ALANINE FORMATION BY RAT MUSCLE HOMOGENATE

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SUMMARY: Rat hind leg muscle homogenates synthesized alanine at a rate of 1.06 μmoles/hr/gm for as long as 4 hours which is comparable to rates reported for *in vivo* perfusion experiments. Alanine synthesis by diaphragm and heart muscle was consistently less than 20% that of hind limb. Alanine formation was not enhanced by the addition of glucose, pyruvate or β-hydroxybutyrate nor was it decreased by proteolytic enzyme inhibitors. Homogenates were analyzed for concentrations of free amino acids and related intermediates (glutamate, α-ketoglutarate, lactate and pyruvate) with and without added NADH and lactic dehydrogenase. The results of these experiments suggest that the *de novo* synthesis of alanine in hind limb muscle may be derived from sources other than pyruvate or proteolysis.

INTRODUCTION: Several reports of perfusion studies in man (1,2,3) and in rats (4) have shown that alanine and glutamine are released by skeletal muscles in quantities far in excess of the amount available based on muscle protein content. Felig and co-workers also demonstrated the continual production of alanine after 4-6 weeks of starvation in man and have postulated that an alanine cycle is the key mechanism in gluconeogenesis (5). The pattern of amino acid release and the continual uptake of these amino acids by the liver, suggest a substantial *de novo* synthesis of alanine by muscle. Several possibilities have been offered to explain the unique synthesis of these amino acids in skeletal muscle including a labile protein rich in alanine and glutamine content, the selective uptake of certain amino acids by muscle in preference to alanine and glutamine and the production of alanine from pyruvate (4).

Although the perfusion techniques have yielded important information, a critical analysis of the mechanism of alanine production requires a more defined system. It is the purpose of this report to describe an *in vitro*

model system which is designed for the direct measurement of alanine production by skeletal muscle, and to describe experiments using this system, which suggest that the de novo synthesis of alanine might be unrelated to glycolysis and ketone body oxidation.

MATERIALS AND METHODS: Male adult Wistar rats maintained on Purina Lab Chow (Ralston Purina Co., St. Louis, Mo.) were sacrificied by decapitation. Hind leg muscles were rapidly excised and homogenized in 9 volumes of Krebs-Henseleit bicarbonate buffer, pH 7.4 (KHBB) in a Sorvall Omni-Mixer at 13,000 rpm for 2 minutes in an ice-water bath. The homogenate was filtered through gauze and kept in ice until utilized within the next hour. In some experiments, small pieces of longitudinally cut hind leg muscles, heart, or cut diaphragm were incubated in the KHBB. Homogenates of these tissues were prepared as described above. Incubations were carried out at 37°C in open vessels, in a Dubnoff shaking water bath with a final concentration of 50 mg fresh muscle per ml of KHBB with or without the addition of NADH and crystalline lactic dehydrogenase (rabbit muscle). Aliquots were removed at 0, 0.5, 1,2, and 4 hours and deproteinized with an equal volume of 2M $HClO_4$. The acid supernatant was neutralized with the same volume of a mixture of 2M $KHCO_3$:2M KCl (2:1). Neutralized protein-free samples were assayed for alanine and pyruvate by a modification of the method of Karl et al (6) and for glutamate, α-ketoglutarate and lactate by enzymatic, spectrophotofluorometric procedures (7). Amino nitrogen was determined with the ninhydrine reaction in an automated system (8) using alanine as a standard.

RESULTS AND DISCUSSION: In the absence of added substrate, pieces or homogenates of rat hind leg muscle in KHBB were found to synthesize alanine at an average rate of 1.06 μmoles/g muscle/hr for as long as 4 hours (Table 1). This is in good agreement with the value of 0.90 μmoles/g muscle/hr reported for perfused rat hind quarter by Ruderman and Lund (4). The

Table 1

RATE OF ALANINE FORMATION

μMoles/g muscle wet weight/hr at 37°; Mean ± S.E. and (n) number of experiments

CONDITION	NO ADDITION	GLUCOSE $2X10^{-2}M$	β-HYDROXYBUTYRATE $10^{-2}M$	PYRUVATE $10^{-3}M$
Homogenate, ad lib fed rats	1.06 ± 0.09 (13)	1.10 ± 0.21 (4)	1.0 ± 0.12 (6)	0.77 ± 0.25 (4)
Homogenate, 48-72 hrs. starved rats	1.42 ± 0.34 (4)	1.48 ± 0.40 (4)	1.45 ± 0.45 (4)	0.85 ± 0.35 (3)
Homogenate, 5 day starved rats	0.66 (2)	1.01 (2)	0.66 (2)	----
Muscle slices, ad lib fed rats	0.70 ± 0.05 (3)	0.80 ± 0.15 (3)	0.78 ± 0.20 (3)	----
Muscle pieces, 48-72 hrs. starved rats	0.25 ± 0.05 (3)	0.02 ± 0.04 (3)	0.22 ± 0.02 (3)	----
Muscle pieces, 5 day starved rats	0.15 (2)	0.22 (2)	0.17 (2)	----

Alanine formation by fed or starved rat's muscle. Homogenate refers to the incubation of a 50 mg/ml rat hind leg muscle homogenate in open vessels at 37°, in KHBB with or without additions as indicated. Muscle pieces refer to the incubation of longitudinally cut small hind leg muscle pieces in KHBB in open vessels at 37°.

addition of β-hydroxybutyrate or pyruvate did not enhance alanine formation. However, when pyruvate and glutamate were added together, a rapid equilibrium was established within the first 10 minutes of incubation, leading to a higher initial level of alanine but without further increase in the rate of alanine production. Alanine production by the homogenate was not due to bacterial contamination, since ampicillin (0.4 gm/ml) did not alter this rate.

Extended periods of starvation (120 hours) caused a significant reduction in the rate of alanine formation by both homogenates and muscle pieces (Table 1). However, after short periods of starvation (48-72 hours) the results were variable. There was a significant reduction in the rate using muscle slices, but little or no change using muscle homogenates. A reduction in final alanine content was also observed in muscle obtained from animals starved for 120 hours as compared with fed animals (0.2 versus 1.0 μmoles/g muscle). Significant rates of alanine production appear to be limited to only leg skeletal muscle since heart muscle and cut diaphragm preparations from the same animals under identical conditions were much less active (0.08 μmoles/g/hr and 0.15 ± 0.02 μmoles/g/hr respectively (n=3). No changes in these values were observed with the addition of either glucose or β-hydroxybutyrate.

These results establish the use of muscle homogenates as a model for the investigation of the mechanism of alanine production and they also support the findings of Felig et al (1) who observed a decrease in the alanine released from fasting adult forearm.

During a 4-hour incubation period of muscle homogenate, there was a steady increase in alanine production, while lactate increased only during the first hour and then fell progressively for the next 3 hours (Fig. 1). Although no free pyruvate could be measured, there was a steady accumulation of glucose-6-phosphate (0.4 μmoles/g/4 hrs) and phosphoenol pyruvate (3.0 μmoles/g/4 hrs). In a similar experiment the addition of excess NADH and lactic dehydrogenase resulted in a striking decrease in lactate production

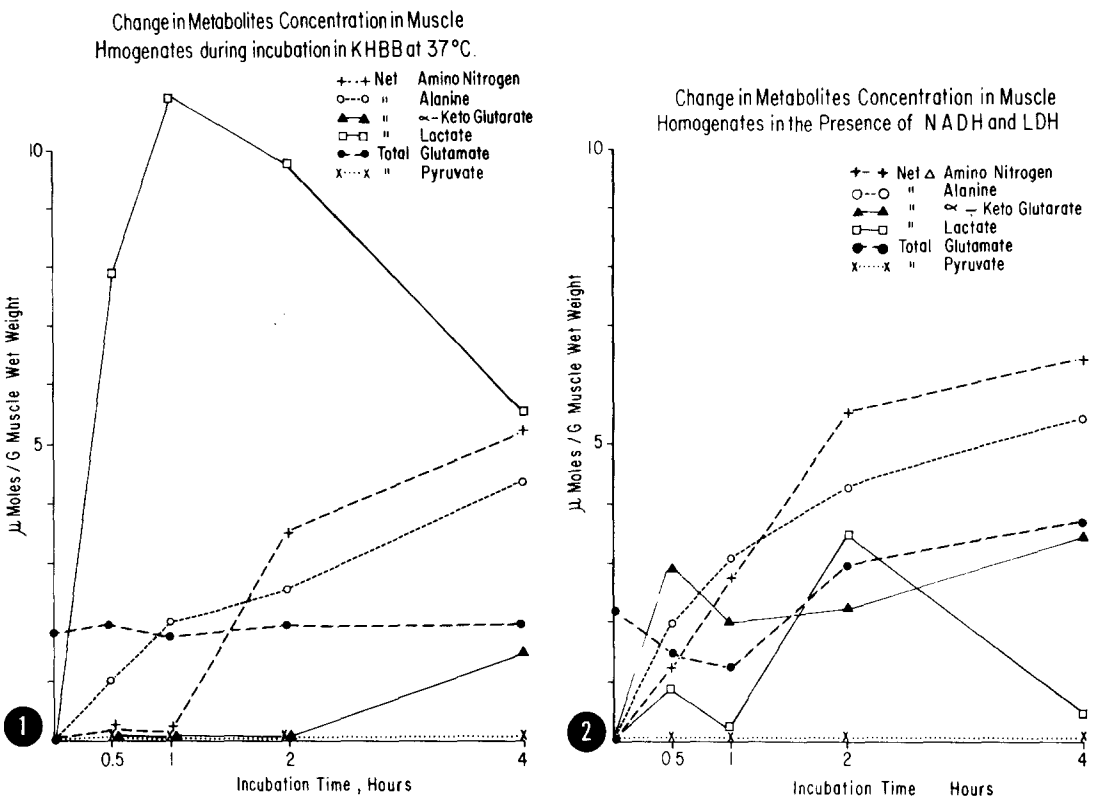

Figure 1. Alanine production by muscle homogenate. 50 mg muscle/ml in KHBB, incubated at 37°C, (▲—▲) total α-ketoglutarate (x····x) total pyruvate, (o---o) net change in alanine over the initial concentration of 0.41 μmoles/g, (□—□) net change in lactate over the initial concentration of 25.7 μmoles/g and (+- - -+) net change in amino nitrogen over the initial concentration of 44.7 μmoles/g, (●—●) total glutamate.

Figure 2. Alanine production by a muscle homogenate (50 mg wet weight/ml KHBB) at 37°C in the presence of 2 x 10^{-3} M NADH and 0.5 units/ml crystalline rabbit muscle lactic dehydrogenase. The loss of NADH during the 4 hr incubation period was 30%. The symbols are the same as in Figure 1, but the initial concentration for alanine, lactate and amino nitrogen was 0.21, 32.6 and 50.2 μmoles/g wet weight muscle respectively.

(Fig. 2) and increased accumulation of glucose-6-phosphate (2.6 μmoles/g/4 hrs). Under these conditions pyruvate production would be expected to be blocked since NADH is known to inhibit pyruvate kinase (9) and the excess NADH and LDH would be expected to convert alanine into lactate stoichiometrically with a concomitant reduction in alanine production. Instead, however, alanine formation followed the same pattern (Fig. 2).

Glutamate concentration did not change significantly during these experiments, although a gradual increase in glutamine content accompanied alanine formation in the presence of excess NADH. However, in both experiments α-ketoglutarate content was consistently low (Figs. 1 and 2), but an accumulation of this metabolite was somewhat concomitant with the increasing alanine formation.

Since muscle pyruvate kinase has a high V_{max} (3.3 mMoles/g/hr at 37°), the possibility of its being rate limiting seems remote. Furthermore, the addition of excessive crystalline rabbit muscle pyruvic kinase or the addition of a pyruvic kinase inhibitor, 10^{-3} M acetyl CoA (9) did not change the rate of alanine formation. Similarly, addition of excess crystalline pig heart transaminase to muscle homogenate did not alter the rate of alanine formation. These results open serious questions to the hypothesis that pyruvate is the major precursor for de novo muscle alanine, and they tend to substantiate the conclusion by Pozefsky and Tancredi (2) who showed that only 7% of the infused pyruvate could account for the alanine formed by perfused human forearm.

The identity of the amino nitrogen donor remains to be established. However, the accumulation of α-ketoglutarate in many experiments paralleling alanine formation suggests a role for glutamate in this process. The α-amino nitrogen content of muscle homogenate, i.e. $HClO_4$ non-precipitable material, was 100 times higher than the alanine concentration. Muscle protein is reported to contain 5 to 7% alanine (4). Therefore, if the alanine production was the result of proteolysis, at least 10 to 20 μmoles of α-amino nitrogen should have been observed per μmole of alanine formed, whereas the actual ratio was close to 1:1.

The contribution by proteolysis also seems partially ruled out by the fact that neither pepstatin 10^{-6} M nor the competitive tryptic activity inhibitor benzoyl arginine ethyl ester (5×10^{-3} M) had any effect on alanine production. Bovine serum albumin also failed to increase alanine formation.

These findings suggest that if the de novo synthesis of alanine does involve proteolysis, it is catalyzed by a relatively specific enzyme.

The expanded utilization of the in vitro system described above provides a useful tool for the elucidation of the mechanism of alanine synthesis by muscle and should permit the rigorous definition of the precursor for the synthesis of the gluconeogenic amino acids.

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