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THE EFFECT OF PYRUVATE ON CYCLIC OXIDATIONS BY HEART SARCOSOMES

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SUMMARY

1. The stoichiometry of oxidation of pyruvate and glutamate by heart mitochondria has been reinvestigated.

2. The inhibition by pyruvate of its own oxidation and that of glutamate is shown to be due not to a side reaction, but to a trace amount of contaminant (probably parapyruvate) which inhibits reversibly the reoxidation of cyclicly generated α-oxoglutarate.

3. Enzymatically generated pyruvate and purified pyruvate are oxidised at a constant rate by heart mitochondria in the absence of added malate without significant accumulation of intermediates, whereas crystalline sodium pyruvate is very poorly oxidised, and causes the accumulation of α-oxoglutarate.

4. Glutamate oxidation is optimal without addition of malate. In the presence of endogenous substrates or very low concentration of malate, glutamate stimulates pyruvate disappearance. These observations are explained by a very slow net synthesis of circulating intermediates from glutamate.

INTRODUCTION

Pyruvate is oxidised very rapidly by isolated mitochondria *via* reactions of the citric acid cycle when small amounts of citric acid cycle intermediates are added, but only very slowly in the absence (see, *e.g.*, refs. 1–3). It was suggested by earlier workers[1] that inadequate endogenous intermediates of the cycle were retained by isolated mitochondria for the continuous oxidation of pyruvate.

BORST AND SLATER[4] and BORST[3] discovered, however, that heart and pigeon-breast mitochondria are essentially devoid of glutamate dehydrogenase (EC 1.4.1.3) activity and that glutamate is oxidised in these preparations exclusively *via* glutamate oxalacetate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) yielding α-oxoglutarate, which can subsequently be oxidised to oxaloacetate. Such a cyclic pathway for glutamate oxidation clearly does not affect the total amount of citric acid cycle intermediates. It became apparent that, since glutamate is very rapidly oxidised by heart and pigeon-breast mitochondria, adequate endogenous intermediates of the citric acid cycle must be present. These facts, along with other evidence, led SLATER[2] to postulate that pyruvate is not oxidised in these preparations

without an added source of oxaloacetate because of depletion of the endogenous oxaloacetate, presumably by a decarboxylation catalyzed by acetyl-CoA derived from pyruvate.

Later SLATER, TAMBLYN-HAGUE AND DAVIS-VAN THIENEN[6] studied in detail the inhibition by pyruvate and acetoacetate of glutamate oxidation by heart sarcosomes, and concluded that these inhibitions are caused by decarboxylation of oxaloacetate, catalysed by acetyl-CoA and acetoacetyl-CoA respectively.

In parallel studies, however, the present author[3] demonstrated that acetate can be oxidised rapidly by heart sarcosomes without an added precursor of oxaloacetate. In order for SLATER's original hypothesis[2] to be correct it was necessary to propose that the acetyl-CoA derived from acetate was not readily available to the enzymes catalysing the hypothetical decarboxylation of oxaloacetate. Indirect evidence was presented that the latter suggestion may be correct[3].

SLATER's proposal[2,6] that pyruvate oxidation led to a side reaction and to depletion of citric acid cycle intermediates formed the starting point of the present investigation. The inhibition by pyruvate of cyclic oxidations was found to be due not to a removal of intermediates, but to an inhibition of the oxidation of α-oxoglutarate. Glutamate was found to stimulate the disappearance of purified pyruvate. This is explained by a net synthesis of α-oxoglutarate from glutamate. The results are discussed in view of previous work on pyruvate and glutamate oxidation by heart mitochondria, and of possible physiological significance of the data presented.

METHODS AND MATERIALS

Sarcosomes were isolated from guinea-pig hearts as described earlier[3], except that the bacterial alkaline proteinase treatment was omitted. The sarcosomes were finally suspended in 0.25 M sucrose–10 mM EDTA (pH 7.4) to a final concentration of 15–25 mg mitochondrial protein per ml.

O_2 consumption was measured at 25° polarographically with a Gilson Medical Electronics "oxygraph", or at 30° manometrically with a Gilson differential respirometer, using vessels of approx. 7 ml capacity and two side-arms. The basic reaction media contained 225 mM sucrose, 10 mM KCl, 10 mM Tris–HCl buffer (pH 7.4) and 0.3–1.0 mM EDTA (derived from the mitochondrial suspension), and other additions as indicated in tables and figures.

Analysis for α-oxoglutarate was made spectrophotometrically with glutamate dehydrogenase on deproteinized extracts after stopping reactions by the addition of 0.1 ml 2 M $HClO_4$. Pyruvate was determined spectrophotometrically at pH 7.0 using NADH and lactate dehydrogenase in phosphate buffer. Protein was determined by the biuret method.

Sodium pyruvate was obtained from three sources: Boehringer and Soehne, Calbiochem, and Sigma Chemical Co. ("dimer-free"). Lactate was prepared as lithium salt from lactic acid (Sigma). NAD^+, NADH, ADP, ATP, L-glutamate dehydrogenase and lactate dehydrogenase were obtained from Sigma; sodium [2-^{14}C]pyruvate and sodium [1-^{14}C]acetate were obtained from Calbiochem.

Preparation of purified pyruvic acid

"Dimer-free" sodium pyruvate (Sigma) was dissolved in 2 vol. of water and

brought to pH 1.5 with 10 M HCl. The resulting solution of free pyruvic acid was distilled *in vacuo*. After removal of water, a constant-boiling fraction (30.5° at approx. 10 mm pressure) was collected. The pyruvic acid thus obtained was subjected to a second vacuum distillation to remove any possible traces of water and stored in a sealed vial at −20°.

RESULTS

Preliminary experiments with 2-^{14}C-labeled pyruvate and [1-^{14}C]acetate

The present investigation was initiated to attempt to identify some unique radioactive material which was formed during the cyclic oxidation of [2-^{14}C]pyruvate. The pattern of radioactivity was compared to that obtained using [1-^{14}C]acetate. Several sets of conditions were used. A typical incubation contained 1.0–1.5 mg mitochondrial protein in the basic incubation medium, 15 μmoles ADP, 15 μmoles potassium phosphate buffer (pH 7.4), 1 μmole sodium pyruvate and 1 μC [2-^{14}C]-pyruvate, in a final volume of 1.0 ml. Control vessels contained the same except that 1 μmole sodium acetate was substituted for pyruvate. After the rate of respiration in presence of pyruvate had almost ceased (usually 10 min) the reactions were stopped by the addition of 0.1 ml 2 M $HClO_4$. An appropriate aliquot (0.01–0.10 ml) of the deproteinized extracts was subjected to two-dimensional thin-layer chromatography on silica gel G using several solvent systems. Solvent systems which gave good resolution of citric acid cycle intermediates and amino acids were usually employed[7,8]. After chromatography X-ray film was placed on the plates and exposed for 3–10 days, after which the films were developed. Although traces of citric acid cycle intermediates were always present no unique radioactive material derived from the oxidation of labeled pyruvate was detected. However, although no attempt was made for the quantitative extraction of citric acid cycle intermediates, a radioactive spot corresponding to α-oxoglutarate was several times more intense in radiograms of extracts from labeled pyruvate than that from labeled acetate. This result was obtained with freshly prepared solutions of sodium pyruvate from all three sources.

These data, though preliminary, led to a reconsideration of the manner in which the supply of intermediates is "depleted" by pyruvate. MONTGOMERY AND WEBB[9] showed in 1956 that parapyruvate, the dimer of pyruvic acid, was often present in crystalline preparations of pyruvate, and that parapyruvate is a specific inhibitor for the oxidation of α-oxoglutarate[10]. Although freshly prepared solutions of sodium pyruvate were routinely used in our present and previous studies[3], and although these solutions were oxidised linearly for considerable lengths of time in the presence of "sparker" amounts of malate (0.5 mM), we could not unambiguously exclude the presence of parapyruvate in our incubations. Several approaches were undertaken to decide this possibility.

The effect of concentration on the oxidation of pyruvate in the absence of added malate

Fig. 1 shows polarographic tracings of O_2 uptake by guinea-pig-heart sarcosomes in the presence of increasing concentrations of pyruvate which was obtained from three sources: A, free pyruvic acid redistilled from "dimer-free" sodium pyruvate (Sigma); B, freshly prepared solution of untreated "dimer-free" sodium pyruvate; and C, freshly prepared solution of sodium pyruvate (Calbiochem) which had been

stored at −20° as dry sodium pyruvate for 1 year before use. These data clearly indicate the presence of contaminant(s) in B and C which is absent from, or present in only very low quantity, in A. These results are compatible with the interpretation that a contaminant, probably parapyruvate, is present in B and C which would account for the increasing degree of inhibition of O_2 uptake with time and with increasing concentrations of pyruvate.

The oxidation of enzymatically generated pyruvate

In order to test the hypothesis that parapyruvate (or some other impurity) in our preparations of pyruvate is responsible for the effect of pyruvate on cyclic oxidations requiring oxaloacetate, it was decided to attempt the design of a system which generates pyruvate at a constant rate. Therefore, an enzymatic system using lactate dehydrogenase, lactate and NAD^+ was devised for this purpose. Since the equilibrium of this reaction is very unfavorable for the production of pyruvate in more than trace concentrations, high concentrations of lactate and NAD^+ and non-limiting amounts of lactate dehydrogenase were employed. It was found that, on varying the concentrations of reactants, a low but constant rate of oxidation of pyruvate could be obtained. Fig. 2 and Table I show the results of a typical manometric experiment comparing the oxidation of enzymatically generated pyruvate with

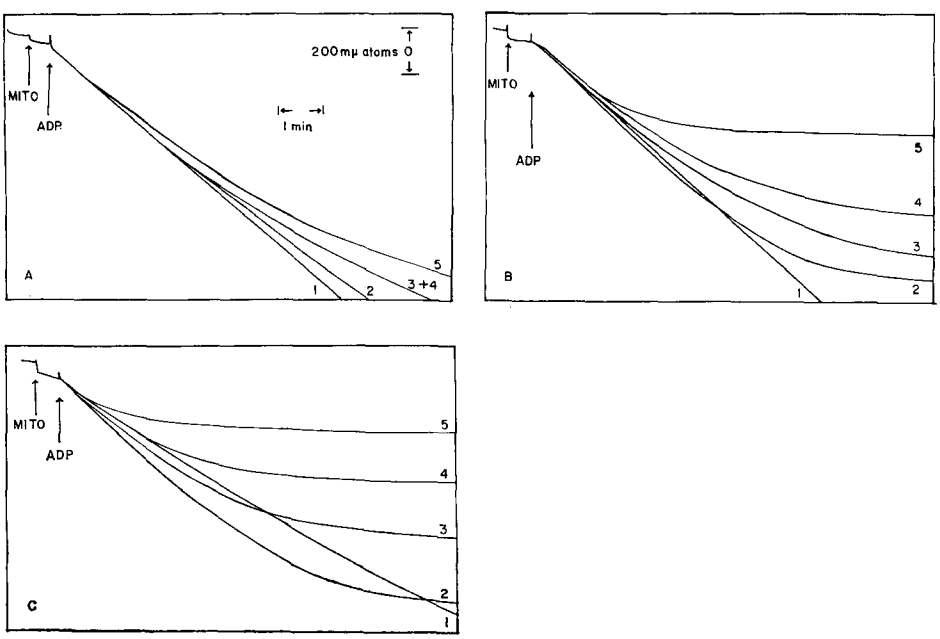

Fig. 1. The effect of concentration of various samples of pyruvate on O_2 uptake in the presence of endogenous substrates. Superimposed tracings of Gilson Medical Electronics oxygraph records. Mitochondria (0.57 mg protein) were added to a solution containing the basic medium *plus* 20 mM potassium phosphate buffer (pH 7.4) and substrates as follows: A, twice distilled "dimer-free" pyruvic acid (Sigma); B, freshly prepared solution of "dimer-free" sodium pyruvate (untreated); C, freshly prepared solution of sodium pyruvate (Calbiochem). After approx. 30 sec, excess ADP (5 μmoles) was added. Concentrations of pyruvate in tracings (2) through (5) were 0.11, 1.1, 2.2 and 11 mM, respectively. Reactions (1) contained 11 mM pyruvate *plus* 0.5 mM L-malate.

that of a sample of crystalline sodium pyruvate. Fig. 2 shows that enzymatically generated pyruvate is oxidised linearly, but at a low rate throughout the experiment. As much as one-sixth (assuming 5 and 1 atoms of O consumed per mole of pyruvate and NADH, respectively) of the observed O_2 uptake may be due to the reoxidation of NADH if these mitochondria are sufficiently leaky to the latter compound. The rate of oxidation is limited by the steady-state concentration of pyruvate, since added malate has no effect on the rate of O_2 consumption. On the other hand, a much higher concentration of sodium pyruvate is oxidised rapidly in the first minutes, but the rate of O_2 consumption rapidly decreases after a few minutes, even in the presence of added malate. What is perhaps more important, however, is the fact that α-oxoglutarate accumulates as a result of the oxidation of added pyruvate, but does not accumulate when enzymatically generated pyruvate is being oxidised (Table I). These data are compatible with at least two interpretations: (1) the crystalline sodium pyruvate contains parapyruvate, which is known to inhibit the oxidation of α-oxoglutarate; and (2) pyruvate, at unphysiologically high concentrations, inhibits its own oxidation. In any case, these results clearly show that the accumulation of

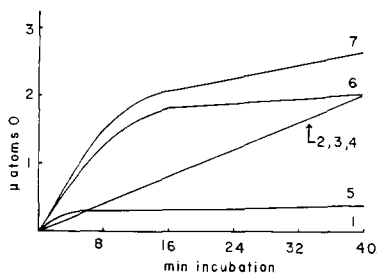

Fig. 2. Oxidation of enzymatically generated and crystalline pyruvate by heart sarcosomes in the absence and presence of added malate. Reaction vessels contained the basic incubation medium, 14 μmoles ADP, 14 μmoles potassium phosphate (pH 7.4), 1.0 mg mitochondrial protein and the following substrates: Curve 1, 0.1 μmole L-malate; Curve 2, 25 μmoles lithium DL-lactate, 1 μmole NAD^+ and 50 units lactate dehydrogenase; Curve 3, same as 2 *plus* 20 mμmoles L-malate; Curve 4, same as 2 *plus* 100 mμmoles L-malate; Curve 5, 10 μmoles sodium pyruvate (source B); Curve 6, same as 5 *plus* 20 mμmoles L-malate; Curve 7, same as 5 *plus* 100 mμmoles L-malate. Final volumes were 1.0 ml. Reactions were started by addition of mitochondria. After a 2-min equilibration period, readings of O_2 uptake were taken at 3-min intervals.

TABLE I

THE ACCUMULATION OF α-OXOGLUTARATE DURING THE OXIDATION OF ENZYMATICALLY GENERATED AND CRYSTALLINE PYRUVATE

Contents of flasks and conditions were those listed in the legend for Fig. 2. Incubation time, 40 min.

Flask	*Substrate*	L-*Malate added* (*mμmoles*)	+*Δα-oxoglutarate* (*mμmoles*)
1	—	100	—
2	Lithium DL-lactate (25 mM)	0	<10
3	50 units lactate dehydrogenase	20	<10
4	1 μmole NAD^+	100	<10
5	Sodium pyruvate (Source B)	0	25
6	(10 mM)	20	38
7		100	105

α-oxoglutarate which results from the oxidation of high concentrations of added pyruvate can account for the apparent depletion of the supply of citric acid cycle intermediates.

Estimation of the enzymatic purity of several samples of pyruvate

Solutions of pyruvate (designated A, B and C, as in Fig. 1) were freshly prepared and a set of twelve spectrophotometric determinations was carried out on aliquots from each sample. Incubations contained approx. 0.3 μmole NADH, 100 μmoles potassium phosphate buffer (pH 7.0) and sample of pyruvate (0.1–0.2 μmole) in a volume of 3.0 ml. Reactions were started with lactate dehydrogenase and were completed within 5 min. Calculations were made using a molar extinction coefficient of $6.22 \cdot 10^3$ for NADH at 340 mμ. Calculation of per cent purities of the three samples based on the weight of dried sodium pyruvate (B and C) and of free pyruvic acid (A) gave the following results ± S.E. from 12 determinations: A, 99.7 ± 1.0; B, 97.5 ± 1.4; and C, 94.6 ± 1.7. It is clear that samples B and C cannot contain more than about 5 % impurity, even if they are completely free of water. Sample A appears to be essentially free of contaminants.

The stoichiometry of oxidation of pyruvate and accumulation of α-oxoglutarate in presence of added malate

Table II summarises the results of two manometric experiments in which the oxidation of three different samples of pyruvate was compared. The redistilled pyruvate (sample A) is oxidised nearly optimally, even in the absence of added malate, whereas samples B and C are oxidised very poorly unless malate is added. Furthermore, there is little accumulation of α-oxoglutarate during the oxidation of sample A,

TABLE II

THE STOICHIOMETRY OF OXIDATION OF PYRUVATE AND ACCUMULATION OF α-OXOGLUTARATE IN THE PRESENCE OF ADDED MALATE

Incubations contained the basic incubation medium, 10 μmoles potassium phosphate buffer (pH 7.4), 1 μmole ATP, 50 mμmoles 2,4-dinitrophenol, 0.8 (Expt. 1) or 1.2 (Expt. 2) mg mitochondrial protein and substrates as listed, in a final volume of 1.0 ml. Reaction time, 30 min.

Expt.	*Substrate*	*Malate added (mμmoles)*	*+Δα-oxoglutarate (mμmoles)*	*−ΔO (μatoms)*	*−ΔO/ −Δpyruvate*	*−Δpyruvate/ +Δα-oxoglutarate*
1	Pyruvate, sample A (5.5 mM)	0	15	3.95	4.7	56
		50	36	3.85	4.8	22
		200	37	5.00	4.9	27
	Pyruvate, sample B (4.85 mM)	0	25	0.85	4.5	7
		50	62	3.25	4.6	11
		200	186	5.45	4.4	7
	Glutamate (10 mM)	200	78	4.05	—	—
2	Pyruvate, sample A (5.8 mM)	0	10	8.68	4.9	177
		50	12	10.80	4.9	184
		200	18	10.90	4.8	126
	Pyruvate, sample C (5.95 mM)	0	18	1.45	4.7	17
		50	61	6.65	4.6	21
		200	180	11.30	4.5	14
	Glutamate (10 mM)	0	—	8.70	—	—

whereas the former accumulates when samples B and C are oxidised. This accumulation is almost stoichiometric with the amount of malate added. The ratio $-\Delta O/-\Delta$pyruvate approaches the theoretical value of 5.0 for the complete oxidation of pyruvate to CO_2 and water in all cases. However, this ratio is slightly lower for samples B and C than A. This is accounted for by the accumulation of α-oxoglutarate.

The determination of α-oxoglutarate in experiments where no malate is added is probably not very precise due to the small amounts being measured. Nevertheless, the ratio $-\Delta$pyruvate$/+\Delta\alpha$-oxoglutarate gives an indication of the effectiveness of the inhibition.

The effect of malate on the course of oxidation of pyruvate and glutamate

Fig. 3 shows the time-course of O_2 uptake in the presence of crystalline pyruvate, purified pyruvate and of glutamate. The time of onset and degree of inhibition of the oxidation of impure pyruvate are both greatly affected by low concentrations of malate. The oxidation of purified pyruvate is linear, but not optimal, since a small amount of malate stimulates O_2 uptake. On the other hand, glutamate is oxidised almost optimally when added alone.

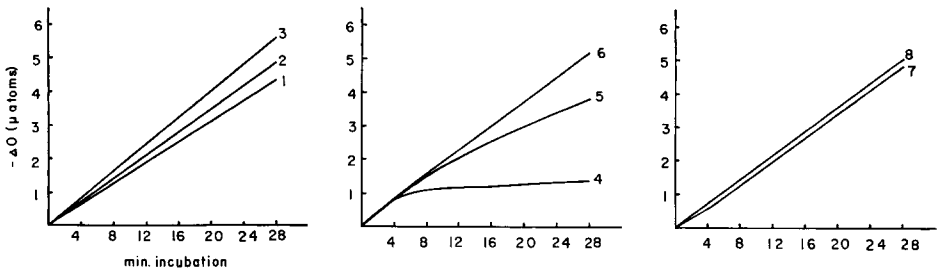

Fig. 3. The effect of malate on the course of oxidation of pyruvate and glutamate. Manometric experiment. Incubation contained the basic incubation medium, 20 μmoles potassium phosphate buffer (pH 7.4), 1 μmole ATP, 50 mμmoles 2,4-dinitrophenol, 0.9 mg mitochondrial protein and substrates as follows: No. 1, 2 and 3, 2.5 μmoles pyruvic acid (sample A) and 0, 50 and 200 mμmoles potassium malate, respectively; No. 4, 5 and 6, 2.5 μmoles sodium pyruvate (sample C) and 0, 50 and 200 mμmoles potassium malate, respectively; No. 7 and 8, 5 μmoles potassium glutamate, and 0 and 200 mμmoles potassium malate, respectively. Reactions were initiated after 3 min of temperature equilibration by tipping substrates from a side-arm. Final volume, 1.0 ml; temperature, 30°.

Competition between pure pyruvate and glutamate for oxidation in presence of endogenous substrates

Table III summarises the results of an experiment designed to show the effects of glutamate and purified pyruvate on their individual rates of oxidation, under conditions in which there might be competition between the two substrates for the low endogenous level of circulating intermediates. The rate of O_2 uptake in the presence of both substrates is much higher than that obtained when either substance is oxidised alone (not indicated in the table is the fact that all substrates were oxidised at a constant rate throughout the experiment). Pyruvate disappearance is increased by glutamate, the former being adequate to account for most of the observed increase in O_2 consumption. The expected result was that glutamate and pyruvate would compete for the available oxaloacetate (*via* the aspartate aminotransferase, EC 2.6.1.1,

TABLE III

THE OXIDATION OF PURIFIED PYRUVATE AND GLUTAMATE IN THE ABSENCE OF ADDED MALATE

Incubations were carried out at 30° in the standard reaction medium *plus* 10 mM potassium phosphate buffer (pH 7.4), 50 μM 2,4-dinitrophenol, 1 mM ATP and substrates as listed in the table. Reactions were initiated by addition of mitochondria (0.8 mg protein) and stopped after 36 min by tipping in 0.1 ml 2 M $HClO_4$. Volume of final reaction mixture was 1.0 ml. Each value is the mean of two separate determinations.

Substrate	$-\Delta O$ (*μatoms*)	$-\Delta$*pyruvate* (*μmoles*)	$-\Delta O / -\Delta$*pyruvate*	$-\Delta O$ *attributable to pyruvate oxidation* (*μatoms*)	$-\Delta O$ *attributable to glutamate oxidation* (*μatoms*)	$+\Delta\alpha$-*oxoglutarate* (*mμmoles*)
Pyruvate, sample A (2.1 μmoles)	4.30	0.89	4.85	4.30	—	*
Glutamate (5.0 μmoles)	5.45	—	—	—	5.45	*
Pyruvate, sample A (2.1 μmoles) *plus* glutamate (5.0 μmoles)	8.70	1.31	6.65	6.55	2.15	24

* Indicates no measurable α-oxoglutarate.

and citrate synthase, EC 4.1.3.7, reactions, respectively), and that the rate of oxidation of each substrate would be diminished, the relative rates being governed primarily by the relative affinities of these two enzymes for oxaloacetate. Other data obtained in the same experiment suggest an explanation for the results actually obtained. A tacit assumption for this experiment was that the sum of all citric acid cycle intermediates is unchanged during the oxidation of pyruvate or glutamate. However, α-oxoglutarate accumulated when both pyruvate and glutamate were oxidised (Table III, last column). The level of α-oxoglutarate is equal to, or even greater than that obtained on oxidation of slightly impure pyruvate (see *e.g.* Table II). Under the latter condition, α oxoglutarate is probably the most abundant intermediate, since its oxidation is blocked, and the conversion of isocitrate to α-oxoglutarate strongly favors α-oxoglutarate formation. These data, therefore, suggested that the level of endogenous intermediates is limiting for pyruvate oxidation, and that there is a very slow but significant net synthesis of an intermediate from glutamate.

A more definitive approach to this problem would be an experiment in which glutamate is oxidised in the presence of impure pyruvate, since the latter is observed to prevent the reoxidation of α-oxoglutarate. The results of an experiment of this type are presented in Table IV. The α-oxoglutarate which accumulates in the presence of pyruvate probably is very nearly equal to the total endogenous intermediates for reasons already mentioned. It is clear, therefore, that there is a net synthesis of α-oxoglutarate from glutamate when impure pyruvate and glutamate are both present. That pyruvate is less effective in inhibiting its own oxidation when being oxidised in the presence of glutamate is apparent from these data. This result is also expected if the level of circulating intermediates is increased by glutamate. Other experiments (not shown), in which glutamate was incubated with mitochondria in the presence of arsenite (to prevent removal of α-oxoglutarate), also demonstrated net α-oxoglutarate synthesis.

TABLE IV

THE EFFECT OF GLUTAMATE ON PYRUVATE DISAPPEARANCE AND THE ACCUMULATION OF α-OXOGLUTARATE IN THE PRESENCE AND ABSENCE OF ADDED MALATE

Incubations contained the basic medium, 10 μmoles potassium phosphate buffer (pH 7.4), 50 mμmoles 2,4-dinitrophenol, 6.07 μmoles sodium pyruvate (source C) and 1.1 mg mitochondrial protein *plus* additions listed in the table, in a final volume of 1.0 ml. Incubations, 40 min at 30°. Not indicated is the fact that O_2 uptake in presence of pyruvate alone is nearly stopped within 10 min, but is stimulated for longer periods in the presence of glutamate or malate.

Other additions	$-\Delta$*pyruvate* (*μmoles*)	$+\Delta$*α-oxoglutarate* (*mμmoles*)
None	0.70	17
Glutamate (10 μmoles)	1.51	78
Malate (50 mμmoles)	1.12	52
Malate (50 mμmoles) *plus* glutamate (10 μmoles)	1.93	120

These data explain the observation (see Fig. 3) that glutamate oxidation by heart mitochondria is near optimal in the absence of added malate, whereas pyruvate oxidation under identical conditions is slow. However, in short-term experiments, SLATER, TAMBLYN-HAGUE AND DAVIS-VAN THIENEN[6] found that malate could stimulate glutamate oxidation as much as 30 % (ref. 6, Table III). Similar results were also obtained in the present study. Indeed, in manometric experiments, a lag-time was often detectable for optimal glutamate oxidation. This is explained by the fact that the supply of oxaloacetate is rate-limiting in the first minutes of incubation, but later glutamate has contributed adequate intermediates for a faster reaction rate.

DISCUSSION

The inhibitory effect of pyruvate on its own oxidation and on the oxidation of glutamate has been reinvestigated. The previous observation[2] that pyruvate oxidation by heart mitochondria is not self-perpetuating, and that the oxidation of glutamate or acetate[3] is rapid and linear, formed the starting point for the present study. Preliminary experiments with radioactive pyruvate and acetate failed to reveal any unusual metabolite derived from pyruvate. However, there was an indication that α-oxoglutarate tended to accumulate during the oxidation of pyruvate. MONTGOMERY AND WEBB[1,9] showed several years ago that pyruvate tends to form its dimer, parapyruvate, which is structurally similar to α-oxoglutarate. Further work by the latter authors[10] showed that parapyruvate inhibits partially purified α-oxoglutarate dehydrogenase in a partly reversible manner with respect to α-oxoglutarate. With the possibility in mind that a small amount of contamination by parapyruvate could explain our previous and present observations, the properties of enzymatically generated and repurified pyruvate were compared to the crystalline material. Although enzymatic assay revealed that all of the samples of pyruvate were at least 95 % pure, the following important differences were noted: (a) both enzymatically generated pyruvate and twice distilled pyruvic acid are oxidised linearly; (b) purified pyruvic acid is oxidised much more rapidly than the crystalline material; and (c)

when oxidised in the presence of very small amounts of added malate, crystalline pyruvate caused the accumulation of α-oxoglutarate, whereas purified pyruvate or enzymatically generated pyruvate did not.

Studies of the competition between pyruvate and glutamate for oxidation revealed that pyruvate did not inhibit O_2 uptake as previously described[2,3,6]. Furthermore, O_2 consumption was much higher in the presence of both substrates than when only one was present, and the disappearance of pyruvate was markedly increased. These observations are attributed to two separate effects: (a) the inhibitory effect of pyruvate on cyclic oxidations requiring oxaloacetate is caused by a small contamination of parapyruvate, which inhibits the oxidation of α-oxoglutarate (and, therefore, of glutamate) in a reversible manner; and (b) when oxidised in the presence of endogenous substrates, glutamate stimulates cyclic oxidations by a slow net synthesis of α-oxoglutarate *via* the glutamate dehydrogenase reaction. It is possible that glutamate could also cause an increase in the supply of citric acid cycle intermediates when pyruvate is present by forming α-oxoglutarate *via* the alanine aminotransferase (L-alanine:2 oxoglutarate aminotransferase, EC 2.6.1.2) reaction. This enzyme was shown by VAN DEN BERGH[11] to be quite active in sarcosomes isolated from housefly thorax muscle. Both glutamate dehydrogenase and alanine aminotransferase activities could increase pyruvate oxidation when glutamate is present due to net synthesis of α-oxoglutarate. The latter enzyme, if present in high enough concentration could lead to significant pyruvate disappearance, without its being further oxidised (*i.e.*, alanine formation). Since the inhibition by parapyruvate of α-oxoglutarate oxidation (and, therefore, of the citric acid cycle) is to a certain degree a competitive one, increasing the level of circulating intermediates by the addition of malate, or by the net synthesis of α-oxoglutarate from glutamate would tend to make parapyruvate much less effective as an inhibitor of cyclic oxidations. A similar effect by malonate on succinate dehydrogenase was observed by KREBS AND EGGLESTON[12] in their classic paper on the oxidation of pyruvate in the citric acid cycle. These authors found that, when a low concentration of malonate was used, fumarate could reverse the inhibition by malonate in a competitive (non-stoichiometric) manner, but if a high concentration of malonate was present, the increase in O_2 uptake due to the addition of fumarate was stoichiometric. In view of the data presented in this communication, it is not surprising that the stoichiometry of pyruvate oxidation previously observed[1,3] is in conformity with its complete disappearance, if provision is made for a relatively high level of intermediates between citrate and α-oxoglutarate. The data summarised in point (a) above, would seem to explain the marked inhibition by high concentrations of pyruvate obtained by HÜLSMANN *et al.*[13] of the oxidation of α-oxoglutarate.

One may ask what possible physiological significance the presence of very low glutamate dehydrogenase activity in heart might have. Since the usual enzymes required for gluconeogenesis and for net production of citric acid cycle intermediates are almost, if not completely, missing in heart and other muscle (see, *e.g.*, ref. 13), glutamate could stimulate the breakdown of fatty acids and carbohydrate in such tissues by providing a source of oxaloacetate. In turn, such a catalytic role by glutamate would tend to inhibit its own breakdown *via* the more active transaminase pathway, presumably due to an unfavorable competition with acetyl-CoA for the available oxaloacetate (see Table III). This interesting effect is being studied in more

detail as a possible regulatory mechanism in the breakdown of protein and carbohydrate in the citric acid cycle.

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