BBA 45 696

ON THE NATURE OF MALONATE-INSENSITIVE OXIDATION OF PYRUVATE AND GLUTAMATE BY HEART SARCOSOMES

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(Received March 8th, 1968)

SUMMARY

1. A quantitative evaluation of respiration of isolated heart sarcosomes due to pyruvate and glutamate has been made when the citric acid cycle is blocked.

2. Malonate inhibits respiration due to added pyruvate 95–97 % when phosphate and ADP are present. Pyruvate is converted quantitatively to acetoacetate and acetate under these conditions.

3. Malonate-insensitive removal of pyruvate and the appearance of acetoacetate and acetate depend on the presence of phosphate and ADP. This requirement is not prevented by 2,4-dinitrophenol, but is circumvented by arsenate.

4. When sarcosomes are incubated in the presence of 2,4-dinitrophenol and arsenite, added glutamate is slowly converted to α-oxoglutarate. Further addition of pyruvate does not stimulate the rate of α-oxoglutarate accumulation. These results are interpreted as showing the presence of glutamate dehydrogenase (EC 1.4.1.3) whereas alanine aminotransferase activity was not detectable.

INTRODUCTION

The key enzymes required for the anaplerotic maintenance of the level of oxaloacetate from pyruvate (*i.e.* CO_2 fixation) are generally believed not to be present in muscle[1,2], whereas in liver and kidney these pathways are very active[3–6]. KREBS AND EGGLESTON[7] showed in 1940 that malonate inhibited the aerobic removal of pyruvate by minces of pigeon breast muscle more than 90 %, and that most of the disappearance of pyruvate which occurred in the presence of high concentrations of malonate was accounted for by anaerobic dismutations of pyruvate with endogenous substrates[8]. FULD AND PAUL[9] reported several years later, however, that malonate inhibited respiration by washed "cyclophorase" preparations from rabbit heart muscle due to added pyruvate by only about 80 %, and that under these conditions there was a stoichiometric conversion of pyruvate to acetate and CO_2. During the course of studies by the present author of pyruvate oxidation by intact phosphorylating guinea-pig and rat heart sarcosomes, it has also been noted that malonate fails to inhibit respiration completely.

The reaction catalysed by glutamate dehydrogenase (EC 1.4.1.3) is generally believed to be obligatory for the disposal of nitrogen from several glucogenic amino acids, and hence in the regulation of the free amino acid pool. Apparently there could

be no net synthesis of oxaloacetate from such amino acids as glutamate, glutamine and proline by tissues which contain no glutamate dehydrogenase activity. BORST[10] reported that respiration of rat heart sarcosomes, when incubated in the presence of glutamate, is inhibited 97 % by malonate, and about 95 % by arsenite, and concluded from these studies that glutamate dehydrogenase activity is very low in this tissue. Recently, the present author reported[11] that guinea-pig heart sarcosomes oxidise added pyruvate linearly, but not optimally, and that glutamate stimulates pyruvate oxidation. It was suggested that this stimulation resulted from an increased availability of oxaloacetate due to glutamate dehydrogenase.

In view of the fact that muscle has little or no capacity for gluconeogenesis[2], and that glutamate dehydrogenase activity is also very low or absent in these tissues, the problem arose as to how the level of citric acid cycle intermediates is regulated in organs which appear to be devoid of anaplerotic reactions known to be of major significance in other tissues. BOWMAN[12] and KRAUPP *et al.*[13] have shown that the levels of citric acid cycle intermediates are markedly elevated in hearts from starved or alloxan-diabetic rats, or in hearts perfused with fatty acids. The manner in which these changes are brought about is still not clear. The drain on the supply of oxaloacetate is probably proportional to the ability of an organ to carry out gluconeogenesis, since this process involves a stoichiometric conversion of oxaloacetate to carbohydrate.

The present report is concerned with a quantitative evaluation of the pathways of oxidation of pyruvate and glutamate by guinea-pig heart sarcosomes when the citric acid cycle is blocked. Under the conditions of these experiments *in vitro*, pyruvate is oxidised quantitatively to acetate and acetoacetate. Both respiration and formation of these products are largely dependent on the presence of ADP and phosphate, even in the presence of uncouplers of oxidative phosphorylation. Arsenate circumvents this requirement. In agreement with reports of GAUTHERON *et al.*[14], who have evaluated the activity of glutamate dehydrogenase in procine heart, glutamate is slowly converted to α-oxoglutarate when arsenite is present.

MATERIALS AND METHODS

Guinea-pig heart sarcosomes were prepared as described previously, omitting the proteinase treatment[15]. When oxygen uptake was measured, reactions were carried out using a Gilson Medical Electronics oxygraph; otherwise incubations were carried out in 25-ml erlenmeyer flasks in a shaking water bath. Incubation media contained 225 mM sucrose, 10 mM KCl, 10 mM Tris-HCl buffer (pH 7.4), 1–3 mM EDTA (derived from the mitochondrial suspension), and other additions as indicated in the legends to the tables. Reaction volume was 2 ml and the incubation temperature 30°. Reactions were terminated by addition of 0.6 mmole of $HClO_4$. After removal of protein by centrifugation, the solutions were neutralised with KOH, and the $KClO_4$ removed in the cold. All determinations of substrates and intermediates were carried out spectrophotometrically: pyruvate was measured with lactate dehydrogenase (EC 1.1.1.27) and NADH; α-oxoglutarate, with glutamate dehydrogenase in the presence of excess NH_4Cl and NADH; and acetoacetate, with β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and NADH. β-Hydroxybutyrate was estimated as described by WILLIAMSON, MELLANBY AND KREBS[16], and acetate by measuring the

appearance of NADH in a system coupled to the formation of citrate, exactly as described by BEINERT *et al.*[17], except that the pH was 8.0. In the assay for acetate, pyruvate was removed by adding lactate dehydrogenase and a slight excess of NADH, since very low concentrations (< 0.1 mM) of pyruvate completely blocked NADH production in this coupled system. β-Hydroxybutyrate dehydrogenase was prepared from *P. lemoignei* as described by DELAFIELD, COOKSEY AND DOUDOROFF[18], and acetyl-CoA synthetase (EC 6.2.1.1) from bovine heart mitochondria following HELE[19]. All other enzymes and coenzymes of the highest purity obtainable were purchased from Sigma Chemical Co., St. Louis, Mo. Pyruvate was purified by distillation[11].

RESULTS

Malonate-insensitive oxidation of added pyruvate

The various possible pathways which we have considered, to explain respiration in the presence of pyruvate *plus* malonate are outlined in Eqns. 1–15. If pyruvate carboxylase (EC 6.4.1.1) were present, as is the case in liver and kidney mitochondria, Reactions 1–5 would be expected to take place due to oxidations known to occur in heart sarcosomes, and the ratio $-\Delta O/-\Delta$pyruvate would be 1.5.

$$\text{Pyruvate} + CO_2 \rightarrow \text{oxaloacetate} \quad (1)$$

$$\text{Pyruvate} + \tfrac{1}{2}\,O_2 + \text{CoASH} \rightarrow \text{acetyl-CoA} + CO_2 + H_2O \quad (2)$$

$$\text{Acetyl-CoA} + \text{oxaloacetate} \rightarrow \text{citrate} + \text{CoASH} \quad (3)$$

$$\text{Citrate} + \tfrac{1}{2}\,O_2 \longrightarrow \alpha\text{-oxoglutarate} + CO_2 + H_2O \quad (4)$$

$$\alpha\text{-Oxoglutarate} + \tfrac{1}{2}\,O_2 \rightarrow \text{succinate} + CO_2 + H_2O \quad (5)$$

Sum:

$$2\ \text{Pyruvate} + {}^{3}/_{2}\,O_2 + CO_2 \rightarrow \text{succinate} + 3CO_2 + 3H_2O \quad (6$$

If Reaction 1 were taking place, albeit very slowly, it would provide a mechanism for a net change in the level of circulating citric acid cycle intermediates.

Other possibilities to explain pyruvate oxidation when the cycle is blocked are outlined in equations which follow.

$$\text{Pyruvate} + \text{CoASH} + \tfrac{1}{2}\,O_2 \rightarrow \text{acetyl-CoA} + CO_2 + H_2O \quad (2)$$

$$\text{Acetyl-CoA} + H_2O \xrightarrow{\text{deacylase}} \text{acetate} + \text{CoASH} \quad (7)$$

Sum:

$$\text{Pyruvate} + \tfrac{1}{2}\,O_2 \rightarrow \text{acetate} + CO_2 \quad (8)$$

As seen in the sum Reaction 8, if Reaction 7 were largely operative, the ratio $-\Delta O/-\Delta$ pyruvate would be 1.0, and there would be a stoichiometric conversion of pyruvate to acetate. An enzyme which catalyses Reaction 7 has been isolated from a particulate fraction from porcine heart[20]. A third reaction (Reaction 10) coupled to pyruvate dehydrogenase and acetoacetyl-CoA thiolase (EC 2.3.1.9) (Reactions 2 and 9) would also provide for a one-step oxidation of pyruvate, with a stoichiometric production of acetoacetate.

Enzyme preparations from extracts of ox or chicken liver have been shown to catalyse Reaction 10, whereas extracts from muscle or kidney had little or no deacylase activity unless succinate was also added[21].

$$2\ \text{Pyruvate} + 2\ \text{CoASH} + O_2 \rightarrow 2\ \text{acetyl-CoA} + 2\ CO_2 + 2\ H_2O \quad (2)$$

$$2\ \text{Acetyl-CoA} \leftrightarrows \text{acetoacetyl-CoA} + \text{CoASH} \quad (9)$$

$$\text{Acetoacetyl-CoA} + H_2O \xrightarrow{\text{deacylase}} \text{acetoacetate} + \text{CoASH} \quad (10)$$

Sum:

$$2\ \text{Pyruvate} + O_2 \rightarrow \text{acetoacetate} + 2\ CO_2 + H_2O \quad (11)$$

In our earlier experiments, the requirements for respiration were studied, and the ratio of oxygen uptake to pyruvate utilisation was determined. In 55 separate incubations using an oxygen polarograph under various conditions, the mean ratio $-\Delta O/-\Delta$pyruvate was 1.06 (range 0.94–1.22). This result seemed to establish the main pathway of malonate-insensitive oxidation as being related to Reactions 8 and/or 11; *i.e.* resulting from acetyl-CoA or acetoacetyl-CoA deacylases.

Table I summarises the results of some additional experiments in which the requirements for respiration were studied, along with those for acetoacetate production. As seen in Table I, acetoacetate production accounted for a large portion of respiration and pyruvate removal (49–77 %), and all three parameters measured were largely dependent on the presence of ADP and inorganic phosphate, even in the presence of 2,4-dinitrophenol. $MgCl_2$ partially reversed the requirement for ADP and phosphate, presumably due to coupling of the Mg^{2+}-stimulated ATPase with nucleoside diphosphate kinase (EC 2.7.4.6). This is effectively a partial uncoupling of substrate-linked phosphorylation of GTP. It was clear from these data that in short-term incubations, malonate-insensitive oxidation of pyruvate by intact sarcosomes is largely accounted for by production of acetoacetate, and that these parameters have a parallel requirement for ADP and phosphate, even in the presence of an uncoupler. However, since acetoacetate production did not completely account for pyruvate

TABLE I

STOICHIOMETRY OF PYRUVATE OXIDATION IN THE PRESENCE OF MALONATE

Reactions were carried out at 30° in a Gilson Medical Electronics Oxygraph. Incubation mixtures contained the basic medium, 20 μmoles of potassium malonate and the following additions where indicated in a final volume of 1.75 ml: potassium phosphate buffer (pH 7.4), 20 μmoles; ADP, 5 μmoles; 2,4-dinitrophenol, 0.1 μmole; $MgCl_2$, 10 μmoles. 0.25 ml sarcosomal suspension (6.1 and 7.9 mg protein in Expts. 1 and 2, respectively) was added. After endogenous respiration had ceased, 0.94 μmole pyruvate was added. Respiration was allowed to proceed for 12.5 min (Expt. 1) or 7.0 min (Expt. 2). Reactions were terminated with 0.6 mmole of $HClO_4$, deproteinised, neutralised with KOH and assayed for pyruvate and acetoacetate as described in METHODS.

Other additions	$-\Delta O$ (*μatoms*)	$-\Delta$*Pyruvate* (*μmoles*)	$+\Delta$*Acetoacetate* (*μmoles*)
Expt. 1			
Phosphate, ADP	0.70	0.62	0.24
Phosphate, ADP, dinitrophenol	0.66	0.62	0.21
Dinitrophenol	0.20	0.15	0.05
Dinitrophenol, $MgCl_2$	0.38	0.31	0.10
Expt. 2			
Phosphate, ADP	0.80	0.65	0.17
Phosphate, ADP, dinitrophenol	0.81	0.65	0.16
Phosphate, ADP, dinitrophenol, $MgCl_2$	0.87	0.69	0.14
Dinitrophenol	0.27	0.17	0.06
Dinitrophenol, $MgCl_2$	0.41	0.29	0.09

removal, additional incubations were carried out in which acetate production was also measured. Tables II and III show the results of two experiments in which both acetate and acetoacetate production were estimated. These two experiments are exactly comparable, except that the incubation time was different. As seen in Table II, in

TABLE II

STOICHIOMETRY OF AEROBIC REMOVAL OF PYRUVATE AND PRODUCTION OF ACETOACETATE AND ACETATE IN PRESENCE OF MALONATE, AND THE DEPENDENCE ON ADP AND INORGANIC PHOSPHATE

Incubations contained the basic medium, 4.5 mg sarcosomal protein, 20 μmoles potassium malonate and other additions as indicated in the table in a final volume of 2.0 ml. After a preincubation period of 6 min, 0.98 μmole of sodium pyruvate was added and allowed to incubate aerobically for an additional 8 min. Reactions were terminated by addition of 0.6 mmole of $HClO_4$. Additions were as follows: 2,4-dinitrophenol, 0.1 μmole; ADP, 5 μmoles; potassium phosphate (pH 7.4), 20 μmoles; potassium arsenate, 30 μmoles; $MgCl_2$, 10 μmoles. The amounts of endogenous acetate and acetoacetate were subtracted from the total to obtain the values listed. Endogenous acetate and acetoacetate at zero time was 0.14 and 0.04 μmole, respectively.

Other additions	*Products formed (μmoles)*		*−Δ Pyruvate (μmoles)*	*Pyruvate accounted for as acetate plus acetoacetate*
	Acetoacetate	*Acetate*		
Dinitrophenol	0.09	0.05	0.28	0.23
ADP, phosphate	0.20	0.16	0.64	0.56
Dinitrophenol, ADP, phosphate	0.19	0.23	0.67	0.61
Dinitrophenol, $MgCl_2$	0.15	0.11	0.59	0.41
Arsenate	0.18	0.53	0.79	0.88

TABLE III

THE EFFECTS OF UNCOUPLERS, ADP AND PHOSPHATE ON THE AEROBIC REMOVAL OF PYRUVATE IN THE PRESENCE OF MALONATE

Reactions were carried out in 25-ml erlenmeyer flasks on a shaking water bath at 30°. Incubation mixtures contained the basic medium, 20 μmoles of potassium malonate, 5.6 mg mitochondrial protein and the following additions where indicated in a final volume of 2.0 ml: 2,4-dinitrophenol, 0.1 μmole; $MgCl_2$, 10 μmoles; potassium phosphate (pH 7.4), 10 μmoles; ADP, 10 μmoles, and arsenate, 20 μmoles. After a preincubation period of 5 min to remove endogenous substrates, 4.96 μmoles of pyruvate were added to each vessel and allowed to react 45 min. Reactions were terminated with $HClO_4$.

Additions	*μmoles*			*Pyruvate accounted for*
	+Δ Acetate	*+Δ Acetoacetate*	*−Δ Pyruvate*	
None	0.38	0.08	0.63	0.54
None	0.43	0.06	0.50	0.55
Dinitrophenol	0.45	0.08	0.62	0.61
Dinitrophenol	0.42	0.11	0.64	0.64
Dinitrophenol, $MgCl_2$	0.85	0.21	1.16	1.27
Dinitrophenol, phosphate	0.69	0.18	1.05	1.05
Dinitrophenol, $MgCl_2$, phosphate	0.97	0.37	1.56	1.71
Dinitrophenol, ADP	0.48	0.18	0.94	0.84
Dinitrophenol, ADP, phosphate	0.65	0.23	1.20	1.11
Dinitrophenol, ADP, $MgCl_2$	0.77	0.25	1.25	1.27
Dinitrophenol, ADP, $MgCl_2$, phosphate	0.89	0.39	1.80	1.67
Arsenate	0.86	0.30	1.30	1.46
Arsenate	0.86	0.31	1.30	1.45

which the incubation time with pyruvate was 8 min, acetoacetate accounts for more than half of pyruvate removal, whereas in the experiment summarised in Table III (incubation time 45 min), the relative contribution by acetate to the over-all disappearance of pyruvate was increased, while the relative contribution by acetoacetate was decreased (in Table III, acetoacetate formation accounted for 24–47 % of pyruvate removal). Arsenate circumvented the requirement for ADP and phosphate, increasing the disappearance of pyruvate and formation of products measured in a parallel manner. In every case, production of acetate *plus* acetoacetate accounted for approximately all of the pyruvate which was consumed. No measurable D(—)-β-hydroxybutyrate was found. The inability to show net production of D(—)-β-hydroxybutyrate can be explained by two considerations: (1) added DL-β-hydroxybutyrate is very poorly oxidised by guinea-pig heart sarcosomes when incubated in the basic medium described in this communication in the presence of 50 μM 2,4-dinitrophenol (< 2 natoms of oxygen were consumed due to β-hydroxybutyrate per min per mg of a protein at 30°), demonstrating that β-hydroxybutyrate dehydrogenase activity is very low in these preparations; and (2) in the presence of uncouplers, mitochondrial NAD is mainly oxidised. The ratio acetoacetate/β-hydroxybutyrate is very high under these conditions[22].

The formation of acetoacetate from pyruvate, the requirement for ADP and phosphate in the presence of 2,4-dinitrophenol, and the lack of this requirement when arsenate is present, are most simply explained by reactions already known to occur in heart sarcosomes (Reactions 2, 9, 12–14). The sum Reaction 15 is essentially an acetoacetyl-CoA deacylase, which required ADP, phosphate and catalytic amounts of succinate and GDP, essentially the acetoacetate activating and cleavage system first described by GREEN *et al.*[23].

$$2\ \text{Pyruvate} + 2\ \text{CoASH} + O_2 \rightarrow 2\ \text{acetyl-CoA} + 2\ CO_2 + 2\ H_2O \qquad (2)$$

$$2\ \text{Acetyl-CoA} \rightleftarrows \text{acetoacetyl-CoA} + \text{CoASH} \qquad (9)$$

$$\text{Acetoacetyl-CoA} + \text{succinate} \rightleftarrows \text{succinyl-CoA} + \text{acetoacetate} \qquad (12)$$

$$\text{Succinyl-CoA} + \text{GDP} + P_i \rightleftarrows \text{succinate} + \text{GTP} \qquad (13)$$

$$\text{GTP} + \text{ADP} \rightleftarrows \text{GDP} + \text{ATP} \qquad (14)$$

Sum:

$$2\ \text{Pyruvate} + \text{ADP} + P_i + O_2 \xrightarrow[\text{GDP}]{\text{succinate}} \text{acetoacetate} + 2\ CO_2 + 2\ H_2O + \text{ATP} \qquad (15)$$

Clearly, when heart sarcosomes are incubated aerobically in the presence of pyruvate and malonate, most if not all of the endogenous citric acid cycle intermediates will be converted to succinate, so that the substrate requirements for Reactions 12 and 13 are present.

The requirement for ADP and phosphate for maximum production of acetate is not clear. One possibility which has been considered is that acetoacetate is first formed, and that the acetoacetate is broken down in some manner to form two molecules of acetate. If this were true, it would be possible to explain the requirement for ADP and phosphate for acetate production. However, as shown in Table IV, this appears not to be the case, since there is little or no disappearance of added acetoacetate on incubation with sarcosomes under a variety of conditions, nor is there a net synthesis of acetate unless pyruvate is also present.

TABLE IV

RECOVERY OF ADDED ACETOACETATE AND FORMATION OF ACETATE ON INCUBATION WITH HEART SARCOSOMES

Reactions were carried out in 25-ml erlenmeyer flasks on a shaking water bath at 30°. Reaction mixtures contained the basic medium, 20 μmoles of potassium malonate, 4.9 mg sarcosomal protein and the following additions where indicated: lithium acetoacetate, 1.05 μmoles; potassium pyruvate, 2.48 μmoles; ADP, 10 μmoles; potassium phosphate buffer (pH 7.4), 10 μmoles; and potassium arsenate, 20 μmoles. Reaction volume, 2.0 ml.

Additions	*Incubation time (min)*	*Acids found (μmoles)*			*−ΔPyruvate (μmoles)*
		Aceto-acetate	*Acetate*	*Pyruvate*	
ADP, phosphate	0	0.01	0.18	0	—
Arsenate, acetoacetate	0	1.03	0.17	0	—
ADP, phosphate, acetoacetate	0	1.05	0.18	0	—
ADP, phosphate, pyruvate	0	0.01	0.17	2.48	—
Pyruvate (no sarcosomes)	0	0	0	2.47	—
ADP, phosphate	45	0.02	0.18	0	—
Arsenate, acetoacetate	45	0.99	0.24	0	—
ADP, phosphate, acetoacetate	45	1.03	0.19	0	—
ADP, phosphate, pyruvate	45	0.57	0.85	0.64	1.84

Glutamate dehydrogenase activity in guinea-pig heart sarcosomes

In an earlier paper, it was shown that glutamate stimulated the oxidation of pyruvate when the latter is oxidised in the presence of endogenous substrates[15]. This result was interpreted as follows: the level of circulating endogenous intermediates was rate-limiting for the oxidation of pyruvate, but glutamate increased the rate of pyruvate oxidation due to a slow net synthesis of α-oxoglutarate *via* glutamate dehydrogenase. HOLTON[24] reported several years ago that heart sarcosomes catalyse the slow production of glutamate from α-oxoglutamate and ammonia. BORST[10] has shown that glutamate dehydrogenase is very low in rat heart sarcosomes, but was unable completely to inhibit glutamate oxidation by malonate. GAUTHERON *et al.*[14] have reported glutamate dehydrogenase activity in porcine heart sarcosomes. In the

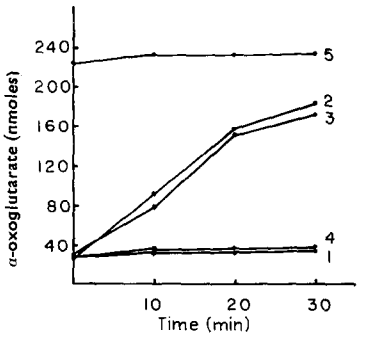

Fig. 1. The effect of glutamate and pyruvate on the accumulation of α-oxoglutarate by heart sarcosomes incubated in the presence of arsenite and 2,4-dinitrophenol. Incubations contained the basic medium, 0.1 μmole 2,4-dinitrophenol, 2 μmoles of sodium arsenite, 3.5 mg sarcosomal protein and substrates as follows: (1) none; (2) sodium L-glutamate, 5 μmoles; (3) sodium L-glutamate, 5 μmoles *plus* potassium pyruvate, 5 μmoles; (4) potassium pyruvate, 5 μmoles; (5) sodium α-oxoglutarate, 0.22 μmole. Final volume, 2.0 ml.

presence of pyruvate, alanine aminotransferase (EC 2.6.1.2), if present, could also lead to a change in the level of citric acid cycle intermediates. VAN DEN BERGH[25] has reported that sarcosomes isolated from housefly thorax contain appreciable alanine aminotransferase activity. Fig. 1 shows the results of an experiment carried out to determine the activities of these two enzymes in intact guinea-pig heart sarcosomes. 2,4-Dinitrophenol was present in order to keep endogenous nucleotides oxidised, and arsenite to prevent oxidation of pyruvate and α-oxoglutarate. When glutamate, or glutamate *plus* pyruvate was added, there was a slow accumulation of α-oxoglutarate (Curves 2 and 3). However, there was no significant increase in α-oxoglutarate when pyruvate alone was added (Curve 4). It is therefore concluded that there is a low but significant activity of glutamate dehydrogenase in these sarcosomal preparations, but that alanine aminotransferase activity is not detectable.

DISCUSSION

Intact heart sarcosomes contain adequate endogenous substrates to support rapid oxidation of acetate or pyruvate without any requirement for added citric acid cycle intermediates[11,15]. The major pathways by which the level of these intermediates in various muscle preparations is regulated is largely unknown. CO_2-fixing reactions leading to the anaplerotic maintenance of oxaloacetate from pyruvate are generally considered to be very low or absent in muscle[1,2]. Glutamate dehydrogenase, which is required for nitrogen disposal from a number of amino acids, as well as for net synthesis of citric acid cycle intermediates from these same amino acids, has been shown to be very low, if not absent, in sarcosomal preparations from rat heart and pigeon breast muscle[10]. A third pathway for the maintenance of the level of oxaloacetate in mitochondria is the synthesis of succinate from propionyl-CoA derived from the breakdown of fatty acids with an odd number of carbon atoms, since the enzymes required for this, propionyl-CoA carboxylase (EC 6.4.1.3), methylmalonyl-CoA racemase (EC 5.1.99.1), and methylmalonyl-CoA mutase (EC 5.4.99.2), are all present in mitochondria[26,27]. Since myocardial substrate levels have been shown to fluctuate widely as a result of dietary variations and diabetes[12,13], it might be inferred that this pathway plays a role in the regulation of myocardial respiration.

The present work is an attempt to evaluate, in a quantitative manner, some of the possible pathways which might lead to a change in the level of citric acid cycle intermediates. Under the conditions of our experiments *in vitro*, malonate inhibited respiration due to added pyruvate 95–97 % in a series of experiments. It seemed of importance, therefore, to ascertain the nature of this residual respiration. A very slow production of oxaloacetate catalysed by pyruvate carboxylase might go undetected in many instances, but could easily account for the low rate of respiration due to pyruvate which we have observed when malonate is present. Since heart muscle has little or no capacity for gluconeogenesis[2,28] there is probably very little drain on the supply of oxaloacetate for biosynthetic reactions in this tissue. We have found, however, that essentially all respiration observed under the present conditions is accounted for by the synthesis of free acetoacetate and acetate. Respiration, pyruvate removal and formation of products are markedly inhibited if ADP and phosphate are absent, even in the presence of an uncoupler of oxidative phosphorylation. The probable route of formation of acetoacetate from pyruvate is suggested to be due solely to reactions

already known to take place in heart sarcosomes, whereas the requirement for ADP and phosphate for maximum production of acetate has not been clarified. The increase in the proportion of total pyruvate removal which is accounted for by production of acetate on longer periods of incubation apparently does not require added ADP and phosphate, and is presumed to result from an emergent activity of an acetyl-CoA hydrolase as described by GERGELEY, HELE AND RAMAKRISHNAN[20]. VON KORFF AND MAC PHERSON[29] and OLSON AND VON KORFF[31] have repeatedly reported the production of labeled acetate from [^{14}C]pyruvate when rabbit heart sarcosomes were incubated in the absence of phosphate acceptor. In any case, the present data indicate to us that there is no mechanism for a net change in the total level of citric acid cycle intermediates by heart sarcosomes oxidising pyruvate. The major pathway for malonate-insensitive oxidation of pyruvate shows a pronounced requirement for ADP and phosphate when respiratory chain-linked oxidative phosphorylation is uncoupled.

When guinea-pig heart sarcosomes were incubated in the presence of 2,4-dinitrophenol, added glutamate was oxidised *via* glutamate dehydrogenase to α-oxoglutarate at a rate of approx. 100 nmoles/h per mg of sarcosomal protein. This pathway might provide one mechanism for catalytic regulation of the level of circulating citric acid cycle intermediates, and therefore of the rate of aerobic energy production.

ACKNOWLEDGEMENTS

The culture of *P. lemoignei* was generously provided by Dr. M. DOUDOROFF. This work was supported by grants from the U.S. Public Health Service HE-06308, Indiana Heart Association and Eli Lilly and Co.

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