

Amino acid catabolism by perfused rat hindquarter

The metabolic fates of valine

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Hindquarters from starved rats were perfused with plasma concentrations of amino acids, but without other added substrates. Release of amino acids was similar to that previously reported, but, if total amino acid changes were recorded, alanine and glutamine were not formed in excess of their occurrence in muscle proteins. In protein balance (excess insulin) there was no net formation of either alanine or glutamine, even though the branched-chain amino acids and methionine were consumed. If [U-¹⁴C]valine was present, radiolabelled 3-hydroxyisobutyrate and, to a lesser extent, 2-oxo-3-methylbutyrate accumulated and radiolabel was incorporated into citrate-cycle intermediates and metabolites closely associated with the citrate cycle (glutamine and glutamate, and, to a smaller extent, lactate and alanine). If 2-chloro-4-methylvalerate was present to stimulate the branched-chain oxo acid dehydrogenase, flux through this step was accelerated, resulting in increased accumulation of 3-hydroxyisobutyrate, decreased accumulation of 2-oxo-3-methylbutyrate, and markedly increased incorporation of radiolabel (specific and total) into all measured metabolites formed after 3-hydroxyisobutyrate. It is concluded that: (a) amino acid catabolism by skeletal muscle is confined to degradation of the branched-chain amino acids, methionine and those that are interconvertible with the citrate cycle; (b) amino acid catabolism is relatively minor in supplying carbon for net synthesis of alanine and glutamine; and (c) partial degradation products of the branched-chain amino acids are quantitatively significant substrates released from muscle for hepatic gluconeogenesis. For valine, 3-hydroxyisobutyrate appears to be quantitatively the most important intermediate released from muscle. A side path for inter-organ disposition of the branched-chain amino acids is proposed.

INTRODUCTION

It is generally held that, during starvation or other conditions of negative nitrogen balance, muscle is the principal source of carbon provided to the liver for gluconeogenesis. This carbon can be that which is formed through release of amino acids from proteolysis, or from the release of partial degradation products of these amino acids. It is well established that alanine and glutamine are the principal amino acids released from human (Felig *et al.*, 1969, 1970) or rat muscles (Ruderman & Berger, 1974). The extent of synthesis of these glucogenic amino acids *de novo* from other amino acids, although a subject of intense investigation in several laboratories (e.g. Garber *et al.*, 1976*a,b*; Chang & Goldberg, 1978*a,b*; Lee & Davis, 1979; Snell, 1980; Harper & Zapalowski, 1981; Snell & Duff, 1984; Palmer *et al.*, 1984), is still disputed. For instance, Garber *et al.* (1976*b*) concluded that the carbon skeletons of at least 13 amino acids could serve as carbon precursors in muscle of both alanine and glutamine. Although it is now known that only a few amino acids (most notably the branched-chain amino acids) are metabolized by skeletal muscles, the extent of their conversion into intermediate oxidation products and CO₂ is still in dispute (for review see, e.g., Harper & Zapalowski, 1981). In a very recent study (Davis & Lee, 1985) we have evaluated the absolute and relative changes in endogenous amino acids by rat hindquarters perfused without interacting or interfering added substrates. It was

concluded from that study that only valine, isoleucine, aspartate and methionine can be catabolized by skeletal muscle as potential carbon precursors for glutamate, glutamine and alanine. Although that study clearly demonstrated catabolism of these amino acids, it could not discriminate to what extent partially degraded products accumulated. The present work, in which hindquarters were perfused with plasma concentration of all amino acids, is an extension of the study cited above. The metabolic fates of valine, together with balance studies of other amino acids, are reported.

EXPERIMENTAL

Hindquarter perfusion

Female Wistar rats weighing 180–200 g were starved for 48 h. The surgical operation and perfusions, a modification of the protocol of Ruderman *et al.* (1971), were carried out exactly as described previously (Lee & Davis, 1979; Davis & Lee, 1985). Normal plasma concentrations of amino acids (Malette *et al.*, 1969) were the only added substrates. L-[U-¹⁴C]-Valine (0.065 μCi/ml) and [*ring*-3,5-³H]tyrosine (0.129 μCi/ml) were added after a 50 ml washout, followed by perfusion for 1 or 2 h by recirculating 100 ml of perfusates at flow rates of 10–12 ml/min under five experimental conditions as described in legends to Tables and Figures.

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Preparation of samples for assay

At the end of perfusions, 7–8 g of muscle from the hindlegs was rapidly excised and clamped with Wollenberger forceps which had been precooled in liquid N₂. Both perfusate and muscle samples were extracted with HClO₄ (1 M). Before neutralization with K₂CO₃, the acid extracts were gassed with N₂ for 20 min to remove labelled CO₂.

To measure incorporation of [³H]tyrosine and [¹⁴C]valine into muscle protein, about 0.4 g of frozen muscle was treated with 1 ml of 2 M-HCl. The protein pellet formed by acid treatment was washed with 5 ml of 30 mM-potassium phosphate buffer (pH 7.0) and later dissolved in 1 ml of 1 M-NaOH. Protein was determined by a standard biuret method (Cleland & Slater, 1953) and 200 μl portions of protein solution were taken for counting radioactivity.

Determination of amino acids and other metabolites

Amino acid analyses were carried out with a Beckman 119CL amino acid analyser. Enzymic determinations of alanine, glutamate, glutamine and aspartate and were also routinely performed (Lee & Davis, 1979; Davis & Lee, 1985) further to document the results of the automatic amino acid analyser. Citrate-cycle intermediates, pyruvate, lactate, adenine nucleotides, phosphocreatine and the four amino acids listed above were measured by standard enzymic assays as individually referenced by Lee & Davis (1979).

Separation of metabolites

Anion-exchange chromatography was performed on Dowex-1 (formate form) columns as described by Lee & Davis (1979). Radioactive peaks were identified by separate assays with authentic carrier substances. Since the first two peaks from this column were not completely resolved on the above column, these two peaks were pooled and separated on a Dowex-1 (acetate form) column (Lee & Davis, 1979). Glutamate and aspartate present in the first peak eluted from the Dowex acetate column were removed from the unknown compound having most of the radioactivity (peak 1) by Dowex-50W cation exchange (please see the Results section for other details). To identify the unknown compound (which migrated essentially identically with 3-hydroxyisobutyrate in a similar chromatographic system; Spydevold, 1979), pooled fractions from this peak were extracted with diethyl ether at pH 1.0. Authentic 3-hydroxyisobutyrate was synthesized from 3-aminoisobutyric acid by treatment with NaNO₂ in the presence of HCl (Landaas, 1975). The identity of 3-hydroxyisobutyrate was further documented by t.l.c. of the ether-extracted peak (on silica gel G-25) in the solvent system 95% (v/v) ethanol/25% (v/v) ammonia/water (50:8:5, by vol.). The radioactivity migrated in a single peak which was identical in mobility with authentic 3-hydroxyisobutyrate ($R = 0.47$).

Measurement of radioactivity incorporated into amino acids

Two separate runs of each sample were carried out on the amino acid analyser. The first run was carried out in the standard manner in the presence of ninhydrin to determine the concentration and exact retention time of each amino acid. A second run was carried out without ninhydrin. Fractions were collected directly into scintilla-

tion vials at 30 s intervals (0.55 ml), and counted for radioactivity. Spot checks were also made of the retention times of glutamate, glutamine, aspartate and alanine by enzymic analysis.

Materials

Liquid perfluorocarbon FC-47 was obtained from the 3 M Co., St. Paul, MI, U.S.A. Pluronic polyols were obtained from BASF, Wyandott Corp., Wyandott, MI, U.S.A. Radioactive compounds, [3,5-³H]-tyrosine (55 Ci/mmol) and [U-¹⁴C]valine (265 m Ci/mmol), were from New England Nuclear, Boston, MA, 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 materials were from Sigma Chemical Co., St. Louis, MO, U.S.A.

RESULTS

Effect of perfusion on key metabolites

To establish viability of each perfused hindquarter, several critical metabolites were routinely measured. These included the adenine nucleotides, phosphocreatine, malate, citrate and 2-oxoglutarate. All of these criteria were essentially identical with those reported by Davis & Lee (1985) and, for brevity, are not separately tabulated. In a few perfusions (< 5%) the phosphocreatine content and the ATP/ADP ratio fell, with attendant changes in other measured metabolites. These perfusions were discarded as being unsatisfactory.

Release of amino acids into the perfusing medium

Fig. 1 shows the net changes in perfusate concentration of 18 amino acids in controls (no other additions) and in the presence of insulin and/or cycloheximide. In control perfusions, the general pattern of amino acid release was very similar to that reported for rat hindquarters perfused with glucose (Ruderman & Berger, 1974). Notably, there was no significant release of the branched-chain amino acids and methionine. For perfusions in the presence of insulin, there was rather marked net uptake of these four amino acids, which is in part due to a change in the balance of protein synthesis and proteolysis (Jefferson *et al.*, 1977) and probably in part to an increase in the rate of their catabolism (Hutson *et al.*, 1980). If cycloheximide was present to block protein synthesis, the release of amino acids was increased, as expected. Insulin decreased, and in some cases abolished, this release, which is in line with the inhibitory effect of insulin on proteolysis.

Net formation and removal of total free amino acids

Measurement of amino acid release, as has been done in several studies as an indicator of net amino acid formation (through synthesis *de novo* and proteolysis), can be misleading. That this is indeed the case with alanine and glutamine is shown in Fig. 2. If total (tissue plus perfusate) amino acids are measured as in Fig. 2, the general patterns of amino acid release, and net formation or consumption, were similar, with these two exceptions. It is apparent, as suggested by our previous study (Davis & Lee, 1985), that alanine and glutamine are more readily washed out of the tissue than are other amino acids. It becomes clear then from Fig. 2 that the net formation of all amino acids, including alanine and glutamine, was essentially abolished by insulin, illustrating that now the

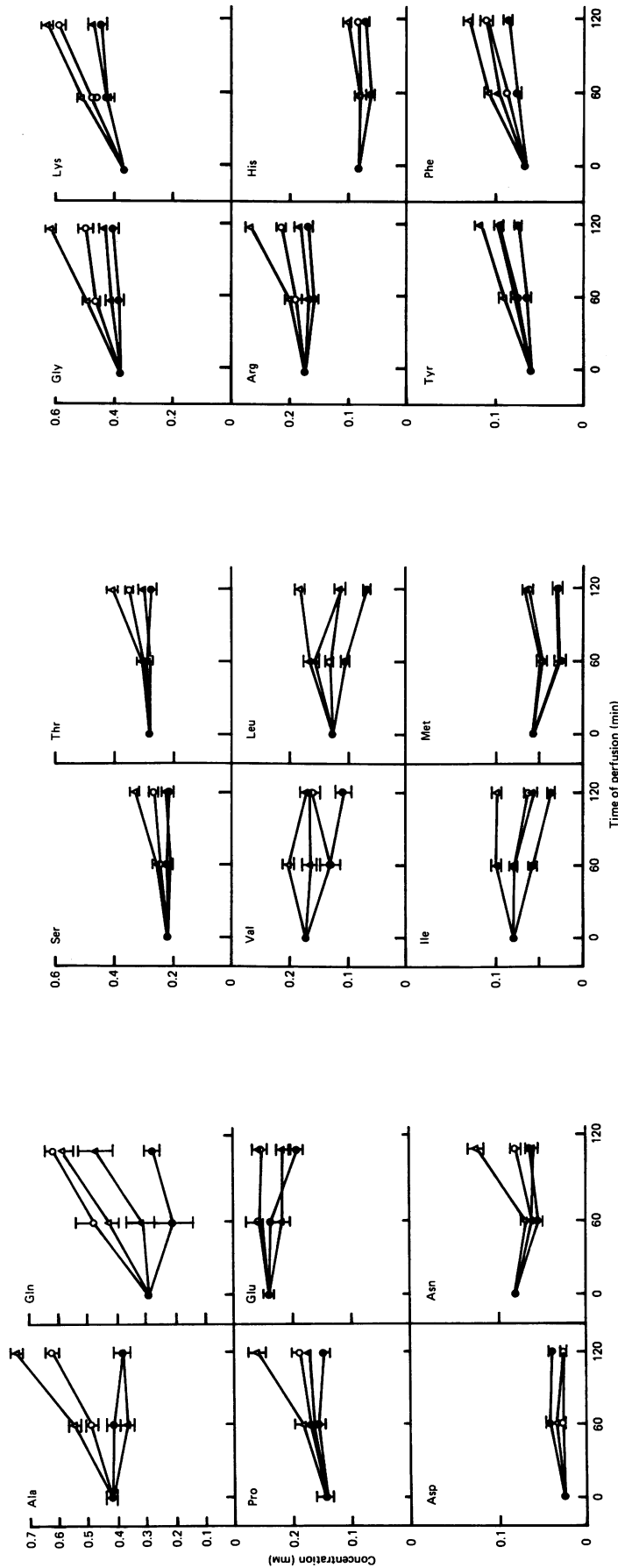


Fig. 1. Release and uptake of amino acids by hindquarters perfused with plasma concentrations of amino acids plus: ○, no addition; ●, 12.5 munits of insulin/ml; △, 100 μM-cycloheximide; ▲, insulin plus cycloheximide

Perfusion volume was 100 ml. Amino acid concentrations at the beginning of perfusions (mM) were: taurine, 0.20; ornithine, 0.09; 2-aminobutyrate, 0.015; citrulline, 0.08; serine, 0.28; methionine 0.07; threonine, 0.30; glycine, 0.40; arginine, 0.20; lysine, 0.40; tyrosine, 0.07; phenylalanine, 0.08; tryptophan, 0.07; histidine, 0.08; glutamine, 0.35; asparagine, 0.07; alanine, 0.45; glutamate, 0.20; aspartate, 0.035; cysteine, 0.07; valine, 0.20; isoleucine, 0.10; leucine, 0.17; and proline, 0.18. For further details see the text. Results are means ± S.E.M. (bars).

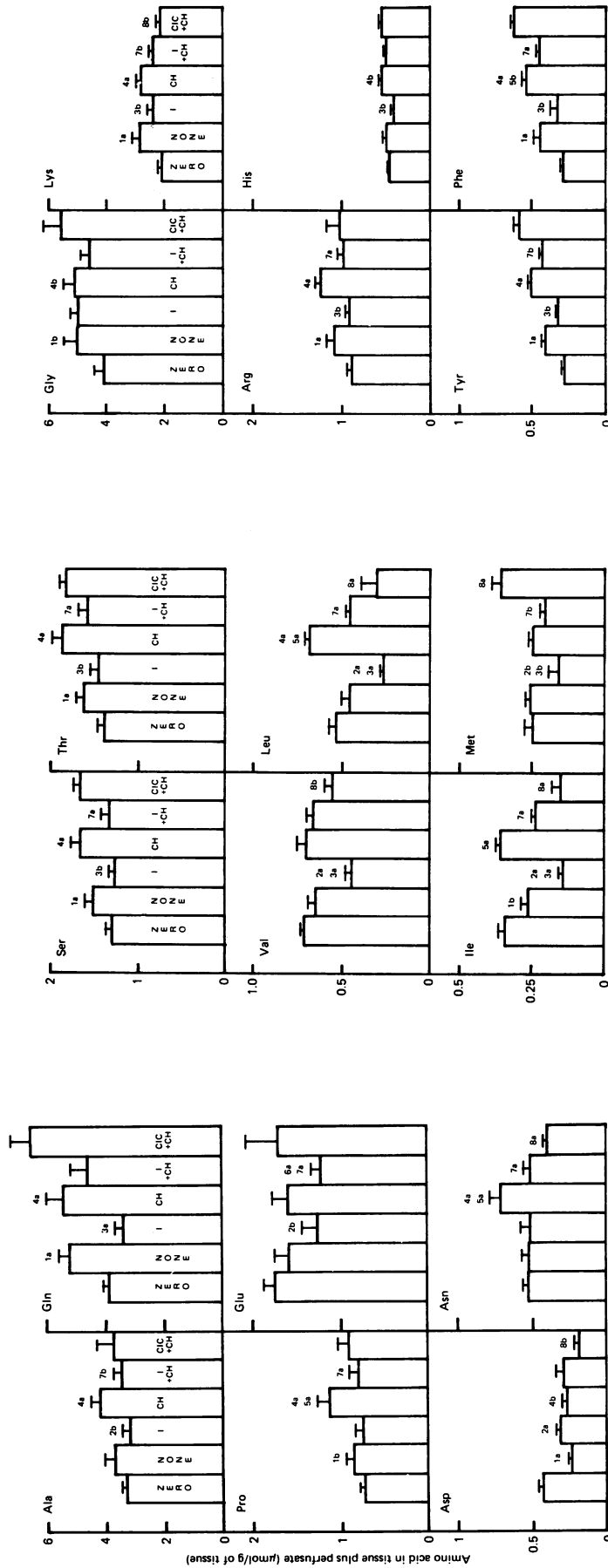


Fig. 2. Changes in total (tissue plus perfusate) amino acids during 2 h perfusion with plasma concentrations of amino acids

Key: zero, zero time after 50 ml washout; none, no other additions; I, insulin added; CH, cycloheximide added; CIC, 2-chloro-4-methylvalerate (0.4 mM) added. The number of perfusions was seven. Statistical significance was as follows: 1, no addition versus zero time; 2, insulin versus zero time; 3, insulin versus no addition; 4, cycloheximide versus zero time; 5, cycloheximide plus insulin versus no addition; 6, cycloheximide plus insulin versus no addition; 7, cycloheximide plus insulin versus cycloheximide; 8, cycloheximide plus 2-chloro-4-methylvalerate versus cycloheximide. Results are means \pm S.E.M. (bars).

muscle was in net nitrogen balance. In addition, the presence of insulin resulted in a marked increase in the net removal of the branched-chain amino acids, methionine and glutamate. Hence, in this situation of nitrogen balance, removal of these five amino acids represents oxidation (partial or complete).

In other studies (e.g. Ruderman & Berger, 1974), when hindquarters were perfused with glucose, lactate and pyruvate, insulin had only marginal effects on the release of glutamine and alanine. Hutson & Zapalowski (1981) reported experiments with hindquarters perfused with a complete amino acid mixture (as in the present study), but the perfusing medium also contained glucose, lactate and pyruvate. In their study insulin somewhat suppressed, but did not prevent, the release of glutamine and alanine. The logical conclusion from the present study and the reports cited above is that, even though the branched-chain amino acids, methionine and glutamate were removed from the system, their rate of catabolism was inadequate to sustain net synthesis of glutamine and alanine.

Effects of 2-chloro-4-methylvalerate on amino acid balance

It has been shown previously that 2-chloro-4-methylvalerate stimulates the branched-chain 2-oxo acid dehydrogenase isolated from heart by inhibiting the kinase which inactivates the dehydrogenase (Harris *et al.*, 1982) and stimulates the removal of endogenous branched-chain amino acids by perfused hindquarter (Davis & Lee, 1985). As shown in Fig. 2 (right-most column in each panel), the principal effect of 2-chloro-4-methylvalerate was on the appearance of the branched-chain amino acids. Even when protein synthesis was blocked, there was net consumption of these amino acids. Compared with the control with cycloheximide alone, the total amounts of valine, leucine and isoleucine were decreased by 2-chloro-4-methylvalerate by about 25, 60 and 60% respectively. This effect was examined in more detail, by using [¹⁴C]valine (see below).

The other rather striking effect of 2-chloro-4-methylvalerate was an increase in the accumulation of methionine. We have no explanation for this effect, and it was not detected previously (Davis & Lee, 1985) when hindquarters were perfused without added substrates.

Measurement of specific radioactivities of tyrosine and valine, and incorporation of ³H and ¹⁴C into protein

In order to obtain the approximate rate of valine catabolism, it is necessary to estimate the tissue specific radioactivity during the perfusions. This is rather complex, since mixing of perfusate amino acids with intracellular amino acids is not instantaneous. It is not experimentally feasible to carry out a detailed time-course of tissue specific radioactivity, owing to the cost of radioactive valine. A rough estimate can, however, be made. The specific radioactivity of [³H]tyrosine in the perfusate at 1 and 2 h (as % of initial specific radioactivity) was 54 ± 3 and 41 ± 2 , respectively, under all perfusion conditions. The average tissue specific radioactivity over this period was 36 ± 3 . For [¹⁴C]valine the tissue specific radioactivity was 40 ± 1 . Hence the latter value was used to calculate the minimum rate of conversions of valine carbon over the 2 h period. We recognize that this value underestimates the true flux rates, since the cellular/specific radioactivity of valine was increasing during at least part of the first 1 h of perfusion. This underestimate of flux will in any case be less than 50%, and probably less than 25%.

Incorporation of radioactivity from tyrosine and valine into tissue protein is recorded in Table 1, by making the same assumption for average specific radioactivity. As expected, insulin increased net incorporation of label by about 50%, whereas cycloheximide inhibited it by about 90%. The calculated ratio of rates of incorporation of valine/tyrosine into protein was about 5. Since this ratio is considerably greater than that in which they occur in muscle proteins (see Davis & Lee, 1985, for references), the probable conclusion is that perfusate valine exchanges with tissue valine more rapidly than the tissue-perfusate tyrosine exchange. Thus the underestimate of average tissue valine specific radioactivity is probably less than 25%.

Oxidation of valine to CO₂

The rate of oxidation of valine carbon was measured indirectly by comparing the total recovery of radioactivity from valine with that for tyrosine, after removal of CO₂. Table 2 shows that essentially 100% of tyrosine radioactivity was accounted for, which is in line with the

Table 1. Incorporation of radioactivity from [³H]tyrosine and [U-¹⁴C]valine into tissue proteins

Perfusions were for 2 h with plasma concentrations of all amino acids together with [³H]tyrosine and [¹⁴C]valine (6.0 and 1.0 d.p.m./pmol respectively for these amino acids in the perfusate at zero time). Minimum rates of incorporation were calculated from the averages of their specific radioactivities at 1 and 2 h. Results are from five to seven perfusions for each condition \pm S.E.M. **P* < 0.005 versus no addition; †*P* < 0.005 versus no addition, or plus insulin. ³H counts in the presence of cycloheximide were very low but not consistent, apparently owing to low counting efficiency and quenching.

Additions	³ H		¹⁴ C	
	(d.p.m./mg of protein)	(nmol of tyrosine incorporated/g)	(d.p.m./mg of protein)	(nmol of valine incorporated/g)
None	228 ± 24	23 ± 1	182 ± 16	110 ± 9
Insulin	392 ± 5*	35 ± 1*	286 ± 17*	154 ± 9*
Cycloheximide	< 30†		20 ± 2†	11 ± 1†
Insulin + cycloheximide	< 30†		25 ± 2†	14 ± 1†

Table 2. Recovery of radioactivity from [³H]tyrosine and [U-¹⁴C]valine after perfusion for 2 h in the presence of cycloheximide

The concentration of cycloheximide was 100 μ M, and that of insulin, when present, was 12.5 m units/ml. Perfusates contained plasma concentrations of amino acids, [³H]tyrosine (0.129 μ Ci/ml) and [U-¹⁴C]valine (0.065 μ Ci/ml). Samples of acid extracts of perfusates and tissue were taken after 2 h and counted for radioactivity. Values are expressed as % of initial radioactivity \pm S.E.M., for four perfusions in each category. Radioactivity incorporated into proteins was insignificant for the recovery calculations.

Other additions	³ H recovery (%)	¹⁴ C recovery (%)
None	101 \pm 1	88 \pm 1
Insulin	98 \pm 2	83 \pm 1

known inability of muscle to catabolize tyrosine. On the other hand, when hindquarters were perfused in the presence of cycloheximide, about 12% of valine carbon was lost as CO₂ and, when insulin was also present, about 17% was oxidized to CO₂. By using the minimum estimate of average tissue specific radioactivity, it was then possible to evaluate the approximate rate of

oxidation of valine carbon to CO₂. According to this estimate, about 5.7 and 8.0 μ mol of valine were oxidized to CO₂ in the absence and presence, respectively, of insulin. These values reduce to 95 and 135 nmol of valine oxidized/h per g respectively.

Separation and identification of organic acids formed from [U-¹⁴C]valine

In order to determine other products derived from valine, some organic acids were separated and identified as described in the Experimental section, and illustrated in Fig. 3. The results of some experiments are summarized in Table 3 (in these early experiments incorporation of label into glutamine, 2-oxo-3-methylbutyrate and alanine was not recorded), showing that some valine carbon reaches the citrate cycle and, hence, has the potential to be oxidized to CO₂, or be converted into other products. The partial oxidation product, 3-hydroxyisobutyrate, accumulated rather substantially (note that in Table 3 the values are not the totals formed, but only those in tissue). There were only marginal differences in the labelling patterns under the four perfusion conditions. It is not surprising that the specific radioactivities of citrate-cycle intermediates were rather low, since those derived from valine would be diluted by many other endogenous substrates.

A rather more detailed analysis of products formed, in both tissue and perfusates, is given for two perfusion

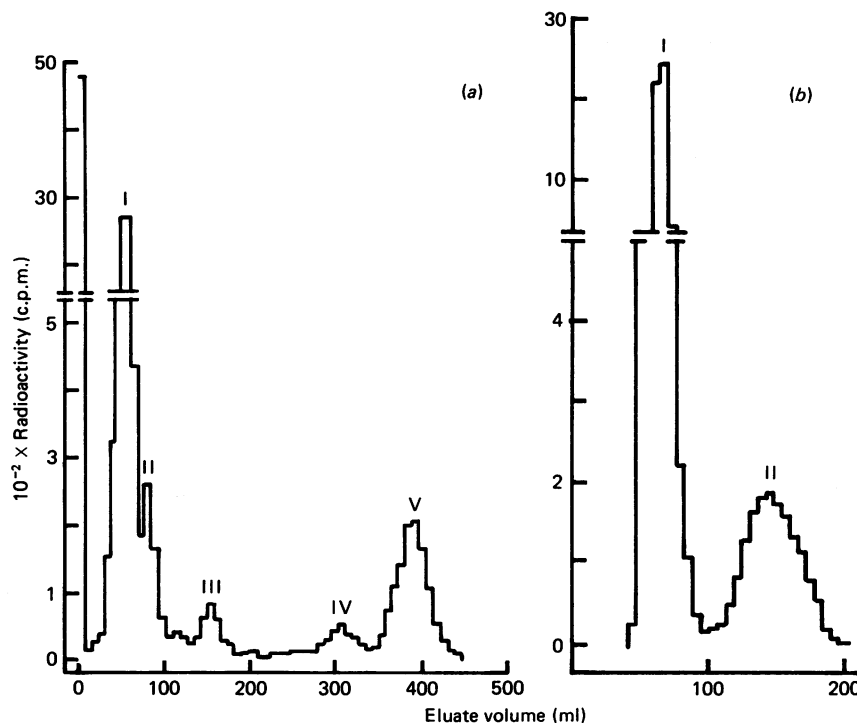


Fig. 3. Identification of main radioactive products in rat hindquarters perfused with plasma concentrations of amino acids and [U-¹⁴C]valine

(a) Neutralized HClO₄ extracts of frozen tissue or perfusate after 2 h of perfusion were chromatographed on a Dowex-1 (formate form) column as described in the Experimental section. Peaks were identified as: I, 3-hydroxyisobutyrate; II, lactate; III, malate; IV, citrate; V, 2-oxoisovalerate. Since peaks I and II were not completely separated on this column, combined fractions from peaks I and II were further separated on a Dowex-1 (acetate form) column. Glutamate and aspartate, which were co-eluted with peak I on the first column, were removed from 3-hydroxyisobutyrate by passage through a Dowex-50W (H⁺ form) cation-exchange column before application to the Dowex-1 (acetate) column (b). Authentic carrier acids were added for identification. The identity of peak I as 3-hydroxyisobutyrate was further documented by t.l.c. as described in the Experimental section, and peak II was identified as lactate.

Table 3. Radioactivity incorporated into metabolites in rat hindquarters perfused with plasma concentrations of amino acids plus [^{14}C]valine

Perfusions were for 2 h, after which approx. 7–8 g of muscle was rapidly freeze-clamped, pulverized and extracted with HClO_4 . Neutralized muscle extracts were analyzed by ion-exchange chromatography. Total radioactivity in each metabolite is expressed as d.p.m. ($\times 10^{-3}$)/30 g of muscle, and specific radioactivity as d.p.m./nmol. Results are means \pm S.E.M. for four observations.

Metabolite	Radioactivity	None	Insulin	Cycloheximide	Insulin + cycloheximide
	Other additions . . .				
Glutamate	Total	87 \pm 25	70 \pm 13	119 \pm 34	101 \pm 28
	Specific	3.5 \pm 1.5	3.5 \pm 0.7	4.8 \pm 1.4	6.1 \pm 1.7
3-Hydroxyisobutyrate	Total	315 \pm 29	219 \pm 32	363 \pm 78	330 \pm 31
	Specific		(Absolute concn. not measured)		
Lactate	Total	24 \pm 6	37 \pm 4	24 \pm 7	43 \pm 14
	Specific	0.5 \pm 0.1	0.2 \pm 0.1	0.3 \pm 0.1	0.4 \pm 0.1
Malate	Total	21 \pm 2	10 \pm 3	31 \pm 6	29 \pm 8
	Specific	5.4 \pm 0.5	3.4 \pm 0.3	6.8 \pm 1.4	7.5 \pm 2.0
Citrate	Total	15 \pm 2	14 \pm 5	18 \pm 3	14 \pm 3
	Specific	9.3 \pm 1.2	9.0 \pm 3.2	12.5 \pm 2.1	8.6 \pm 1.8

conditions in Table 4, which again shows that radioactivity was incorporated into citrate-cycle intermediates as well as glutamate, glutamine, lactate and alanine. Much more 3-hydroxyisobutyrate accumulated than any other intermediate, followed by the transamination product of valine. Now, if the perfusion also contained 2-chloro-4-methylvalerate, rather remarkable changes occurred. The accumulation of 2-oxo-3-methylbutyrate was diminished about 60%, whereas accumulation of 3-hydroxyisobutyrate was elevated about 2.5-fold. Both total and specific radioactivities of all metabolites that were formed subsequent to the oxo acid were greatly elevated, and this coincided with a 60% decrease in the accumulation and release of 2-oxo-3-methylbutyrate. Hence, flux of carbon to all products past the first decarboxylation step was greatly enhanced by 2-chloro-4-methylvalerate, resulting in highly significant fluxes to the citrate cycle and subsequently to glutamine and glutamate, and flux from the cycle to alanine and lactate. The data in Table 5 are calculated as minimum incorporation rates of valine carbon into metabolites, based on the results presented in Table 4 with approximations as defined in the legend. It is noteworthy that approx. 19 and 40% of the total valine pool was recovered in these metabolites when 2-chloro-4-methylvalerate was absent or present respectively. Without 2-chloro-4-methylvalerate, incorporation was mainly into 3-hydroxyisobutyrate, followed by 2-oxo-3-methylbutyrate, lactate, glutamate, glutamine and alanine. With 2-chloro-4-methylvalerate, much more 3-hydroxyisobutyrate accumulated and less 2-oxo-3-methylbutyrate, and, owing to elevated flux through the 2-oxo acid dehydrogenase, much more radioactivity was found also in glutamine, glutamate and alanine. It is concluded then that, when the 2-oxo acid dehydrogenase is stimulated with 2-chloro-4-methylvalerate, this enzyme ceases to be rate-limiting for the overall catabolism of valine. This results in increased flux over the entire pathway to citrate-cycle intermediates (including the 3-hydroxyisobutyrate dehydrogenase reaction, E.C. 1.1.1.31), but now resulting in even more massive accumulation of 3-hydroxyisobutyrate. The rate constriction under these conditions appears to be the latter enzyme.

DISCUSSION

Several important new points appear to be resolved by the present and recently published studies. Firstly, if muscle is perfused in the absence of added substrates (Davis & Lee, 1985) or with only plasma concentrations of amino acids (the present paper), alanine is not formed in excess of that found in muscle proteins, so that there is no net synthesis of alanine carbon from amino acid precursors. The net formation of glutamine, however, appears to be in part from amino acids (Davis & Lee, 1985). Thus, in the absence of a major source of added carbohydrate, there was no net synthesis of alanine, which means that carbon flux from amino acids is inadequate for net alanine synthesis. In this context, it is striking that, when the muscle was in protein balance (pharmacological amounts of insulin present), and no glucose or pyruvate was added, there was no net synthesis of either alanine or glutamine (Fig. 2), even though there was catabolism of the branched-chain amino acids and methionine. On the other hand, if glucose and/or carbohydrate degradation products were also added (Ruderman & Berger, 1974; Hutson & Zapalowski, 1981), net synthesis of alanine and glutamine was only slightly suppressed by insulin. Hence it is concluded, in agreement with our previous report (Davis & Lee, 1985), that amino acid catabolism alone is too slow to sustain net synthesis of the carbon skeleton of alanine.

It was established several years ago that both heart (Davis & Bremer, 1973) and skeletal muscle (Spydevold *et al.*, 1976) catabolize, to a certain extent, all three of the branched-chain amino acids to the level of the citrate cycle, since perfusion with their oxo acid analogues resulted in detection of all of the intermediate acylcarnitine derivatives. However, those studies did not give information on the extent of this interconversion or on the extent to which intermediates in the pathways accumulated. Harper and co-workers (reviewed by Harper & Zapalowski, 1981) and several groups subsequently have shown that the oxo acid analogues of the branched-chain amino acids are released from muscle preparations presented with these amino acids. This has led to the conclusion by Harper's group that, on a

Table 4. Effect of 2-chloro-4-methylvalerate on incorporation of radioactivity into metabolites from [U-¹⁴C]valine in rat hindquarters perfused with normal plasma concentrations of amino acids and cycloheximide

Perfusions were for 2 h. Total radioactivity is expressed as d.p.m. ($\times 10^{-3}$) in total muscle or perfusate. Specific radioactivity is expressed as d.p.m./nmol. Values obtained with cycloheximide alone are means \pm S.E.M. for three perfusions; those with 2-chloro-4-methylvalerate are the means from two perfusions, with ranges given.

Metabolite	Radioactivity	Cycloheximide			Cycloheximide plus 2-chloro-4-methylvalerate		
		Tissue	Perfusate	Sum	Tissue	Perfusate	Sum
2-Oxo-3-methylbutyrate	Total	205 \pm 29	208 \pm 35	431 \pm 64	78 (42-114)	89 (63-115)	167 (157-167)
	Specific	5.6 \pm 0.6			19 (18-20)		
3-Hydroxyisobutyrate	Total	280 \pm 26	1616 \pm 103	1886 \pm 129	667 (648-686)	3907 (3857-3957)	4574 (4505-4643)
	Specific				27 (2.5-2.9)	1.1 (0.8-1.5)	
Malate	Total	23 \pm 4	0	23 \pm 4	22 (18-25)	0	22 (18-25)
	Specific	5.6 \pm 0.6			19 (18-20)		
Lactate	Total	34 \pm 10	99 \pm 26	133 \pm 36	121 (87-154)	117 (98-136)	243 (195-290)
	Specific	0.5 \pm 0.1	0.4 \pm 0.1		27 (2.5-2.9)	1.1 (0.8-1.5)	
Citrate	Total	14 \pm 5	11 \pm 0	24 \pm 5	36 (35-37)	19 (18-21)	55 (53-57)
	Specific	8.8 \pm 0.6	1.5 \pm 0.0		9.5 (9-10)	4.5 (4-5)	
Glutamate	Total	67 \pm 11	63 \pm 20	130 \pm 31	165 (132-197)	51 (46-55)	216 (187-243)
	Specific	2.2 \pm 0.6	2.6 \pm 1.2		8.3 (6.6-10)	2.3 (1.3-3.3)	
Glutamine	Total	77 \pm 8	44 \pm 11	121 \pm 19	499 (374-623)	216 (159-273)	715 (533-890)
	Specific	1.0 \pm 0.0	1.0 \pm 0.3		3.9 (3.2-4.6)	3.7 (3.0-4.3)	
Alanine	Total	16 \pm 2	14 \pm 2	30 \pm 4	41 (39-44)	27 (23-31)	68 (66-90)
	Specific	0.3 \pm 0.1	0.2 \pm 0.1		1.0 (0.7-1.3)	0.5 (0.4-0.6)	

quantitative basis, much more oxo acid is released from muscle than is oxidized (e.g. Zapalowski *et al.*, 1981). On the other hand, Spydevold (1979) reported that rat hindquarters perfused with valine formed approximately as much 3-hydroxyisobutyrate as 2-oxo-3-methylbutyrate, even though these experiments were carried out with fed animals, a situation in which the branched-chain oxo acid dehydrogenase is relatively inactive. If this step was stimulated (with octanoate), much more 3-hydroxyisobutyrate and much less 2-oxo-3-methylbutyrate accumulated (Spydevold, 1979; Spydevold & Hokland, 1981). In a subsequent study (Spydevold & Hokland, 1983) it was also shown that metabolites from isoleucine and leucine beyond the 2-oxoacids were released into the perfusing medium, and that octanoate greatly accelerated their formation. These data cast considerable doubt on the generally accepted conclusion that the branched-chain 2-oxo acid dehydrogenase is the principal (or only) control step in the overall handling of the branched-chain amino acids by muscle under most conditions.

The present results on valine metabolism should be considered especially in view of the data of Spydevold (1979), Spydevold & Hokland (1981, 1983) and the accumulated data of Harper's group (notably Zapalowski

et al., 1981). Spydevold (1979) perfused muscles from fed rats. In this system, approximately equal amounts of 3-hydroxyisobutyrate and 2-oxo-3-methylbutyrate accumulated unless the branched-chain 2-oxo acid dehydrogenase was stimulated with octanoate. In the experiments reported herein, starved animals were used, and the former acid accumulated to a far greater extent than did the latter. This is not surprising, since flux through the branched-chain oxo acid dehydrogenase is increased by starvation (e.g. Goldberg & Odessey, 1972). On close examination of the results of Zapalowski *et al.* (1981), the present results appear to be completely in agreement with their data, except that now we have measured other products of valine metabolism. The principal results of Zapalowski *et al.* (1981) in the present context are as follows. (a) When rat hindquarters from fed rats were perfused with [1-¹⁴C]valine, only about 25% of valine transaminated was α -decarboxylated, and 2-oxo-3-methylbutyrate accumulated. However, if hindquarters from starved rats were used, α -decarboxylation was more than doubled, and the accumulation and release of 2-oxo-3-methylbutyrate was decreased by about 70%. (b) When ¹⁴CO₂ production from [1-¹⁴C]- and [U-¹⁴C]-valine was compared, it was concluded that rather little valine was oxidized to CO₂ beyond the

Table 5. Net incorporation of valine carbon into measured metabolites

Approximations are as follows: the total pool of valine was that added in perfusates plus endogenous (taken from Fig. 2) plus the rate of proteolysis over 2 h perfusion [from tyrosine appearance (Fig. 2) and the estimated relative frequency of occurrence of tyrosine and valine in muscle proteins (Davis & Lee, 1985)]. From this calculation the total pool size of valine was 60 μmol , or 2 $\mu\text{mol/g}$ of muscle tissue. The average specific radioactivity of tissue valine over the 2 h time period (2.5×10^5 d.p.m./ μmol) was estimated as 40% that of [^{14}C]valine added to the perfusate at zero time.

Metabolite	Valine incorporated (nmol/h per g)	
	Cycloheximide (100 μM) present	Cycloheximide (100 μM) + 2-chloro-4-methylvalerate present
2-Oxo-3-methylbutyrate	29	11.2
3-Hydroxyisobutyrate	127	305
Malate	1.5	1.6
Lactate	8.8	16
Citrate	1.5	3.6
Glutamate	8.4	14
Glutamine	8.4	48
Alanine	2.0	4.8
Percentage of total radioactivity recovered in acid-soluble metabolites	18.7	40.5
Conversion of valine carbon into acid-soluble metabolites [μmol (total) in 2 h]	11.2	24.3

α -decarboxylation step. From the indirect measurement of $^{14}\text{CO}_2$ production in the present work, it can be calculated that nearly all of the $^{14}\text{CO}_2$ formed is accounted for by accumulation of 3-hydroxyisobutyrate and other acid-soluble metabolites. In the experiments with 2-chloro-4-methylvalerate present, $^{14}\text{CO}_2$ production was not estimated. Nevertheless, this compound certainly increased $^{14}\text{CO}_2$ production from both the α -decarboxylation step and subsequent oxidations, since it markedly increased the accumulation of 3-hydroxyisobutyrate and the specific radioactivities of citrate-cycle intermediates and other metabolites linked to the cycle.

From the above considerations of the results of ourselves and others, the following conclusions appear to be justified. (a) The carbon flux from amino acids play a minor role in the net synthesis of alanine, which is under certain conditions released from muscle. There is incorporation of label (in this case from [^{14}C]valine) into glutamine, especially when the branched-chain 2-oxo acid dehydrogenase is stimulated. This probably is indicative of the relative rates of flux of carbon to the citrate cycle. However, it is probably not justified to equate incorporation rates with the rates of net glutamine synthesis, since glutamate and glutamine can become labelled by exchange reactions (reversible aspartate

aminotransferase) without net glutamine synthesis. [Note that a remarkable amount of radiolabel from [^{14}C]leucine was reported to be incorporated into glutamine (Hutson & Zapalowski, 1981), but the pathway of leucine metabolism precludes net synthesis of glutamine carbon from this amino acid.] In fact, the balance studies reported herein and the study by Zapalowski *et al.* (1981) suggest that net glutamine synthesis from branched-chain amino acids is relatively minor (but contrast conclusions of Chang & Goldberg, 1978b). (b) The carbon compounds from valine metabolism released by muscle for ultimate uptake by the liver as glucogenic precursors are mainly 3-hydroxyisobutyrate and 2-oxo-3-methylbutyrate, and not alanine and glutamine. Under starvation conditions (when protein amino acids are important carbon precursors for gluconeogenesis), the principal glucose precursor released from muscle appears to be 3-hydroxyisobutyrate.

The situation with isoleucine is less clear, since the overall fate of its carbon skeleton has not been so extensively studied. It is of interest that hydroxy acids derived from the partial degradation of all three of the branched-chain amino acids are excreted in small amounts into the urine by healthy humans, and the amount excreted is elevated many-fold by ketoacidosis (apparently independent of the etiology of the acidosis) (Landaas, 1975). Furthermore, in studies with perfused rat hindquarters (Spydevold & Hokland, 1983) it was reported that isovalerate (from leucine) and 2-methylbutyrate (from isoleucine) were accumulated. If octanoate was present to stimulate the α -oxidation of these amino acids, the release of these carboxylic acids was also stimulated. In contrast with 3-hydroxyisobutyrate formation from valine, isovalerate and 2-methylbutyrate do not exist as free acids in the accepted pathway of their metabolism, but as CoA esters. Nevertheless, the now documented formation of these free acids in humans (Landaas, 1975) and in perfused muscle preparations (Spydevold & Hokland, 1983) makes it tempting to propose a side path for the inter-organ handling of branched-chain amino acids. In addition to the release of 2-oxo acids by muscle and their subsequent uptake and oxidation by the liver, as proposed by Harper and co-workers (summarized in Harper & Zapalowski, 1981), release from muscle and uptake by liver of 3-hydroxyisobutyrate may be quantitatively even more important in the inter-organ handling of valine carbon than is 2-oxo-3-methylbutyrate. For isovalerate and 2-methylbutyrate, hydrolysis of their CoA esters is required to form the free acids. Once these free acids are released from muscle, they could be taken up and re-activated by the liver, which contains a fatty-acid-activating enzyme (medium-chain acid:CoA ligase; EC 6.2.1.2), which is very active in forming CoA esters of hydroxy- and methyl-substituted medium-chain-length fatty acids (Mahler *et al.*, 1953; Lehninger & Greville, 1953). An inter-organ shunt as here suggested has not been demonstrated, perhaps owing to the absence until recently of some of the suggestive evidence enumerated above that this pathway may indeed occur. The existence and quantification of this deacylation/reacylation cycle of intermediates in leucine and isoleucine metabolism between muscle and liver, inferred from recent evidence, will require further study.

It should be emphasized that partial degradation of the branched-chain amino acids (stopping before entry into

the citrate cycle) presents a net drain on the pool of the latter as a result of the primary transamination reaction. For valine and isoleucine the depletion and repletion flux rates balance if part of their carbons reach succinyl-CoA, or if carbon intermediates formed are decarboxylated, with coupled alanine formation through transamination (see Davis *et al.*, 1972, 1980; Davis & Bremer, 1973; Lee & Davis, 1980). The identity and quantitative importance of these anaplerotic reactions (some of which have been identified; see, e.g., Lee & Davis, 1979), in particular with respect to catabolism of the branched-chain amino acids by muscle, also requires further study.

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