Amino acid metabolism by perfused rat hindquarter

Effects of insulin, leucine and 2-chloro-4-methylvalerate

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Hindquarters from starved rats were perfused without substrates but in the presence of an O₂- and CO₂-carrying perfluorocarbon emulsion to evaluate principally the metabolism of individual endogenous and protein-derived amino acids by this muscle preparation. This experimental model was shown, by a battery of metabolite measurements, to maintain cellular homoeostasis for at least 2 h. The net appearance of most amino acids closely approximated their frequency of occurrence in muscle proteins, showing that they are not significantly metabolized. Exceptions were the branched-chain amino acids, methionine and those amino acids that are interconvertible with intermediates of the citrate cycle and pyruvate through coupled transaminations. The evidence indicates that only valine, isoleucine, aspartate and probably methionine can be catabolized by skeletal muscle to provide carbon precursors for glutamate/glutamine and alanine that are formed *de novo* by protein-catabolic muscle. The protein-sparing effects of insulin and leucine were confirmed. Although each decreased proteolysis and the net appearance of free amino acids, they were generally without effect on the ratios of amino acids formed. 2-Chloro-4-methylvalerate selectively stimulated the removal rate for the branched-chain amino acids, confirming the idea that the branched-chain oxo acid dehydrogenase normally limits the rate of their oxidation by muscle. It is also concluded that, since alanine was not formed in excess of that found in muscle proteins when no glucose was added as substrate, the excess of alanine (carbon) released from muscles in other studies is derived to a large extent, but not exclusively, from preformed carbohydrate.

It is well established that, under conditions of negative nitrogen balance, large amounts of alanine and glutamine are released from muscle (Felig *et al.*, 1970) to serve as a major source of carbon for glucose synthesis by the liver. The carbon skeleton of alanine as originally proposed (Mallette *et al.*, 1969; Felig *et al.*, 1970; Ruderman & Berger, 1974) is considered by many workers to be derived principally from preformed carbohydrate, whereas others have presented evidence that a significant portion of the alanine carbon skeleton is formed *de novo* through the breakdown of certain protein amino acids (Davis *et al.*, 1972; Garber *et al.*, 1976; Spydevold *et al.*, 1976; Lee & Davis, 1979; Snell & Duff, 1980). This is a central point since, for net hepatic gluconeogenesis from alanine to occur, the

carbon in alanine must be derived from non-carbohydrate precursors. It is generally accepted, however, that synthesis *de novo* of glutamine by muscle must occur, since more is released from muscle than is found in muscle proteins (Ruderman & Berger, 1974). The extent to which the various protein amino acids are degraded by muscle, and could serve as precursors of glutamine and alanine, has not been satisfactorily resolved.

It is also established that insulin conserves muscle protein both by inhibiting proteolysis and by stimulation of protein synthesis (Jefferson *et al.*, 1977, 1980; Morgan *et al.*, 1981). There are also reports that the branched-chain amino acids, especially leucine, have a similar protein-sparing effect, but there is not agreement concerning the specificity of this effect (Harper & Zapalowski, 1981).

It occurred to us that perfusion of a muscle preparation without added substrates would be

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useful in resolving some of these questions provided that this preparation could survive on endogenous substrates throughout the rather long perfusion period required. Firstly, omission of glucose would minimize the contribution of carbohydrate carbon to amino acid interconversions. Secondly, omission of amino acids would allow more precise measurements of the changes in the amounts of the individual endogenous amino acids, and would preclude effects on their transport into the muscle cells. And, finally, the complexity of the system could be diminished further by the omission of erythrocytes and albumin and, instead, perfusing with a basic Krebs–Henseleit medium fortified with a perfluorocarbon to facilitate transport of gases, and an oncotic agent to substitute for albumin. The results of experiments using the above experimental design are reported in the present paper. Substrate-poor and partially carbohydrate-depleted diaphragms, which do not require perfusion, have previously been used in related studies (Goldstein & Newsholme, 1976; Snell & Duff, 1977).

Experimental

Hindquarter perfusions

Female rats (180–200g) that had been starved for 48h were used. All perfusions were performed as described by Ruderman *et al.* (1971), except as modified in the description below. Perfusions were carried out at 37°C on animals at rest in a humid chamber with the hindlegs skinned. The perfusion medium was composed of Krebs & Henseleit (1932) buffer with 10% (v/v) fluorocarbon FC-80 and 3% (w/v) pluronic polyol. Preparation of the fluorocarbon emulsion was as described by Lee & Davis (1979). After a wash-out with 50ml of medium, time was set at zero and hindquarters were perfused, with the use of a recycling system, with 120ml of medium for periods up to 2h. Throughout the perfusions, the flow rate of medium remained constant at 12–15ml/min and the mean pressure in the tubing leading to the aortic catheter was 90/70mmHg. The basal system contained no added substrates or other additions. When present, insulin was added to 10munits/ml, cycloheximide to 100µM, leucine or valine to 1mM, and 2-chloro-4-methylvalerate to 0.4mM. These values refer to the initial concentrations in the perfusing medium.

Sampling of perfusates and tissue

Samples (10ml) of medium were taken from the lower reservoir at 60 and 120min. The samples were each added to 2ml of 6M-HClO₄ and centrifuged at 17000g for 30min to remove fluorocarbon. The supernatant fluids were neutral-

ized with 4M-K₂CO₃. At the end of perfusion portions of muscle (mainly quadriceps and biceps) from hindlegs were freeze-clamped with Wollenberger forceps that had been precooled in liquid N₂. Between 7 and 8g of tissue from each rat was frozen and extracted. To pulverized frozen tissues, 3vol. of 2M-HClO₄ was added, followed by about 1min of ultrasonic homogenization. After centrifugation of the acid precipitates, the fluids were neutralized with 4M-K₂CO₃. The temperature was maintained near 0°C throughout the extraction and neutralization. Usually, the frozen tissue was stored at –70°C, and then on the day of assay of metabolites the frozen tissue was pulverized and extracted as described above.

Assays of intermediates and products

Acid-soluble components were assayed in 0.05–1.0ml of samples of the neutralized tissue extracts and perfusates. Enzymic determinations were carried out by standard procedures used in this laboratory, as referenced individually by Lee & Davis (1979). Amino acid analyses were carried out with a Beckman 119CL amino acid analyser. Glutamate, glutamine, alanine and aspartate were also routinely determined by enzymic assay (Lee & Davis, 1979). Cross-checks on the accuracy of amino acid analyses were carried out in two ways: (a) independent enzymic analyses of glutamate, glutamine, alanine and aspartate corresponded closely to column analysis; (b) in addition to external standards of all amino acids, periodic separate runs with the amino acid analyser were carried out with internal standards of six amino acids, which included glutamate and glutamine. All external and internal standards gave satisfactory results. No significant hydrolysis of glutamine to glutamate was observed.

Materials

Liquid fluorocarbon FC-80 was obtained from the 3M Co., St. Paul, MI, U.S.A. Pluronic polyols were obtained from BASF, Wyandotte Corp., Wyandotte, MI, U.S.A. Zinc insulin was provided by Eli Lilly Co., Indianapolis, IN, U.S.A. 2-Chloro-4-methylvalerate, prepared by Sandoz, was a gift from Dr. Robert A. Harris of this department. All other chemicals, enzymes and co-enzymes were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Presentation of data

Concentrations of tissue metabolites are expressed as µmol/g of muscle. For uniformity, perfusate content of metabolites is not given as concentrations, but is referred to amount/g of tissue perfused. It is assumed that the rather large mixed-muscle sample extracted (about 30% of

total) was characteristic of total musculature. Estimation of the weight of muscle perfused was based on the ratio of body weight to muscle mass as determined by Ruderman *et al.* (1971). Results are presented as the means $\pm$ S.E.M. for five to seven perfusions. The significance of the differences between means was determined where appropriate by the Student's *t* test.

Results

Effect of substrate-free perfusion on key metabolites

In order to establish the viability of this muscle preparation perfused without substrates, several critical metabolites were measured in muscle samples taken immediately after a preliminary wash-out (3–4 min) and after perfusion for 2 h. Probably the most critical indicator for inadequate oxidizable substrate or O₂ would be a drastic decrease in the concentration of phosphocreatine, accompanied by a more moderate decline in the concentration of ATP and of the [ATP]/[ADP] ratio. As shown in Table 1, the concentrations of these metabolites, which reveal the energy status of

the muscle cells, are well maintained throughout at least 2 h of perfusion. Perhaps surprisingly, there was no significant glycogenolysis, which also attests to the very satisfactory viability of the preparation.

Another critical indicator is the [lactate]/[pyruvate] ratio, which was maintained at a value near that found *in vivo*. As reported previously (Lee & Davis, 1979), there was net synthesis of citrate, much of which was released into the medium. The concentration of tissue malate + fumarate, and therefore aspartate (via transamination), decreased. About 2 μ mol of glucose/g of tissue was not removed in the initial wash-out. This glucose was very slowly glycolysed to lactate, but apparently very little or none was oxidized to CO₂. Hence the muscle was oxidizing principally endogenous fatty acids for fuel.

Changes in the concentrations of individual amino acids during perfusion without substrates

When no substrates were included in the perfusion medium, the total amounts (tissue plus perfusate) of all amino acids increased with time,

Table 1. Concentrations of critical metabolites in muscle before and after perfusion in several conditions
For full experimental details see the text. 'Wash-out' indicates that the tissue was freeze-clamped immediately after an initial washing with 50 ml of perfusate. Results are expressed as means $\pm$ S.E.M. for five to seven perfusions. Abbreviation: N.M., not measured.

		Concn. of metabolite ($\mu\text{mol/g}$ of muscle)					
(a) Tissue	After wash-out	After 120min perfusion plus addition					
		None	Insulin	Cycloheximide			
ATP	9.2 $\pm$ 0.4	8.6 $\pm$ 0.7	7.5 $\pm$ 0.4	9.3 $\pm$ 0.6			
ADP	0.93 $\pm$ 0.02	0.83 $\pm$ 0.04	0.95 $\pm$ 0.06	0.89 $\pm$ 0.04			
AMP	0.22 $\pm$ 0.02	0.15 $\pm$ 0.03	0.19 $\pm$ 0.01	0.18 $\pm$ 0.02			
[ATP]/[ADP] ratio	9.9	10.4	7.9	10.5			
Phosphocreatine	17.0 $\pm$ 1.1	17.7 $\pm$ 0.4	15.7 $\pm$ 3.5	21.3 $\pm$ 3.7			
Lactate	7.6 $\pm$ 1.5	7.7 $\pm$ 1.0	5.0 $\pm$ 1.5	2.3 $\pm$ 0.4			
Citrate	0.15 $\pm$ 0.02	0.13 $\pm$ 0.03	0.13 $\pm$ 0.01	0.15 $\pm$ 0.01			
Malate + fumarate	0.25 $\pm$ 0.05	0.14 $\pm$ 0.04	0.16 $\pm$ 0.03	0.12 $\pm$ 0.03			
Glucose	2.0 $\pm$ 0.4	0.84 $\pm$ 0.14	0.86 $\pm$ 0.16	0.80 $\pm$ 0.30			
Glutamine	2.7 $\pm$ 0.3	2.0 $\pm$ 0.2	1.8 $\pm$ 0.2	2.9 $\pm$ 0.2			
Alanine	2.1 $\pm$ 0.2	1.7 $\pm$ 0.3	1.8 $\pm$ 0.3	2.0 $\pm$ 0.2			
Aspartate	0.40 $\pm$ 0.06	0.10 $\pm$ 0.02	0.10 $\pm$ 0.02	0.14 $\pm$ 0.02			
Glycogen	11.9 $\pm$ 1.7	13.0 $\pm$ 3.3	11.9 $\pm$ 0.8	13.0 $\pm$ 1.0			
		Concn. of metabolite ($\mu\text{mol/g}$ of muscle perfused)					
(b) Perfusate	Addition ...	None		Insulin		Cycloheximide	
		1 h	2 h	1 h	2 h	1 h	2 h
Lactate		4.5 $\pm$ 0.6	6.5 $\pm$ 1.2	6.4 $\pm$ 1.0	10.8 $\pm$ 2.2	7.6 $\pm$ 1.0	13.4 $\pm$ 1.1
Pyruvate		0.45 $\pm$ 0.06	0.59 $\pm$ 0.12	N.M.	N.M.	0.67 $\pm$ 0.08	1.2 $\pm$ 0.1
[Lactate]/[pyruvate] ratio		9.9	11.1	—	—	11.3	11.3
Citrate		0.17 $\pm$ 0.02	0.28 $\pm$ 0.02	0.21 $\pm$ 0.02	0.28 $\pm$ 0.07	0.22 $\pm$ 0.02	0.37 $\pm$ 0.04
Glucose		0.88 $\pm$ 0.12	0.57 $\pm$ 0.15	0.25 $\pm$ 0.07	0.13 $\pm$ 0.04	1.1 $\pm$ 0.1	0.55 $\pm$ 0.08

showing, as expected, that the muscles were in a net protein catabolic state (Fig. 1, first three bars). The net appearance of all amino acids, except those that are interconvertible, was roughly constant over the first and second hours of perfusion. These data show that the rate of net proteolysis was essentially constant during 2h. Therefore for all other conditions only 2h perfusions were done.

Effects of insulin, leucine and valine

Insulin markedly decreased the net accumulation of at least 12 amino acids, with less significant effects on several others (Fig. 1). Hence the known effects of insulin on muscle protein metabolism can be demonstrated in this preparation with a high degree of precision. The insulin effects are, in this case, clearly independent of its effects on amino acid transport, since all of the amino acids present were initially intracellular. Leucine also had a very significant protein-sparing effect, which was quantitatively similar to that of insulin, whereas valine was without any significant effect. In two experiments (results not shown) hindquarters were perfused with 1 mM-isoleucine. No effect of isoleucine on the appearance of amino acids was evident. Hence, of the branched-chain amino acids added singly to perfusions, leucine appears to be specific in its protein-sparing effect.

Net production of individual amino acids in the presence of cycloheximide

Cycloheximide was employed to block protein synthesis in order to discriminate between effects of other additions on protein synthesis and proteolysis. At the concentration used, cycloheximide blocked incorporation of label from [^3H]tyrosine into protein by more than 95% during both the first and second hours of perfusion (results not shown).

As summarized in Fig. 2 (cf. Fig. 1), net appearance of the essential amino acids, as well as some others, was generally increased when cycloheximide was present. This was expected, since net appearance of a given amino acid is now equivalent only to protein breakdown, together with interconversions of certain amino acids with intermediates of the citrate cycle and pyruvate. The net production of glycine was not significantly altered by cycloheximide, whereas insulin decreased glycine accumulation (change not significant, however, when cycloheximide was present). Chang & Goldberg (1978a) reported that glycine is not oxidized (to CO_2) by several muscle types. We therefore have no convincing explanation for this apparent discrepancy of the effect of cycloheximide. However, in this case the standard errors of the individual experiments were rather large, so that an effect of cycloheximide may have gone

undetected. The general picture that emerges is that proteolysis is suppressed by insulin or leucine, although there are some exceptions in which changes in some of the essential (or not interconvertible) amino acids by cycloheximide are not significant. But now, as opposed to perfusions in the absence of cycloheximide, valine significantly decreased the formation of the aromatic amino acids, methionine and proline, and tended to diminish the appearance of some other amino acids. Thus it cannot be excluded that valine also suppresses proteolysis, but, if so, it is less effective than is leucine.

Effects of 2-chloro-4-methylvalerate

Many workers believe that the metabolism of the branched-chain amino acids by muscles is limited by the activity of the branched-chain α -oxo acid dehydrogenase. It has been shown that 2-chloro-4-methylvalerate stimulates the enzyme isolated from heart by inhibiting the kinase that inactivates the dehydrogenase (Harris *et al.*, 1982). We therefore decided that it would be useful to check the effects of this compound in skeletal muscle. As shown in Fig. 2 (last column), the net accumulation of all three of the branched-chain amino acids was markedly suppressed, showing that the catabolism of these three amino acids was stimulated, and gives support to the idea that the branched-chain α -oxo acid dehydrogenase is normally rate-limiting for their catabolism. Another major effect of 2-chloro-4-methylvalerate was an increase in glutamate. The most likely explanation of this effect on glutamate formation is by the stimulated flux through the primary transaminase reaction (note that the net formation of the sum of the branched-chain amino acids was decreased by 2-chloro-4-methylvalerate by about $0.9\ \mu\text{mol/g}$ of tissue, whereas glutamate appearance was increased by about the same amount).

2-Chloro-4-methylvalerate rather markedly increased the net appearance of asparagine, whereas it apparently decreased the appearance of tryptophan. We can provide no explanation at present for these effects, but, perhaps, they are due to unknown non-specific effects of 2-chloro-4-methylvalerate in addition to its known effect on branched-chain oxo acid dehydrogenase.

Comparison of the ratios of individual amino acids formed with those in muscle proteins

Table 2 gives the median amounts of 17 amino acids that accumulated during 2h of perfusion in the presence of cycloheximide, and, from these data, the ratios of individual amino acid/tyrosine formed are tabulated. It has previously been shown that the frequencies of occurrence of all amino acids in myosin, actin and several other muscle

Fig. 1. Net changes in the total amounts (tissue plus perfusate) of 17 amino acids in muscle during 2 h of perfusion without added substrates

Effects of insulin (Ins), leucine (Leu) and valine (Val) are shown. Control perfusions with no additions are presented in the first three columns. The numbers at the bottom of each column indicate the duration of perfusions (h). Abbreviation: N.M., not measured. Statistical significance: *a*, $P < 0.001$ versus zero time (wash-out); *b*, $P < 0.01$ versus zero time; *c*, $P < 0.02$ versus zero time; *d*, $P < 0.05$ versus zero time; *e*, $P < 0.001$ versus control (no additions) at 2 h; *f*, $P < 0.01$ versus control, 2 h; *g*, $P < 0.02$ versus control, 2 h; *h*, $P < 0.05$ versus control, 2 h.

Fig. 2. Net changes in the total amounts (tissue plus perfusate) of 17 amino acids in muscle during 2h of perfusion in the presence of cycloheximide

Effects of insulin (Ins), leucine (Leu), valine (Val) and 2-chloro-4-methylvalerate (Cmv) are shown. See the legend to Fig. 1 and the Experimental section for other details.

Table 2. *Ratios of net accumulation of individual amino acids by perfused muscle compared with those found in muscle proteins*
Data are taken from perfusions in the presence of cycloheximide, but no other additions (Fig. 2).

Amino acid	Amount formed ($\mu\text{mol/g}$ in 2 h)	Ratio of amino acid/ tyrosine formed	Amino acid/tyrosine ratio found in muscle proteins		
			(Bárány <i>et al.</i> , 1964)	(Köminz <i>et al.</i> , 1954)	(Caldwell <i>et al.</i> , 1978)
Leucine	0.68	1.9	4.1	4.4	3.2
Isoleucine	0.29	0.8	2.2	2.3	1.4
Valine	0.40	1.1	2.5	2.3	1.8
Alanine	0.85	2.4	3.9	4.3	3.3
Glutamine	3.10	8.9	8.1	8.6	5.4
Glutamic acid	0.68	1.9			
Tyrosine	0.35	—	—	—	—
Phenylalanine	0.38	1.1	1.4	1.5	1.3
Tryptophan	0.09	0.3	0.3	0.2	—
Methionine	0.18	0.5	1.2	1.2	1.0
Lysine	1.47	4.2	5.2	4.7	3.1
Threonine	0.75	2.2	2.2	2.3	2.0
Serine	0.71	2.0	1.9	2.3	2.2
Proline	0.50	1.4	1.2	1.2	1.7
Glycine	2.15	6.1	4.0	2.2	3.2
Aspartic acid	0.00	0	4.5	4.7	3.8
Asparagine	0.29	0.8			

Table 3. *Calculation of metabolism in the presence of 2-chloro-4-methylvalerate of four amino acids formed by proteolysis in perfused muscle*

Data are taken from 2 h perfusions in the presence of $100\mu\text{M}$ -cycloheximide and 0.4mM -2-chloro-4-methylvalerate. Percentage catabolized is calculated from the ratio of net amino acid formed compared with tyrosine, and the range of these ratios reported for muscle protein (see Fig. 2 and Table 2).

Amino acid	Net amount formed ($\mu\text{mol/g}$)	Amino acid/tyrosine formed	Percentage metabolized
Leucine	0.24	0.70	60–85
Isoleucine	0.09	0.25	80–89
Valine	0.27	0.77	57–70
Methionine	0.13	0.37	63–70
Tyrosine	0.35	—	—

proteins are nearly identical (Köminz *et al.*, 1954). Therefore we have compared the net appearance of free amino acids with their relative occurrence in muscle proteins. A general picture that emerges is that, other than the amino acids that can freely interact directly with intermediates of the citrate cycle or pyruvate (and therefore are interconvertible), only the branched-chain amino acids and methionine were significantly catabolized. According to such an estimation, more than half of these four amino acids that were derived from protein were metabolized. It is of further interest to consider the fate of these four amino acids when 2-chloro-4-methylvalerate was also present. These data, taken from Fig. 2 and the reported occurrence of amino acids in muscle proteins (Köminz *et al.*, 1954; Bárány *et al.*, 1964; Caldwell *et al.*, 1978),

are given in Table 3. From these data it is estimated that about 60–90% of these four amino acids that were derived from muscle protein were catabolized.

Release of amino acids into the perfusing medium

All of the amino acids reported were released into the perfusing medium. This is to be expected, since their concentrations in the perfusate were initially zero. Their rates of release were in most cases attenuated in the second hour of perfusion (Table 4). With only three exceptions the ratios of amino acids released over both time intervals were very close to the ratios of total amino acids formed (compare Tables 2 and 4). The three exceptions were glutamine, glycine and alanine. For the first two of these, less were released than formed. Since

Table 4. Release by perfused muscle of amino acids into the perfusing medium: comparison with tyrosine release. Data are from perfusions in the presence of cycloheximide, but no other additions. The amounts released are given as means $\pm$ S.E.M. for six perfusions.

Amino acid	Amount released (μ mol/g of tissue)		Amino acid/ tyrosine ratio	
	1 h	2 h	1 h	2 h
Leucine	0.40 $\pm$ 0.03	0.58 $\pm$ 0.03	2.0	1.8
Isoleucine	0.21 $\pm$ 0.01	0.29 $\pm$ 0.02	1.0	0.9
Valine	0.37 $\pm$ 0.05	0.48 $\pm$ 0.04	1.8	1.4
Alanine	1.10 $\pm$ 0.21	1.55 $\pm$ 0.15	5.5	4.4
Glutamine	1.10 $\pm$ 0.32	2.10 $\pm$ 0.18	5.5	6.3
Glutamic acid	0.27 $\pm$ 0.05	0.52 $\pm$ 0.09	1.3	1.6
Tyrosine	0.20 $\pm$ 0.02	0.33 $\pm$ 0.04	—	—
Phenylalanine	0.22 $\pm$ 0.02	0.35 $\pm$ 0.04	1.1	1.1
Tyrosine	0.04 $\pm$ 0.00	0.08 $\pm$ 0.01	0.2	0.3
Methionine	0.12 $\pm$ 0.02	0.18 $\pm$ 0.02	0.6	0.5
Lysine	0.70 $\pm$ 0.11	1.25 $\pm$ 0.18	3.5	3.8
Threonine	0.49 $\pm$ 0.09	0.73 $\pm$ 0.14	3.0	2.2
Serine	0.42 $\pm$ 0.06	0.66 $\pm$ 0.12	2.1	2.0
Proline	0.26 $\pm$ 0.05	0.45 $\pm$ 0.07	1.3	1.4
Glycine	0.65 $\pm$ 0.08	1.30 $\pm$ 0.15	3.3	4.0
Aspartic acid	0.02 $\pm$ 0.00	0.05 $\pm$ 0.00	0.1	0.2
Asparagine	0.17 $\pm$ 0.02	0.22 $\pm$ 0.04	0.8	0.7

there was net synthesis of these, apparently there was a disequilibrium in their release after their intracellular synthesis. In the case of alanine the reverse was true: more alanine was released than formed, so that the cellular concentration of alanine declined.

It is noteworthy that, uniformly, with the same exceptions, the net appearance of all of the individual amino acids was almost identical with the amounts released into the perfusing medium over a 2 h period (compare data in columns 1 and 2 in Tables 2 and 4). Hence the intracellular concentrations of the individual amino acids were maintained at their initial, physiological, values. The above correlations were also observed in all other perfusion conditions (results not shown).

Discussion

When a muscle preparation *in situ* is perfused without inhibitors of any metabolic process, the net appearance (or consumption) of free amino acids is the sum reaction of three possible interacting processes (as in Fig. 1): protein degradation, protein synthesis and amino acid catabolism. Many workers consider that most amino acids, other than the branched-chain amino acids and those that are interconvertible via transamination reactions linked to the citrate cycle and to pyruvate, are not significantly metabolized by muscle (see e.g., Manchester, 1965; Odessey & Goldberg, 1972; Odessey *et al.*, 1974; Chang &

Goldberg, 1978*a,b*) and therefore cannot be carbon precursors of alanine and/or glutamine, which are released from muscle. However, this conclusion is not agreed upon by other workers, who have suggested that several other (glucogenic) amino acids may also be catabolized to citrate-cycle intermediates and, subsequently, to pyruvate and alanine (Davis *et al.*, 1972; Davis & Bremer, 1973; Goldstein & Newsholme, 1976; Garber *et al.*, 1976; Spydevold *et al.*, 1976; Snell & Duff, 1977; Lee & Davis, 1979). In the first set of experiments reported in the present paper (in which both proteolysis and protein synthesis were occurring), the results were generally in accord with the conclusion that only the branched-chain amino acids, methionine and those that are interconvertible via the citrate cycle (Tables 2 and 3) were significantly metabolized. In the experiments referred to above (without an inhibitor of protein synthesis) there were some apparent inconsistencies, or lack of significance of changes inflicted by insulin or leucine: i.e. leucine decreased the net appearance of tyrosine and phenylalanine and of tryptophan, but the latter was without statistical significance; on the other hand insulin decreased the appearance of phenylalanine and tryptophan, and appeared to lower the accumulation of tyrosine, but the latter was not statistically significant. The trends are all as expected for non-metabolizable amino acids. Significance of changes due to additions of insulin or leucine were

calculated at a probability level of $>95\%$, so that it is not surprising that not all apparent or expected changes met this rather rigid statistical requirement. For instance, the anomalous lack of significant effect of insulin on the net appearance of lysine may be due to limitation in the accuracy of the measurements (Table 2). The data on lysine in Table 4 also indicate that this is the correct explanation.

In the experiments carried out with an inhibitor of protein synthesis, the net accumulation of amino acids was increased, so that trends resulting from a given treatment were somewhat more amenable to statistically significant changes in individual quantities. Thus the effects of insulin and leucine on the accumulation of the branched-chain amino acids, the aromatic amino acids, methionine and proline were statistically significant (with exceptions, but still with trends in the same direction for these and some other amino acids).

The present report is the first not to show that alanine is formed by a muscle preparation from starved animals in large excess over that found in muscle proteins. The principal difference is that, both *in vivo* and *in vitro*, nearly all of these studies were carried out under conditions in which glucose was present in physiological or supraphysiological concentrations. Hence the principal difference would appear to be that, when pyruvate derived from glycolysis is present in relative abundance, there will be a tendency to form alanine via coupled transamination reactions (see Lee & Davis, 1980). On the other hand, when carbohydrate-derived pyruvate is absent or in short supply, as in the present experimental design, pressure to form alanine (by mass action) through coupled transaminase reactions will be removed. In fact, in the present study the net appearance of alanine was even less than that expected from proteolysis (Table 2), indicating that alanine catabolism has occurred. Thus it is likely that this is due to removal of alanine by mass action, faced with lower than normal pyruvate concentration, via coupled transaminase reactions, with the result that glutamate concentration will be elevated and that there will be a tendency for net depletion of citrate-cycle intermediates. This is the result obtained (see Table 1). Such a process of alanine depletion requires simultaneous anaplerotic reaction(s) to prevent complete depletion of citrate-cycle intermediates.

Notably, two groups (Goldstein & Newsholme, 1976; Snell & Duff, 1977) have reported that, when isolated rat diaphragms were preincubated to deplete endogenous substrates partially, addition of rather high concentrations of glutamate, valine, leucine or isoleucine resulted in accelerated formation and release of alanine. This increased alanine

release can be the metabolic consequence of both the initial transamination reaction and the conversion of the oxo acid into pyruvate (see also Lee & Davis, 1980). The results reported by the above workers and those reported in the present paper appear superficially to be inconsistent. However, we do not believe that this is a difficulty for interpretation, for the following reasons. (a) Goldstein & Newsholme (1976) and Snell & Duff (1977) used rather higher concentrations of amino acids (3 mM), which will tend to promote alanine formation by mass action (transamination). In the present study, leucine and valine (1 mM) caused depletion of aspartate through transamination, but were without significant effect on net alanine production. The latter is probably due to paucity of pyruvate necessary for coupled transamination and alanine production. (b) Pyruvate, formed from any source, has the option of being metabolized to lactate, alanine or acetyl-CoA, so that net production or utilization of alanine will ultimately depend on the relative rates of pyruvate synthesis and degradation. Therefore (c), if the supply of pyruvate is confined solely to amino acid breakdown (glycogenolysis and glycolysis minimized), its rate of removal by oxidation may equal or exceed the rate of its production, with the result that the rate of removal of alanine may equal or exceed the rate of its formation from proteolysis.

We were perhaps the first (see, e.g., Davis *et al.*, 1972; Davis & Bremer, 1973) to suggest that protein-derived amino acids provide some of the carbon in alanine released from muscle. Rather convincing evidence from several laboratories has accrued that this is the case (for review see Snell, 1980). The principal question that remains is how rapidly are amino acids broken down by muscle, and to what extent the various amino acids can contribute to formation of pyruvate + alanine and glutamine. The present paper shows (e.g. Table 2) that, at least on a quantitative basis, only the branched-chain amino acids, methionine and aspartate (+ asparagine) are significantly catabolized by this mixed-muscle hindquarter preparation. From the frequency of their occurrence in muscle proteins, isoleucine, valine and aspartate are quantitatively the most important candidates as carbon precursors of glutamine and alanine, since the frequency of occurrence of methionine in muscle proteins is low, and since the pathway of leucine catabolism precludes it as a net precursor of alanine or glutamine carbon. Knowledge of methionine metabolism in muscle is rather scanty. However, there is rather strong evidence that methionine is metabolized at least in part by a pathway independent of *S*-adenosylmethionine. This pathway involves first a transamination catalysed by the branched-chain amino acid

aminotransferase(s) followed by oxidative decarboxylation by the branched-chain α -oxo acid dehydrogenase (for references see Livesey, 1984). The remainder of this pathway has not been fully described.

With respect to aspartate (+asparagine), the balance sheet suggests that aspartate is converted into net glutamate (and thus glutamine synthesis), since there is production of the latter in excess of that found in muscle proteins. The evidence suggests the following pathway for these interconversions:

where reaction (1) is catalysed by aspartate aminotransferase (EC 2.6.1.1) and reaction (2) is catalysed by enzymes generating acetyl-CoA precursors, together with citrate-cycle oxidations. Since the branched-chain amino acids were oxidized, and since carbon atoms from both isoleucine and valine feed into the citrate cycle at the level of succinyl-CoA (3-carboxypropionyl-CoA), a similar scheme can be written for net conversion of part of the carbon skeleton of these amino acids into glutamate + glutamine:

where reaction (4) is catalysed by the branched-chain amino acid transaminase(s) and reaction (5) is catalysed by the various enzymes involved in the conversion of the oxo analogues of isoleucine and valine into succinyl-CoA. Thus, in both schemes above, three of the carbon atoms of (excessively formed) glutamate + glutamine are derived from aspartate, valine or isoleucine, and the remaining two from acetyl-CoA.

The only amino acids appearing in large excess of those found in muscle proteins were glutamate + glutamine and glycine (Table 2). In view of the amino acid balance studies reported in the present paper, the only known pathways that could contribute significantly to net synthesis of glutamate + glutamine are those depicted in eqns. (1)–(6). As indicated in eqn. (2), carbohydrate could provide part of the carbon for this synthesis

through formation of acetyl-CoA, but this is obligatorily linked to breakdown of aspartate, valine or isoleucine. An ancillary pathway that in theory could provide the remainder of glutamate + glutamine carbon atoms is from carbohydrate through the action of pyruvate carboxylase. Although this enzyme has been shown to be present in skeletal muscle (Spydevold *et al.*, 1976; Lee & Davis, 1979; Davis *et al.*, 1980), its activity is too weak to play a significant role in the observed rate of glutamine synthesis. The glycine formed in excess of that derived from proteolysis

was apparently not formed from serine (Table 2), so that the data indicate that it was formed *de novo* by CO₂ and ammonia fixation via the glycine synthase pathway.

We recognize that the perfused hindquarter is a mixed-muscle preparation also containing small amounts of adipose cells. However, the contribution of non-muscle tissues to the results reported in the present paper is almost certainly less than the small amount originally estimated by Ruderman *et al.* (1971), since (a) the hindquarters were skinned

and (b) the rats were starved for 2 days before the perfusions, so that fat-cell metabolism was also minimized. The relative rates of protein turnover of the various muscle types are not known with certainty, so that the present results represent an average of the protein and amino acid metabolism principally of the muscle types present in this model. It should perhaps be mentioned that it is probably very similar to the mixed muscle of human forearm, in which the arteriovenous amino acid changes measured led to the original proposal of the 'alanine cycle' (Felig *et al.*, 1970).

Over several years it has been pointed out by several workers (Davis *et al.*, 1972; Garber *et al.*, 1976; Spydevold *et al.*, 1976; Lee & Davis, 1979; Snell & Duff, 1980) that, in order to achieve net gluconeogenesis from alanine, its carbon skeleton must be derived from non-carbohydrate precursors

(amino acids). Indeed, it has been shown that pathways adequate for the production of pyruvate (and thus alanine) from citrate-cycle intermediates do exist in muscle (Lee & Davis, 1979). However, on a quantitative basis, the present data strongly suggest that the carbon skeleton of alanine released from muscle is to a large extent linked to carbohydrate metabolism and is therefore principally derived from preformed carbohydrate. From the data given in the present paper, valine and isoleucine are the only essential amino acids metabolized to the extent that they could be significant precursors of alanine.

Finally, the present data clearly confirm in a rather quantitative way the muscle-protein sparing effects of both insulin and leucine. The data also provide evidence that proteolysis is suppressed by valine, although this effect was not evident in experiments in which protein synthesis was also occurring.

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References

- Bárány, M., Gaetjens, E., Bárány, K. & Karp, E. (1964) *Arch. Biochem. Biophys.* **106**, 280–292
- Caldwell, M. D., Lacy, W. W. & Exton, J. H. (1978) *J. Biol. Chem.* **253**, 6837–6844
- Chang, T. W. & Goldberg, A. L. (1978a) *J. Biol. Chem.* **253**, 3677–3684
- Chang, T. W. & Goldberg, A. L. (1978b) *J. Biol. Chem.* **253**, 3685–3695
- Davis, E. J. & Bremer, J. (1973) *Eur. J. Biochem.* **38**, 86–97
- Davis, E. J., Lin, R. C. & Chao, D. (1972) in *Energy Metabolism and the Regulation of Metabolic Processes in Mitochondria* (Mehlman, M. A. & Hanson, R. W., eds.), pp. 211–238, Academic Press, New York
- Davis, E. J., Spydevold, Ø. & Bremer, J. (1980) *Eur. J. Biochem.* **110**, 255–262
- Felig, P., Pozefsky, T., Marliss, E. & Cahill, G. J., Jr. (1970) *Science* **167**, 1003–1004
- Garber, R. J., Karl, I. E. & Kipnis, D. M. (1976) *J. Biol. Chem.* **251**, 836–843
- Goldstein, L. & Newsholme, E. A. (1976) *Biochem. J.* **154**, 555–558
- Harper, A. E. & Zapalowski, C. (1981) in *Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids* (Walser, M. & Williamson, J. R., eds.), pp. 195–203, Elsevier, New York
- Harris, R. A., Paxton, R. & DePaoli-Roach, A. A. (1982) *J. Biol. Chem.* **257**, 13915–13918
- Jefferson, L. S., Li, J. B. & Rannels, S. R. (1977) *J. Biol. Chem.* **252**, 1476–1483
- Jefferson, L. S., Boyd, T. S., Flaim, K. E. & Daniel, E. P. (1980) *Biochem. Soc. Trans.* **8**, 282–283
- Kominz, D. R., Hough, A., Symonds, P. & Laki, K. (1954) *Arch. Biochem. Biophys.* **50**, 148–159
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Lee, S.-H. & Davis, E. J. (1979) *J. Biol. Chem.* **254**, 420–430
- Lee, S.-H. & Davis, E. J. (1980) *Biochem. Int.* **1**, 201–206
- Livesey, G. (1984) *Trends Biochem. Sci.* **9**, 27–29
- Mallette, L. E., Exton, J. H. & Park, C. R. (1969) *J. Biol. Chem.* **244**, 5713–5723
- Manchester, K. L. (1965) *Biochim. Biophys. Acta* **100**, 295–298
- Morgan, H. E., Chua, B. H., Boyd, T. A. & Jefferson, L. S. (1981) in *Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids* (Walser, M. & Williamson, J. R., eds.), pp. 217–226, Elsevier, New York
- Odessey, R. & Goldberg, A. L. (1972) *Am. J. Physiol.* **223**, 1376–1383
- Odessey, R., Khairallah, E. A. & Goldberg, A. L. (1974) *J. Biol. Chem.* **249**, 7623–7629
- Ruderman, N. B. & Berger, M. (1974) *J. Biol. Chem.* **249**, 5500–5506
- Ruderman, N. B., Houghton, C. R. S. & Hems, R. (1971) *Biochem. J.* **124**, 639–651
- Snell, K. (1980) *Biochem. Soc. Trans.* **8**, 205–213
- Snell, K. & Duff, D. A. (1977) *Biochem. J.* **162**, 399–403
- Snell, K. & Duff, D. A. (1980) *Biochem. Soc. Trans.* **8**, 501–504
- Spydevold, Ø., Davis, E. J. & Bremer, J. (1976) *Eur. J. Biochem.* **71**, 155–165