Glycolytic origin of alanine formed in rat diaphragm muscle *in vitro*

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In quarter-diaphragms from 40 h-starved rats the rate of glycogen mobilization is sufficient to account for the rate of lactate + pyruvate + alanine production. It is concluded, therefore, that alanine derives its carbon skeleton predominantly via glycolysis and not via synthesis *de novo* from tricarboxylic acid-cycle intermediates and related amino acids.

INTRODUCTION

Alanine and glutamine account for more than 50% of the amino acids released by muscle in starvation, whereas they comprise less than 10% of the component amino acids in skeletal-muscle proteins. Alanine, which is widely accepted (cf. glutamine; see Kovacević & McGivan, 1983) to have a key role as a precursor in hepatic gluconeogenesis (Felig, 1975; Snell, 1980), is formed in the alanine aminotransferase (EC 2.6.1.2) reaction. The branched-chain amino acids (valine, leucine and isoleucine) contribute amino nitrogen to alanine formation via glutamate produced in the branched-chain amino acid aminotransferase (EC 2.6.1.42) reaction, but it is controversial whether they may also contribute carbon. It has been proposed that in skeletal muscle there is alanine synthesis *de novo* from certain amino acid precursors (namely glutamate, valine, isoleucine, aspartate, asparagine). The proposed pathway for alanine synthesis involves the conversion of the amino acids into oxaloacetate and then to pyruvate via phosphoenolpyruvate carboxykinase (EC 4.1.1.32) and pyruvate kinase (EC 2.7.1.40) or NADP⁺-linked malate dehydrogenase (malic enzyme; EC 1.1.1.40) (Garber *et al.*, 1976; Goldstein & Newsholme, 1976; Snell & Duff, 1977). An alternative proposal ('the glucose-alanine cycle') is that glucose taken up by muscle from the bloodstream produces pyruvate via glycolysis for alanine synthesis (Felig, 1975). Alanine formation by this mechanism serves to transport potentially toxic amino nitrogen from muscle to liver but makes no net contribution to the total body glucose pool.

Studies in this laboratory have led us to conclude that flux via the pathway of alanine synthesis *de novo* is quantitatively insignificant in rat hemidiaphragm muscle *in vitro* (Palmer *et al.*, 1984a, b). Firstly, the addition of unlabelled glucose does not decrease (via radiolabel dilution) ¹⁴CO₂ production from [U-¹⁴C]valine, [U-¹⁴C]isoleucine, [U-¹⁴C]glutamate and [U-¹⁴C]aspartate, as would be predicted if the pathways of glucose and amino acid oxidation were to share a common pyruvate pool (Palmer *et al.*, 1983). Secondly, neither 3-mercaptopicolinate (an inhibitor of phosphoenolpyruvate carboxykinase) nor dichloroacetate (an activator of the

pyruvate dehydrogenase complex) has effects on amino acid oxidation and alanine release, which observations are consistent with operation of the pathway of synthesis *de novo* (Palmer *et al.*, 1984a,b). The present paper presents data that demonstrate that glycogenolysis occurs at a rate sufficient to account for the rates of production of alanine, lactate and pyruvate in rat quarter-diaphragm *in vitro* and therefore that it is unnecessary to invoke neogenic pyruvate synthesis as a source of alanine carbon.

MATERIALS AND METHODS

Materials

Sources of reagents were as given in Palmer *et al.* (1984a,b).

Animals

Male Wistar rats (100–120 g) were maintained at approx. 17 °C on a 12 h-day/12 h-night cycle (day started at 08:00 h). The rats were starved for 40 h before the experiments, which were commenced at 11:00–13:00 h.

Experimental

Rat quarter-diaphragms were preincubated (30 min at 37 °C) in 2 ml of substrate-free Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), equilibrated with O₂/CO₂ (19:1). The purpose of this preincubation was to deplete muscle glycogen. Glycogen content was determined after preincubation in one quarter-diaphragm from each diaphragm. The remaining quarter-diaphragms were incubated (Palmer *et al.*, 1983) for 2 h in 2 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, either with no added substrate or with 3 mM-[1-¹⁴C]-valine, 3 mM-[1-¹⁴C]leucine or 3 mM-[U-¹⁴C]isoleucine. ¹⁴CO₂ production from the radiolabelled substrates during the incubation period was determined by entrapment in Hyamine (BDH Chemicals, Poole, Dorset, U.K.) after acidification of the media with 0.2 ml of 60% (w/v) HClO₄ (Palmer *et al.*, 1983). The remaining quarter-diaphragms were removed from their incubation media before acidification, freeze-clamped with aluminium tongs precooled in liquid N₂ and stored at –23 °C until use; the incubation media were then acidified with 0.2 ml of 60% (w/v) HClO₄.

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The KOH-neutralized media were assayed for lactate, pyruvate, alanine, glutamate and glutamine as described elsewhere (Palmer *et al.*, 1984a,b). The conversion of the 1-¹⁴C-labelled amino acids into their 2-oxo derivatives was quantified as ¹⁴CO₂ release from the incubation media after addition of H₂O₂ (Chang & Goldberg, 1978a). Total transamination of [1-¹⁴C]valine and [1-¹⁴C]leucine is calculated as the sum of the rate of 1-¹⁴C-labelled 2-oxo acid production plus the rate of 2-oxo acid 1-decarboxylation in the branched-chain 2-oxo acid dehydrogenase reaction. Glycogen content of diaphragm muscle (expressed as μ mol of glucose equivalents/g wet wt.) was determined by using a combined amyloglucosidase/glucose oxidase reagent after extraction (100 °C, 5 min) with 0.3 ml of 30% (w/v) KOH and either isolation by ethanol precipitation (Palmer *et al.*, 1983) or after dialysis against distilled water (Palmer *et al.*, 1982). Glycogen mobilization as triose equivalents is calculated from the difference $\times 2$ in glycogen content between the start and end of the 2 h incubation.

Results are expressed as means $\pm$ S.E.M. with the numbers of observations in parentheses. Statistical analysis was by Student's *t* test.

RESULTS AND DISCUSSION

Quarter-diaphragms from 40 h-starved rats produced lactate + pyruvate + alanine at similar rates irrespective of whether they were provided with 3 mM-leucine, -valine or -isoleucine or incubated in the absence of substrate (Table 1). The provision of the branched-chain amino acids, however, increased alanine release (by 45%, 26% and 49% by leucine, valine and isoleucine respectively), presumably at the expense of release of lactate and pyruvate, although the [lactate]/[pyruvate] ratio (an index of the cytosolic [NAD⁺]/[NADH] ratio) was not significantly changed except on addition of valine (decreased by 30%). The following results suggest that alanine derives its carbon skeleton predominantly, if not exclusively, via glycolysis.

1. Alanine release was increased by leucine, isoleucine and valine to the same extent (Table 1). Since leucine is metabolized to acetyl-CoA and acetoacetate, it cannot act as a carbon precursor in neogenic alanine synthesis, and this suggests that it promotes alanine synthesis solely via acting as an amino-group donor in the branched-chain amino acid aminotransferase reaction. There was no evidence that alanine release in the presence of isoleucine and valine was greater than that in the presence of leucine, suggesting that the stimulatory effects of these latter amino acids may also be secondary to amino-group donation rather than carbon.

2. Addition of valine or isoleucine did not increase total lactate + pyruvate + alanine release, as would be expected if these amino acids were to contribute carbon to alanine synthesis *de novo*.

3. The proposed pathway of alanine synthesis *de novo* involving phosphoenolpyruvate carboxykinase implies a stoichiometry of 1 mol of alanine produced per mol of valine (or isoleucine) utilized (Snell, 1980; Palmer & Sugden, 1983). In Table 2 it is shown that flux through the branched-chain 2-oxo acid dehydrogenase reaction (estimated as ¹⁴CO₂ production from [1-¹⁴C]valine) occurs at a rate only sufficient to account for less than 25% of total lactate + pyruvate + alanine release

(Table 1), implying that the remainder of the lactate + pyruvate + alanine must presumably be derived from glycogenolysis and/or endogenous amino acid catabolism. Interestingly, the rates of transamination of valine and leucine significantly exceeded the corresponding rates of 1-decarboxylation in the branched-chain 2-oxo acid dehydrogenase reaction, with the result that substantial amounts of the 2-oxo acids 3-methyl-2-oxobutanoate and 4-methyl-2-oxopentanoate were released (Table 2).

Goldstein & Newsholme (1976) reported that there was no net glycogenolysis in hemidiaphragms incubated *in vitro* with amino acid substrates, and concluded on the basis of this that glycogenolysis makes no contribution to muscle alanine synthesis under these circumstances. In our laboratory, however, despite preincubation to minimize the contribution of glycogen to alanine synthesis (see the Materials and methods section), we consistently observe that diaphragm muscle incubated *in vitro* with amino acid substrates (namely valine, isoleucine, leucine, glutamate, aspartate) mobilizes its glycogen reserves (Odedra & Palmer, 1981; Palmer *et al.*, 1983, 1984a,b; see also Table 1), and, depending on the method of glycogen isolation, this mobilization can quantitatively account for the rate of lactate + pyruvate + alanine release. In these experiments glycogen was extracted with 30% (w/v) KOH and either isolated by ethanol precipitation (Palmer *et al.*, 1983) or assayed directly after dialysis (Odedra & Palmer, 1981). Immediately after preincubation, the glycogen content of the quarter-diaphragm was 2.9 ± 0.3 (17) μ mol/g wet wt. (as glucose equivalents) measured by the ethanol precipitation method as opposed to 9.6 ± 0.8 (22) μ mol/g wet wt. measured by the dialysis method. On subsequent incubation in media containing no added substrate or 3 mM-leucine, -valine or -isoleucine, diaphragm glycogen was mobilized (Table 1), but glycogen mobilization measured by the ethanol precipitation method was markedly less than that measured by the dialysis method (Table 1), equivalent to < 45% or 89–171% of the total lactate + pyruvate + alanine released respectively. The results indicate that ethanol precipitation does not achieve quantitative recovery of glycogen at low glycogen concentrations and suggest that methodological problems may account for the failure to correlate glycogen mobilization with alanine release. The present work strongly suggests that glycogenolysis provides the bulk of the carbon for lactate + pyruvate + alanine production. Addition of leucine had a glycogen-sparing effect, presumably via citrate-mediated inhibition of glycolytic flux (Chang & Goldberg, 1978c), and glycogenolysis in this instance cannot totally (by 11%; $P < 0.05$) account for lactate + pyruvate + alanine release. The discrepancy does not imply neogenic flux (i.e. leucine is metabolized via acetyl-CoA and cannot contribute carbon to alanine synthesis), and the deficit may be provided by alanine derived from proteolysis.

CONCLUSIONS

The results presented indicate that valine and isoleucine do not specifically promote lactate + pyruvate + alanine synthesis in a manner consistent with neogenic flux via phosphoenolpyruvate carboxykinase or malic enzyme. Glycogen mobilization in quarter-diaphragms *in vitro* proceeds at a rate sufficient to

Table 1. Metabolite production and glycogen mobilization in quarter-diaphragms from 40 h-starved rats

$^{14}\text{CO}_2$ production in these experiments is shown in Table 2. Glycogen mobilization (expressed as triose equivalents) is calculated from the difference in diaphragm glycogen content at the beginning and end of the 2 h incubation. Glycogen was determined after either (a) ethanol precipitation or (b) dialysis (see the Materials and methods section). * $P < 0.05$, *** $P < 0.001$, for differences between no added substrate and plus substrate.

Substrate added	Metabolites ($\mu\text{mol}/2 \text{ h per g wet wt.}$)			
	Alanine	$\frac{[\text{Lactate}]}{[\text{Pyruvate}]}$ ratio	Lactate + pyruvate + alanine	Glycogen mobilization (as triose equivalents)
				(a) (b)
None	1.93 ± 0.09 (50)	10.87 ± 1.14 (50)	10.57 ± 0.39 (50)	4.40 ± 0.63 (17) 15.40 ± 1.61 (22)
3 mM-[1- ^{14}C]Leucine	2.80 ± 0.18 (30)***	13.05 ± 1.08 (30)	11.50 ± 0.56 (30)	5.21 ± 1.22 (8) 10.26 ± 0.77 (12)*
3 mM-[1- ^{14}C]Valine	2.43 ± 0.14 (30)***	7.60 ± 0.69 (30)*	11.25 ± 0.56 (30)	3.64 ± 0.62 (9) 19.24 ± 1.64 (21)*
3 mM-[U- ^{14}C]Isoleucine	2.87 ± 0.23 (23)***	9.33 ± 1.06 (23)	12.02 ± 0.72 (23)	— 11.59 ± 1.57 (12)

account on an approximate basis for the lactate + pyruvate + alanine produced (but see Goldstein & Newsholme, 1976). It is concluded, therefore, that alanine derives its carbon skeleton primarily via glycolysis ('the glucose-alanine cycle'; Felig, 1975) and that neogenic flux is quantitatively unimportant. This conclusion (see also Chang & Goldberg, 1978a,b; Palmer *et al.*, 1984a,b) is based on studies on diaphragm muscle *in vitro*, and whether it is equally valid *in vivo* requires corroboration. If, as the present results imply, alanine does not constitute a vehicle for the net transfer of carbon from muscle to liver for gluconeogenesis, then the question again arises as to how the total body glucose pool is sustained in starvation (see the Introduction section). To maintain this pool it is necessary to compensate for net glucose oxidation by the brain and other oxidative tissues via net glucose production by gluconeogenesis and glycogenolysis. In this context it is important to differentiate between glucogenesis via recycling of products of incomplete glucose metabolism (e.g. lactate, alanine) by peripheral tissues, and net gluconeogenesis from intermediates of non-carbohydrate origin released by peripheral tissue ('neogluconeogenesis'). In considering the role of muscle in the provision of gluconeogenic substrates, both glutamine and intermediate products of branched-chain amino acids may be important. Glutamine is synthesized from glutamate and NH_3 in the glutamine synthase (EC 6.3.1.2) reaction [NH_3 is presumed to be produced in the AMP deaminase (EC 3.5.4.6) reaction, having originated from aspartate in the adenylosuccinate synthase (EC 6.3.4.4) and adenylosuccinate lyase (EC 4.3.2.2) reactions of the purine nucleotide cycle (Lowenstein, 1972)]. The branched-chain amino acids valine and isoleucine may contribute part of the carbon of 2-oxoglutarate and hence glutamine synthesis via their conversion into tricarboxylic acid-cycle intermediates. Although glutamine as such is a poor substrate for hepatic gluconeogenesis, it may be converted in the kidney and intestinal epithelial cells into alanine and lactate, and these may act as net gluconeogenic precursors in the liver (Chang & Goldberg, 1978b). Since in rat muscle flux via branched-chain amino acid aminotransferase exceeds that via branched-chain 2-oxo acid dehydrogenase, there is net release of the branched-chain 2-oxo acids (Livesey & Lund, 1980; see Table 2). Hence 3-methyl-2-oxobutanoate and 3-methyl-2-oxopropanoate may act as precursors in hepatic gluconeogenesis (Lund, 1981). It is commonly assumed that the branched-chain 2-oxo acid dehydrogenase complex catalyses the flux-generating step in branched-chain amino acid oxidation to CO_2 . There are indications, however, that intermediates subsequent to the branched-chain 2-oxo acid dehydrogenase complex accumulate *in vitro* and *in vivo*. Valine metabolism in muscle produces substantial amounts of the intermediate 3-hydroxyisobutyrate (Spydevold, 1979). This and other products of partial oxidation of valine and isoleucine (including 3-hydroxy-2-methylbutyrate) may act as precursors in hepatic gluconeogenesis.

The respective roles of glutamine, the branched-chain 2-oxo acids and the products of their partial oxidation as precursors in 'neogluconeogenesis' remain to be established.

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Table 2. Metabolism of branched-chain amino acids by quarter-diaphragms from 40 h-starved rats

$^{14}\text{CO}_2$ released on treatment of the acidified media with H_2O_2 is a measure of the amount of 4-methyl-2-oxo[1- ^{14}C]pentanoate (derived from [1- ^{14}C]leucine) or 3-methyl-2-oxo[1- ^{14}C]butanoate (derived from [1- ^{14}C]valine). Transamination is calculated as the sum of 1- ^{14}C -labelled oxo acid and $^{14}\text{CO}_2$ production. *** $P < 0.001$ for differences between valine and leucine as substrate.

Substrate	No. of observations	Metabolites produced ($\mu\text{mol}/2\text{ h}$ per g wet wt.)		
		$^{14}\text{CO}_2$ production	$^{14}\text{CO}_2$ released by H_2O_2	Calculated transamination
3 mM-[1- ^{14}C]Leucine	21	3.95 ± 0.23	1.84 ± 0.13	5.77 ± 0.26
3 mM-[^{14}C]Valine	21	$2.59 \pm 0.14^{***}$	$1.14 \pm 0.09^{***}$	$3.70 \pm 0.17^{***}$

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