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The Release of Alanine by Rat Diaphragm Muscle *in vitro*

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Alanine release by rat diaphragm muscle *in vitro* is stimulated by glutamate, valine, leucine and glucose. The stimulation by glutamate and valine (but not leucine) is inhibited by 3-mercaptopicolinate. These results suggest a metabolic route involving phosphoenolpyruvate carboxykinase which directs amino acid carbon skeletons towards pyruvate synthesis for alanine formation.

In situations of dietary insufficiency, substrates for gluconeogenesis must be derived from endogenous sources. Because muscle represents a major fraction of body mass and body protein it would be expected to play a significant role in furnishing amino acid glucogenic precursors to the liver. Measurements *in vivo* in the rat (Aikawa *et al.*, 1973) and in man (see Felig, 1973) indicate that this is so and that, of the amino acids released by skeletal muscle, alanine makes a disproportionately large contribution relative to its abundance in muscle proteins (Pozefsky *et al.*, 1969; Ruderman & Berger, 1974; Odessey *et al.*, 1974; Garber *et al.*, 1976*a*). This has led to the hypothesis that alanine may be formed within muscle by transamination of pyruvate, produced by glycolysis, with a number of other amino acids (Mallette *et al.*, 1969; Felig, 1973; Odessey *et al.*, 1974), either directly or via initial transamination with 2-oxoglutarate.

The pyruvate required for alanine formation must be derived, at least partially, from glycolysis, and evidence for this has been reported (Odessey *et al.*, 1974; Grubb, 1976). However, alanine formation from pyruvate derived by this route cannot contribute to the net replenishment of body carbohydrate (via glucose formation in the liver) that occurs in situations associated with increased gluconeogenesis, such as starvation (Cahill *et al.*, 1966). That process could be served, however, by amino acids and other precursors that can generate tricarboxylic acid-cycle intermediates to provide a potential source of pyruvate for alanine formation. Oxaloacetate or malate from the cycle could be converted into pyruvate via the enzymes phosphoenolpyruvate carboxykinase and pyruvate kinase or NADP-dependent malate dehydrogenase respectively, which are present in muscle (Opie & Newsholme, 1967). On the basis of metabolite changes in the perfused heart, Davis & Bremer (1973) have proposed a role for NADP-dependent malate dehydrogenase in this process. Goldstein & Newsholme (1976) have presented evidence consistent with the generation of pyruvate for alanine formation from isoleucine in diaphragm muscle, and they favour the participation of phosphoenolpyruvate carboxykinase and pyruvate kinase. The branched-chain amino acids valine and isoleucine (but not leucine) may be particularly important, because oxidation of their carbon chains results in the formation of the tricarboxylic acid-cycle intermediate, succinyl-CoA (3-carboxypropionyl-CoA). Oxidation of these amino acids takes place largely in muscle in the body (Miller, 1961; Buse & Buse, 1967; Goldberg & Odessey, 1972). They are also important as sources of amino groups for alanine synthesis because, along with aspartate, they are the best substrates for transamination with 2-oxoglutarate in muscle (Krebs, 1975). Branched-chain amino acids increase the rate of release of alanine by various muscle preparations (Ruderman & Berger, 1974; Odessey *et al.*, 1974; Goldstein & Newsholme, 1976; Garber *et al.*, 1976*b*).

In the present work we have investigated the pathway by which branched-chain amino acids may furnish both the amino nitrogen and carbon for alanine formation in diaphragm muscle.

Materials and Methods

Chemicals

Enzymes and coenzymes were from Boehringer Corp. (London) Ltd., London W.5, U.K. L-Amino acids, D-glucose, dichloroacetic acid and inorganic chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K. L-Cycloserine was given by Dr. D. H. Williamson (Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford, U.K.) and 3-mercaptopicolinic acid by Dr. H. L. Saunders (SKF Laboratories, Philadelphia, PA, U.S.A.). Amino-oxyacetic acid was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Animals

Male rats of the Wistar albino strain were bred in and obtained from the Animal House of the University of Surrey (Director: Mr. P. Scobie-Trumper). Animals were starved for 40h unless otherwise indicated and were used at a body wt. of 80–100g.

Incubation procedure

Rats were killed by cervical dislocation, and hemidiaphragms free of ribs were prepared as described by Goldberg *et al.* (1975). Hemidiaphragms were preincubated in 2ml of Krebs–Henseleit (1932) saline for 30min at 37°C under O_2/CO_2 (19:1, v/v). The hemidiaphragms were then transferred into flasks containing fresh saline, with additions as indicated, at a final concentration of 50mg of tissue/ml of medium and incubated as above for 2h. At the end of the incubation period, hemidiaphragms were removed and ice-cold 20% (w/v) $HClO_4$ (0.1ml/ml of medium) was added to the medium. Neutralized extracts were used (within 3h) for the determination of alanine (Williamson, 1974), lactate (Hohorst, 1963) and pyruvate (Bucher *et al.*, 1963).

A paired experimental design was used in every set of experiments, e.g. one hemidiaphragm from each rat was incubated in the control medium, and its pair in the experimental medium.

Results and Discussion

Alanine release by hemidiaphragms from starved rats was stimulated by the addition of glutamate, leucine or valine or by glucose (Table 1). Similar observations were made with hemidiaphragms from fed rats (results not shown). The stimulation of alanine release by each of the amino acids was accompanied by a decrease in pyruvate release, although only for leucine was there equivalence in the metabolite changes (Table 1). For the other amino acids the decrease in pyruvate release was less than would be expected simply on the basis of increased alanine release, and this is consistent with the possibility that valine and glutamate may serve as carbon sources for pyruvate formation. The stimulation of alanine release by glucose indicates that pyruvate availability is a factor influencing alanine production, at least *in vitro*. This is further substantiated by observations made with dichloroacetate, which is known to stimulate pyruvate oxidation in rat muscle *in vitro* by activating pyruvate dehydrogenase (McAllister *et al.*, 1973; Whitehouse & Randle, 1973; Whitehouse *et al.*, 1974). Dichloroacetate (10mM), in incubations without exogenous substrate, decreased release of pyruvate and lactate by 86% and 65% respectively, compared with that found in the absence of dichloroacetate ($P<0.001$ in both cases) and alanine release was decreased by 45% ($P<0.01$). The increased release of pyruvate and lactate in the presence of 10mM-glucose was diminished by 99% and 73% respectively by dichloroacetate, and the extra alanine release was decreased by 85% ($P<0.001$ in all cases). Similar findings with dichloroacetate have been reported by Blackshear *et al.* (1975) in anaesthetized, functionally hepatectomized and nephrectomized starved rats *in vivo*, although in these experiments contributions to metabolite release by tissues other than muscle are also included. The stimulation of alanine release by valine, in the present experiments, was decreased by 94% by dichloroacetate ($P<0.001$).

Evidence that the alanine released is largely formed *de novo* by pyruvate transamination rather than by enhanced proteolytic release of alanine residues from muscle proteins was obtained by using aminotransferase inhibitors. The increased alanine release in the presence of valine, glutamate or glucose was inhibited by 60, 92 and 86% respectively by 10mM-L-cycloserine (results not shown), an inhibitor of cytoplasmic transamination and particularly of alanine aminotransferase (Wong *et al.*, 1973; Williamson *et al.*, 1974). Comparable inhibitions were observed with 1mM-amino-oxyacetate

Table 1. *Rates of metabolite release by hemidiaphragms from starved rats*

Hemidiaphragms prepared from rats starved for 40h were incubated as described in the Materials and Methods section. Values represent the means of measurements from different rats and are given ±S.E.M. The number of observations is given in parentheses. Statistical analysis (Student's *t* test) was carried out on paired observations, and differences with respect to incubations in the absence of any additions are shown by: *$P<0.05$, ***$P<0.001$.

Additions to incubation medium	Metabolite formation (μmol/2h per g of tissue)		
	Alanine	Pyruvate	Lactate
None	1.06±0.12 (12)	1.43±0.10 (12)	5.11±0.35 (12)
3mM-Glutamate	3.25±0.33 (4)***	0.79±0.11 (4)***	7.26±0.95 (4)
3mM-Leucine	2.07±0.11 (4)***	0.35±0.07 (4)***	4.49±0.41 (4)
3mM-Valine	1.93±0.15 (7)***	1.19±0.13 (7)*	4.64±0.42 (7)
10mM-Glucose	1.67±0.20 (8)***	4.97±0.34 (8)***	30.4±2.13 (8)***
10mM-Glucose+3mM-valine	3.21±0.29 (5)***	3.94±0.22 (5)***	22.4±1.91 (5)***

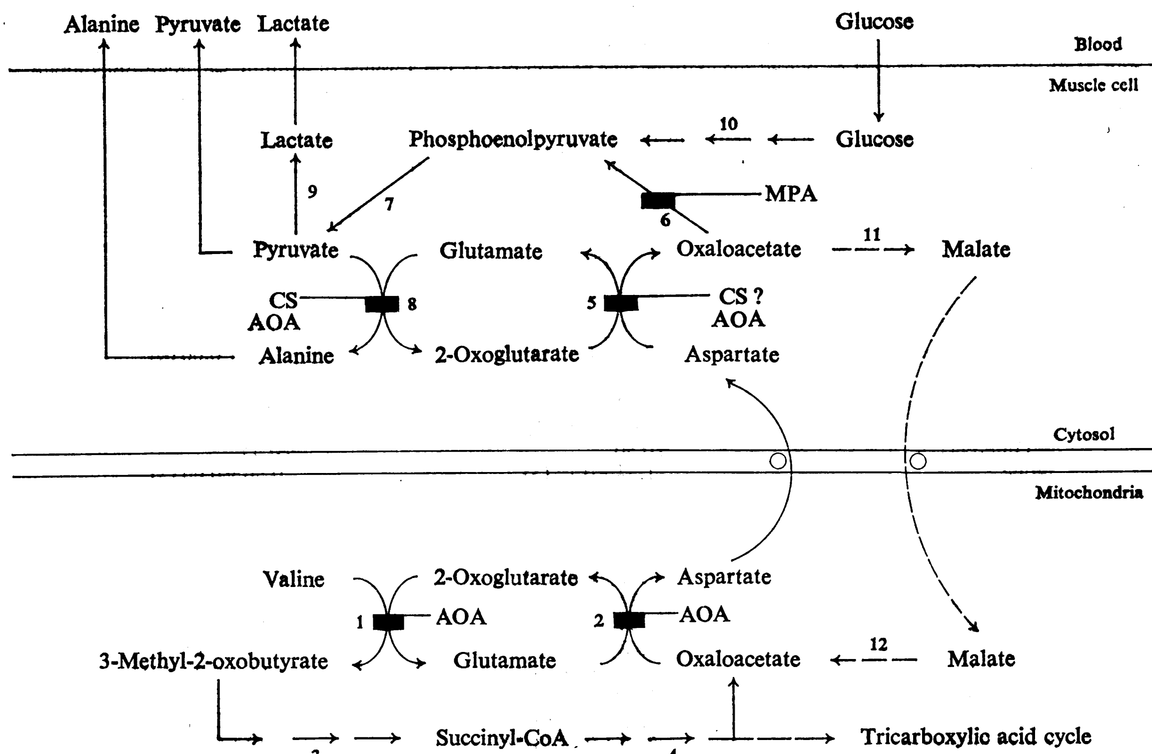
Table 2. *Alanine release by hemidiaphragms incubated with amino acids and 3-mercaptopicolinate*
Hemidiaphragms prepared from fed or 40h-starved rats (as indicated) were incubated as described in the Materials and Methods section. One set of paired incubations was carried out in the absence of sodium 3-mercaptopicolinate (control) and another set of paired incubations was made in the presence of 1mM-sodium mercaptopicolinate. Alanine release into the medium is expressed as μmol/2h per g of tissue. Values are means±S.E.M. for the number of observations in parentheses, and the difference in the means of paired observations given below. Statistical analysis (paired Student's *t* test) comparing incubations with and without added substrate is expressed by $^{**}P<0.01$, $^{***}P<0.001$.

Additions to incubation medium	Alanine formation (μmol/2h per g of tissue) Control	+3-Mercaptopicolinate
Starved		
None	1.48±0.02 (4)	0.99±0.14 (4)
3mM-Leucine	2.07±0.12 (4)***	1.55±0.06 (4)**
Difference	+0.59	+0.56
None	1.16±0.14 (6)	1.07±0.11 (6)
3mM-Valine	2.06±0.15 (6)***	1.42±0.09 (6)**
Difference	+0.90	+0.35
None	1.34±0.14 (3)	0.96±0.06 (3)
3mM-Glutamate	3.27±0.42 (3)***	1.88±0.09 (3)***
Difference	+1.93	+0.92
Fed		
None	1.37±0.05 (4)	0.87±0.03 (4)
3mM-Leucine	2.34±0.20 (4)***	1.86±0.03 (4)***
Difference	+0.97	+0.99
None	1.48±0.15 (5)	1.23±0.17 (5)
3mM-Valine	2.28±0.20 (5)**	1.96±0.24 (5)**
Difference	+0.80	+0.73
None	1.43±0.06 (3)	1.33±0.11 (3)
3mM-Glutamate	3.59±0.09 (3)***	3.50±0.25 (3)***
Difference	+2.16	+2.17

(results not shown), a less specific aminotransferase inhibitor that affects both mitochondrial and cytoplasmic transaminations (Longshaw *et al.*, 1972; Williamson *et al.*, 1974). Similar observations have been made with other muscle preparations (Ruderman & Berger, 1974; Garber *et al.*, 1976*b*) and with the hepatectomized rat (Blackshear *et al.*, 1975).

The dependence of alanine formation on pyruvate availability and the possibility (see above) that part of the pyruvate may be derived from the carbon skeletons of certain amino acids prompted experimentation with 3-mercaptopicolinate. 3-Mercaptopicolinate is a potent inhibitor of the cytosolic and mitochondrial forms of phosphoenolpyruvate carboxykinase (Robinson & Oei, 1975; Kostos *et al.*, 1975) and so would block the conversion of oxaloacetate (derived from the tricarboxylic acid cycle) into phosphoenolpyruvate and prevent pyruvate formation by this route. Alanine release stimulated by valine and glutamate was inhibited by 61 and 52% respectively by mercaptopicolinate (1mM) with diaphragms from starved rats (Table 2). The leucine-stimulated increase in alanine release was unaffected by the inhibitor. With diaphragms from fed rats, mercaptopicolinate had no effect on alanine release with any of the amino acid substrates (Table 2).

These results suggest that glutamate and valine can serve as a source of carbon for pyruvate synthesis (for alanine formation) by a metabolic route involving phosphoenolpyruvate carboxykinase in starved rats. This is shown for valine in Scheme 1, which takes into account the intracellular distribution of the enzymes involved. Thus alanine aminotransferase is located predominantly (>90%) in the cytosol fraction of diaphragm and other muscle tissues (K. Snell, unpublished work), whereas Ichihara *et al.* (1975) reported most of the branched-chain aminotransferase activity (72%) to be mitochondrial in muscle. This implies a transfer of branched-chain amino acid nitrogen from mitochondria to cytosol for alanine synthesis and, by invoking a role for aspartate in this transfer, the Scheme allows for a simultaneous transfer of mitochondrial oxaloacetate, from the valine carbon skeleton, to the cytosol for use in pyruvate synthesis. The presence of aspartate aminotransferase in both mitochondrial and cytosol fractions (46 and 54% of total aminotransferase activity respectively in diaphragm muscle; K. Snell, unpublished work) is, of course, necessary for this role of aspartate in the co-ordinated transfer of carbon and nitrogen derived from mitochondrial amino acid catabolism.



Scheme 1. *Proposed metabolic scheme for alanine synthesis from valine in muscle*

The enzymes catalysing the reactions are: 1, leucine (branched-chain) aminotransferase (EC 2.6.1.6); 2, mitochondrial aspartate aminotransferase (EC 2.6.1.1); 3, enzymes converting 3-methyl-2-oxobutyrate into succinyl-CoA; 4, enzymes of the tricarboxylic acid cycle converting succinyl-CoA into oxaloacetate; 5, cytosolic aspartate aminotransferase (EC 2.6.1.1); 6, phosphoenolpyruvate carboxykinase (EC 4.1.1.32); 7, pyruvate kinase (EC 2.7.1.40); 8, alanine aminotransferase (EC 2.6.1.2); 9, lactate dehydrogenase (EC 1.1.1.27); 10, enzymes of glycolysis converting glucose into phosphoenolpyruvate; 11, cytosolic malate dehydrogenase (EC 1.1.1.37); 12, mitochondrial malate dehydrogenase (EC 1.1.1.37). The broken lines indicate a possible alternative fate for oxaloacetate if it is not decarboxylated to form phosphoenolpyruvate. Reactions inhibited by 3-mercaptopicolinic acid (MPA), L-cycloserine (CS) or amino-oxyacetate (AOA) are indicated.

In conclusion, the results indicate that the diaphragm of the starved rat has the metabolic capability to utilize the carbon and nitrogen of valine and glutamate (and presumably other amino acids whose catabolism results in a net formation of tricarboxylic acid-cycle intermediates) for the formation of alanine. The metabolic route for directing amino acid-derived carbon towards pyruvate synthesis for alanine formation involves phosphoenolpyruvate carboxykinase. The extent to which this metabolic route operates during starvation *in vivo* is unknown, but it does provide an explanation for the net conversion of amino acids, arising from muscle proteolysis, into alanine, which after release into the circulation can be used by the liver for gluconeogenesis. Diaphragm muscle would not be expected to be a quantitatively important source of alanine in this situation, although a net loss of protein from diaphragm during starvation has been observed (K. Snell, unpublished work), but the biochemical pathways in this tissue may be representative of muscles in general.

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