Rapid Papers

The Formation of Alanine from Amino Acids in Diaphragm Muscle of the Rat

By LEON GOLDSTEIN* and ERIC A. NEWSHOLME

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K.

(Received 26 November 1975)

Alanine production and pyruvate content of the isolated rat hemidiaphragm are increased by isoleucine or glutamate. These results support the hypothesis that amino acids are converted into pyruvate before oxidation and that some pyruvate is transaminated to alanine, which is released from the muscle.

Miller (1961) observed that several amino acids were oxidized in the eviscerated rat, which suggested that oxidation occurred in muscle. Further work established that isolated diaphragm or muscles from the hindlimb of the rat oxidize valine, leucine, isoleucine, glutamate, aspartate and alanine (Manchester, 1965; Goldberg & Odessey, 1972; Beatty *et al.*, 1974). Recently it has been shown that oxidations of the branched-chain amino acids in the diaphragm (Odessey *et al.*, 1974) and branched-chain and other amino acids in the isolated perfused hindlimb of the rat (Ruderman & Berger, 1974) are accompanied by increases in the rates of alanine release from the muscle. Arteriovenous differences of alanine in human subjects have demonstrated that skeletal muscle releases large amounts of alanine, especially in the early stages of starvation (Felig & Wahren, 1974). Of the amino acids released by muscle, 30–40% can be accounted for as alanine, although the content of alanine in muscle protein is only 7–10%. The theory has been developed that, when amino acids are oxidized by muscle, pyruvate, which is produced from glucose via glycolysis, is transaminated into alanine. Since the alanine that is released by the muscle is transported to the liver to be reconverted into glucose by gluconeogenesis, the overall process has been termed 'the glucose-alanine cycle' (see Felig *et al.*, 1970; Felig & Wahren, 1974; Felig, 1975).

The oxidation of the amino acids by muscle is considered to be via transamination with α -oxoglutarate and ultimate oxidation of the keto acid via the citric acid cycle. A large proportion of these keto acids or their metabolic derivatives enter the cycle at the levels of α -oxoglutarate, succinate, fumarate or oxaloacetate (see, e.g., Lehninger, 1970). However, contrary to the tacit assumption in some textbooks, the carbon skeleton of these keto acids

cannot be oxidized directly by the citric acid cycle. The latter oxidizes only the carbon skeleton of acetate, via acetyl-CoA. Consequently, the keto acids derived from the following amino acids raise a problem of the pathway of oxidation: arginine, histidine, glutamate, glutamine and proline (which give rise to α -oxoglutarate), methionine, isoleucine and valine (which give rise to succinate), phenylalanine and tyrosine (which give rise to fumarate), and aspartate and asparagine (which give rise to oxaloacetate). Nonetheless there is evidence that many of these amino acids can be completely oxidized by muscle (see above). We suggest that this is achieved by conversion of the keto acids via oxaloacetate (or malate) into pyruvate and hence into acetyl-CoA (see Scheme 1). The formation of pyruvate may be catalysed by the enzymes phosphoenolpyruvate carboxylase or NADP-linked malate dehydrogenase, which are present in muscle (see Opie & Newsholme, 1967; Crabtree *et al.*, 1972).

This metabolic scheme not only explains how some amino acids can be oxidized in muscle (or other tissues), but it also provides a simple metabolic explanation for the formation and release of alanine by muscle. In addition, it suggests that the latter should occur in the absence of pyruvate formation from glucose. Thus the amino acid that is degraded by muscle will produce pyruvate, some of which may be oxidized, but, since the activity of glutamate-pyruvate transaminase is high in muscle, some pyruvate will be transaminated to produce alanine (see Scheme 1). Since the activity of pyruvate dehydrogenase is decreased (see Garland *et al.*, 1964) and amino acid catabolism is increased (see Cahill & Owen, 1968) in starvation, alanine formation by muscle should increase under these conditions. This is indeed the case (see Felig & Wahren, 1974).

This metabolic scheme predicts that, in the absence of glucose, certain amino acids should be converted into alanine in muscle, and that these amino acids should increase the intracellular concentration of

* Present address: Division of Biomedical Sciences, Brown University, Providence, RI, U.S.A.

Scheme 1. Reactions by which amino acids are converted into pyruvate and hence into alanine

The enzymes catalysing the reactions are as follows: 1, an amino acid transaminase; 2, enzymes converting the keto acid into a cycle intermediate and the latter into oxaloacetate; 3, phosphoenolpyruvate carboxylase; 4, pyruvate kinase; 5, glutamate-pyruvate transaminase; 6, pyruvate dehydrogenase.

pyruvate. These predictions have been tested with the isolated rat hemidiaphragm preparation, and the results are now reported.

Experimental

Animals

Male rats (Wistar strain) were purchased from Oxfordshire Laboratory Animal Colonies and kept in the Departmental animal house before use.

Materials

All chemicals and enzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., except for the following. L-Isoleucine and monosodium glutamate were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7HB, U.K. All inorganic chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.

Incubation procedure

Hemidiaphragms were prepared from fed rats weighing 80–100g and incubated as described by Randle & Smith (1958), except that the diaphragms were preincubated for 30min in 5.0ml of Krebs & Henseleit (1932) bicarbonate-buffered saline gassed with $O_2 + CO_2$ (95:5) for 30min to lower the concentrations of endogenous amino acids and glucose. After incubation for 1 or 2h in 5ml of bicarbonate-buffered saline at 37°C, the hemidiaphragms were removed from the flasks, blotted quickly on filter paper moistened with saline and freeze-clamped. The frozen diaphragms were extracted with $HClO_4$ after powdering of the frozen muscle at $-70^\circ C$ in a percussion mortar. After

centrifugation of the precipitated protein, the supernatant was neutralized with 5M- $KHCO_3$ -KCl. A 0.25ml volume of 60% (w/v) $HClO_4$ was added to the incubation medium, which was then centrifuged, and the supernatant was neutralized with $KHCO_3$ -KCl.

Determinations

Glucose and glycogen were measured as described by Ford & Candy (1972). Alanine was determined in both the medium and the tissue extracts by the method of Williamson (1974), and the contents of pyruvate and lactate in the tissue extracts were assayed by the methods of Czok & Lamprecht (1974) and Gutman & Wahlefeld (1974) respectively.

Results

Glutamate and isoleucine were chosen as substrates in these experiments since they produce degradation products that enter the cycle at different positions and isoleucine produces two degradation products. Nonetheless the results obtained with the two amino acids are almost identical. Thus alanine production *in vitro* is increased by the presence of isoleucine or glutamate (Table 1). The increased production is observed after both the 1h and 2h periods of incubation, and the alanine concentration is increased in both the tissue and the medium. These experiments were performed in the absence of glucose to prevent the formation of pyruvate (and hence alanine) from this source. The contents of glucose and glycogen in the diaphragm after 30min preincubation were 0.17 ± 0.034 (10) and 12.1 ± 1.1 (12) $\mu mol/g$ of fresh muscle respectively (mean values $\pm$ s.e.m.), and after 60min

Table 1. Rates of alanine formation and contents of pyruvate and lactate in rat hemidiaphragms incubated with isoleucine or glutamate

Diaphragms were incubated and alanine, pyruvate and lactate measured as described in the Experimental section. Rates and contents are presented as means (of at least ten separate experiments) together with s.e.m. values. The concentration of the amino acids used was 3 mM. Values that are statistically different (Student's *t* test) from incubations in the absence of added amino acids are shown by * ($P < 0.05$), † ($P < 0.01$) or ‡ ($P < 0.001$).

Amino acid added	Incubation time (min)	Alanine produced ($\mu\text{mol/g}$ of fresh muscle)			Contents of intermediates ($\mu\text{mol/g}$ of fresh muscle)	
		Tissue	Medium	Total	Pyruvate	Lactate
None	60	0.35 ± 0.04	0.74 ± 0.09	1.09 ± 0.11	0.078 ± 0.007	1.68 ± 0.26
None	120	0.42 ± 0.10	1.45 ± 0.09	1.87 ± 0.13	—	—
L-Isoleucine	60	$0.58 \pm 0.10^*$	$1.21 \pm 0.10^\dagger$	$1.79 \pm 0.11^\dagger$	$0.128 \pm 0.014^\dagger$	1.48 ± 0.19
L-Isoleucine	120	$0.84 \pm 0.14^*$	$1.89 \pm 0.17^*$	$2.73 \pm 0.21^\dagger$	—	—
L-Glutamate	60	$0.50 \pm 0.06^*$	$1.47 \pm 0.14^\dagger$	$1.97 \pm 0.16^\dagger$	$0.129 \pm 0.019^*$	1.97 ± 0.41
L-Glutamate	120	0.35 ± 0.04	$2.31 \pm 0.29^*$	$2.66 \pm 0.31^*$	—	—

incubation with glutamate they were 0.22 ± 0.04 (10) and 11.3 ± 1.3 (11) $\mu\text{mol/g}$ of fresh muscle respectively. Thus, although the individual variation is large in the case of glycogen, there is no statistically significant difference between glucose or glycogen concentrations before and after the incubation, so that it is unlikely that the rate of glycolysis from either glucose or glycogen could account for the rate of alanine formation. Further, starvation of the rats for 24 h before they were killed lowered the glycogen content by 30%, but the rates of alanine formation from glutamate and isoleucine were unchanged. In addition, 10 mM-NaF, which in other similar experiments decreased maximum glycolytic rate by 70%, did not decrease but increased alanine formation. The percentage increases in the rate of total alanine formation were 61, 24 and 24 for incubations in the absence of added amino acid, in the presence of isoleucine and in the presence of glutamate respectively (results not shown). The larger increase in alanine formation in the absence of added amino acids suggests that NaF increases proteolysis in this tissue. Nonetheless, alanine formation from the added amino acids is still considerably greater than that in the absence of added amino acids.

The contents of pyruvate in the diaphragms were increased by the presence of isoleucine or glutamate (by approx. 60%), but the contents of lactate were unchanged (Table 1).

Discussion

The amino acids isoleucine and glutamate markedly increase the rate of alanine formation by the diaphragm in the absence of glucose. In experiments reported by Odessey *et al.* (1974), quarter-diaphragms were incubated *in vitro*, with branched-chain amino acids and [^{14}C]glucose. The results indicated that approx. 60% of the carbon

in alanine was provided from glucose, with 40% obtained from other sources. The rate of alanine synthesis in the present work (in the absence of glucose) was approx. 47% of that reported by Odessey *et al.* (1974), in the presence of glucose. Thus in the latter experiments branched-chain amino acids may be responsible for the formation of about 40% of the carbon skeleton of alanine, so that, even in the presence of glucose, it is likely that significant quantities of alanine are produced from amino acids.

It is important to note that the release of alanine by muscle is a metabolic consequence of both the initial transamination reaction and the conversion of the keto acid into pyruvate via the normal oxidation pathway for the amino acids. The initial transamination should increase the concentration of glutamate, and the high activity of glutamate-pyruvate transaminase should ensure that a considerable proportion of the pyruvate is converted into alanine. The fact that alanine can be released by the muscle and removed by the bloodstream ensures that the overall reaction is maintained in the direction of alanine formation (see Scheme 1).

The glucose-alanine cycle was developed to explain the release of large quantities of alanine from the muscle and the conversion of this alanine into glucose in the liver. However, a large proportion of the glucose production in the liver under conditions of starvation is required for oxidation in the brain (see Krebs, 1964; Cahill & Owen, 1968). The glucose-alanine cycle would provide glucose for utilization and conversion into alanine only in the muscle, so that the role of alanine in this cycle is to transfer only amino N from the muscle to the liver. The present work demonstrates that a considerable proportion of the carbon skeleton of the alanine released by the muscle may be obtained not from glucose but from other amino acids. Consequently

this alanine transfers both the carbon and nitrogen of certain amino acids to the liver, so that the glucose synthesized from the alanine will be available for oxidation in the brain.

We thank Professor R. R. Porter, F.R.S., for his interest and encouragement and Mrs. Jill Lang for her expert technical assistance. This research was supported by U.S. Public Health Service Grant AM12443 and National Science Foundation Grant GB-30357X2.

References

- Beatty, C. H., Curtis, S., Young, M. K. & Boeak, R. M. (1974) *Am. J. Physiol.* **227**, 268–272
- Cahill, G. F. & Owen, O. E. (1968) in *Carbohydrate Metabolism and its Disorders* (Dickens, F., Randle, P. J. & Whelan, W. J., eds.), vol. 1, pp. 497–522, Academic Press, London and New York
- Crabtree, B., Higgins, S. J. & Newsholme, E. A. (1972) *Biochem. J.* **130**, 391–396
- Czok, R. & Lamprecht, W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 1446–1451, Verlag Chemie, Weinheim, and Academic Press, London
- Felig, P. (1975) *Annu. Rev. Biochem.* **44**, 933–955
- Felig, P. & Wahren, J. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 1092–1097
- Felig, P., Pozefsky, T., Marliss, E. & Cahill, G. F. (1970) *Science* **167**, 1003–1004
- Ford, W. C. L. & Candy, D. J. (1972) *Biochem. J.* **130**, 1101–1112
- Garland, P. B., Newsholme, E. A. & Randle, P. J. (1964) *Biochem. J.* **93**, 665–678
- Goldberg, A. L. & Odessey, R. (1972) *Am. J. Physiol.* **223**, 1384–1391
- Gutman, I. & Wahlefeld, A. W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 1464–1468, Verlag Chemie, Weinheim, and Academic Press, London
- Krebs, H. A. (1964) in *Mammalian Protein Metabolism* (Munro, H. N. & Allison, J. B., eds.), vol. 1, pp. 125–177, Academic Press, New York and London
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Lehninger, A. L. (1970) *Biochemistry*, pp. 440–449, Worth Publishers, New York
- Manchester, K. L. (1965) *Biochim. Biophys. Acta* **100**, 295–298
- Miller, L. L. (1961) *Amino Acid Pools*, pp. 708–721, Elsevier, Amsterdam
- Odessey, R., Khairallah, E. A. & Goldberg, A. L. (1974) *J. Biol. Chem.* **249**, 7623–7629
- Opie, L. H. & Newsholme, E. A. (1967) *Biochem. J.* **103**, 391–399
- Randle, P. J. & Smith, G. H. (1958) *Biochem. J.* **70**, 490–500
- Ruderman, N. B. & Berger, M. (1974) *J. Biol. Chem.* **249**, 5500–5506
- Williamson, D. H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 1679–1682, Verlag Chemie, Weinheim, and Academic Press, London