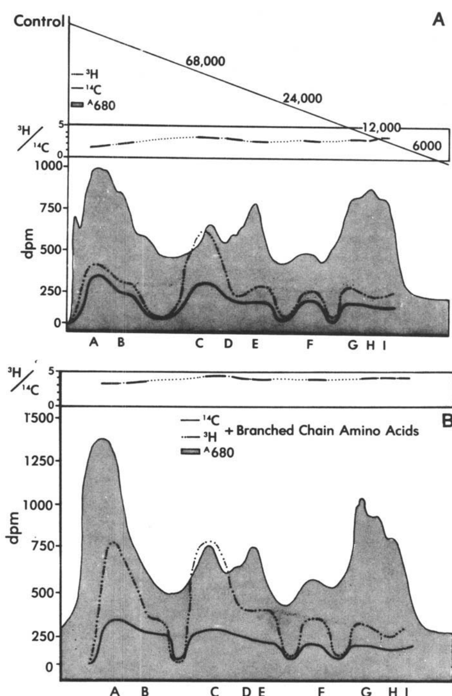

FIG. 1. Disc gel electrophoresis of myofibrillar proteins. Myofibrillar proteins from control (1a) and branched chain amino acid treated (1b) muscles were separated on polyacrylamide gels containing SDS as described by John (9). Procedures for labeling proteins and analysis of gels are described in Methods. In the experiment shown in (1b) branched chain amino acids were added to the medium containing [^{3}H]tyrosine, but not to the one containing [^{14}C]tyrosine. Uptake of [^{3}H]tyrosine into myofibrillar proteins is indicated by (·····), that of [^{14}C]tyrosine by (——); shaded areas represent densitometric scans of the gels, A_{680}. Letters A–I indicate major proteins of the myofibrillar fraction. Marker proteins of known molecular weight are shown in Fig. 1a. Inset above absorbance tracing indicates ^{3}H/^{14}C ratio for specific gel slices. Solid portion of the ^{3}H/^{14}C ratio line indicates protein slice width. ^{3}H/^{14}C ratios are shown only in those areas of the gel where the slices contained counts greater than background.

ence of branched chain amino acids did not alter the isoelectric focusing pattern of muscle proteins.

When branched chain amino acids were included in the ^{3}H-labeled incubation medium, increased incorporation of [^{3}H]tyrosine into all soluble proteins was observed (Fig. 2). Comparing the ^{3}H/^{14}C ratios of control and branched chain treated slices, the latter are consistently higher than the corresponding controls.

The effects caused by incubation with branched chain amino acids shown in Figs. 1 and 2 are typical of results obtained in eight studies. No consistent, preferenas detemulation of tyrosine uptake w; :cted; rather protein synthesis appeared to be generally stimulated. An attempt was made to detect an effect of branched chain amino acids on the synthesis of individual proteins by statistical analysis. The ^{3}H/^{14}C ratio of each branched chain treated gel slice was divided by the ^{3}H/^{14}C ratio of the corresponding control slice. Control and branched chain amino acid treated samples were matched on the basis of relative front value. Analysis of variance showed no significant differences among the branched chain/control quotients in any region of the gel. Identical results were obtained when the ^{3}H/^{14}C ratio of each individual branched chain treated gel slice was divided by the mean ^{3}H/^{14}C labeling ratio of the corresponding control gel. The coefficient of variation for the entire series was 30%.

Discussion. Studies by Millward (14) and Waterlow and Stephen (15) showed that the myofibrillar protein fraction turned over at a slower rate than the soluble protein fraction. The turnover of the myofibrillar fraction also appeared especially sensitive to starvation or a low protein diet. In the present study the synthesis of the soluble and myofibrillar proteins was stimulated equally after 2-hr exposure to branched chain amino acids. The synthesis half lives of the soluble and myofibrillar fractions have been reported as 3.9 and 7.2 days, respectively (15). The two hr incubation used in the present study was too short to detect differences of this magnitude.

Isoelectric focusing distinctly resolved the complex mixture of proteins of the soluble fraction of muscle. Despite the fact that many proteins were separated by this powerful technique, we could not detect any proteins that were selectively affected by branched chain amino acids. Thus, the stimulation of protein synthesis elicited by branched chain amino acids appears to involve many soluble and contractile proteins, suggesting a general effect. However, the possibility that the synthesis of a protein, which is present in relatively low concentrations, may have been preferentially stimulated by branched chain amino acids cannot be excluded.

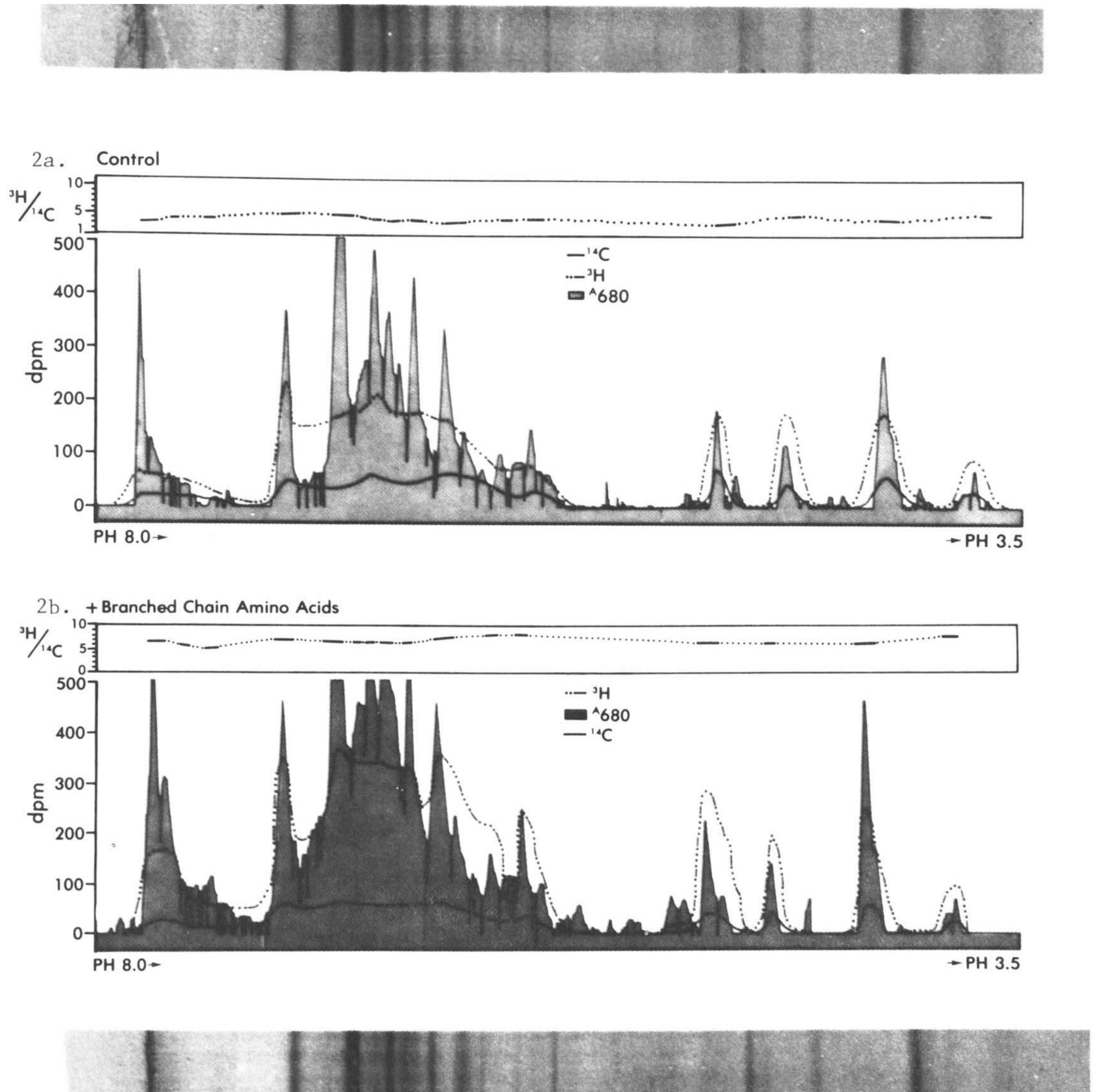

FIG. 2. Effect of branched chain amino acids on uptake of tyrosine into soluble muscle proteins. Soluble proteins from control (2a) and branched chain amino acid treated (2b) muscle preparations were separated by isoelectric focusing. One hundred and 50 μg samples of protein were applied to 5% slab gels. Proteins were separated using 15 W for 30 min, 20W for 30 min, 25W for 25 min, and 35W for 10 min. Branched chain amino acids when present were added to the medium containing [^{3}H]tyrosine only. Slab gels were sliced and [^{3}H]tyrosine (·····) and [^{14}C]tyrosine (——) incorporation into proteins was determined in the slices. The concentration of radioactivity is superimposed on densitometric tracings (shaded areas), A_{680}. The inset above each tracing shows ^{3}H/^{14}C for particular gel slices; solid portion of the line indicates gel slide width. ^{3}H/^{14}C ratios are shown only for gel slices with counts greater than background. The photographs of the gels from which the graphs were prepared are shown above 2a for controls and below 2b for the branched chain amino acid treated muscle.

The branched chain amino acids may be rate-limiting in muscle protein turnover (4–6). Of the three branched chain amino acids, leucine appears to be the specific effector (5, 6). The *in vitro* effects of insulin and leucine are additive (5), and leucine is effective in muscles from fed, fasted and diabetic animals (5, 6). The nature of the regulation is not understood, but mRNA translation may be involved (5, 18). Incubation with actinomy-

cin-D did not decrease the magnitude of the leucine stimulus, indicating that RNA synthesis is not required (5). The fact that the synthesis of all identified proteins was accelerated by branched chain amino acids in the present study also suggests enhanced translation of existing mRNA.

Insulin regulates protein turnover in muscle, presumably by acting at the cell membrane. In a recent review (16), Manchester noted that while modulation of protein turnover by the intracellular levels of amino acids is an attractive hypothesis, no such role for amino acids has been demonstrated. Several recent reports suggest that the branched chain amino acids, particularly leucine, may modulate muscle protein turnover *in vivo* in catabolic states, such as fasting and diabetes. Branched chain amino acids stimulate protein synthesis by muscles of the perfused hindlimb of fasted rats (17). An ip injection of the three branched chain amino acids or of leucine alone results in increased *in vivo* polyribosome formation by muscles of starved rats, treated with glucose and insulin (18). The infusion of small amounts of branched chain α-keto acids, which are reversibly transaminated to branched chain amino acids *in vivo* induces prolonged N_2 retention in fasting patients (19). Finally, following a 2-day fast the addition of branched chain amino acids to a 6-hr constant infusion of glucose increases the incorporation of [^{14}C]tyrosine into rat skeletal muscle protein in vivo (Buse and Peeler, unpublished).

Summary. The effect of branched chain amino acids on precursor incorporation into soluble and myofibrillar protein fractions of muscle was examined with a double-label technique, polyacrylamide gel electrophoresis and isoelectric focusing. Rat hemidiaphragms were incubated in medium containing [^{14}C]-tyrosine or [^{3}H]tyrosine with or without 0.5 m*M* leucine, isoleucine and valine. Branched chain amino acids stimulated synthesis of soluble and myofibrillar protein fractions equally, approximately 60%, $p < .001$. They promoted tyrosine incorporation into individual proteins of both fractions without preferential stimulation of the synthesis of particular proteins. The lack of selectivity of the stimulatory effect is compatible with an effect on translation.

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