Biochimica et Biophysica Acta, 347 (1974) 168–182
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 46744

MITOCHONDRIAL OXALOACETATE DECARBOXYLASE FROM RAT LIVER

ANNA B. WOJTCZAK and ELŻBIETA WAŁAJTYS

Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warszawa (Poland)

(Received November 26th, 1973)

SUMMARY

1. The occurrence of oxaloacetate decarboxylase (EC 4.1.1.3) in rat liver was confirmed. The decarboxylation was found in both mitochondria and the soluble cytoplasm.

2. The mitochondrial enzyme was partially purified. It appeared not to be activated by divalent cations, nor to be inhibited by chelating agents. It was inhibited up to 50% by 0.5 mM *p*-chloromercuribenzoate. The K_m value was 0.55 mM and pH optimum between 6.5 and 7.5.

3. It was found that the enzyme–substrate complex could be reduced by borohydride to a stable compound, thus showing a Schiff base formation. Parallel to this reduction was a loss of the enzymatic activity.

4. Oxaloacetate decarboxylase was also found in mitochondria of kidney and brain but not in mitochondria from heart and skeletal muscles.

5. It was shown that in intact mitochondria, the substrate for the decarboxylase could be both added oxaloacetate and oxaloacetate formed by the oxidation of malate.

INTRODUCTION

There is little information on the enzymatic decarboxylation of oxaloacetate in animal tissues. Indirect evidence for such decarboxylation comes from the experiments of Oestreicher et al. [1] and Lopes-Cardozo and van den Bergh [2] who have shown that pyruvate is produced when liver or heart mitochondria oxidize succinate or malate. Corwin [3] reported the presence of oxaloacetate decarboxylase (EC 4.1.1.3) in rat liver mitochondria. He partially purified the enzyme and described some of its properties. Later on, oxaloacetate decarboxylase was found by Schmitt et al. [4] in cod fish muscle and the mechanism of its action has been studied extensively by Kosicki and Westheimer [5]. From these studies it seemed that both rat liver and cod fish enzymes were catalytically similar, being metal-activated and biotin-independent β-decarboxylases.

In this report we provide more information about the mitochondrial oxalo-

acetate decarboxylase, its distribution in the liver cell and its occurrence in other tissues. We also re-investigated the mechanism of action of the enzyme from rat liver mitochondria and present the evidence that Schiff base formation rather than a metal-activated process is involved in this enzymatic decarboxylation.

Some of these results have already been briefly communicated [6].

MATERIALS AND METHODS

Mitochondria and other subcellular fractions

Liver mitochondria were prepared as described by Hogeboom [7]. Heart and brain mitochondria were obtained according to the procedures described by Chappell and Hansford [8]. Kidney mitochondria were isolated by homogenizing the whole organ in 250 mM sucrose–0.1 mM EDTA and centrifuging the homogenate as in the case of liver mitochondria. Rat skeletal muscle mitochondria were prepared by the method of Max et al. [9] including heparin in the homogenizing medium.

Inner membrane-matrix particles (mitoplasts) were obtained by the digitonin procedure of Schnaitman and Greenawalt [10]. Total disruption of mitochondria was performed by sonication in 50 mM phosphate buffer, pH 7.2, containing 0.5 mM dithioerythritol, for 2 min in 30 s intervals, using a 60 W sonicator (Measuring and Scientific Equipment Ltd., London), and the soluble fraction was obtained by subsequent centrifugation at 100 000 $\times g$ for 1 h.

In the enzyme distribution studies the subfractions of liver homogenate were prepared according to de Duve et al. [11] except that lysosomes were not separated and they contaminated the mitochondrial and microsomal fractions.

Determination of oxaloacetate decarboxylase

The standard incubation medium contained 5 mM oxaloacetate, 20 mM Tris–HCl buffer and 50 mM phosphate buffer, pH 7.2, 0.5 mM dithioerythritol, 5 mM sodium arsenite and either sonicated mitochondria, tissue extracts or the purified decarboxylase. Incubation was performed in test tubes without shaking during 10 min at 30 °C. After incubation the samples were placed in the ice-cold bath and deproteinized with perchloric acid. After removal of perchlorate by $KHCO_3$ the pyruvate produced and oxaloacetate remaining were estimated in the extracts by enzymatic procedures [12]. Both substances were determined in the same sample, first oxaloacetate being reduced by NADH to malate after addition of malate dehydrogenase (EC 1.1.1.37), followed by the addition of lactate dehydrogenase (EC 1.1.1.27) to reduce pyruvate. In some experiments with purified oxaloacetate decarboxylase the spectrophotometric assay was used. It depends upon following the decrease in the absorbance at 255 nm of the enol form of the equilibrated tautomeric mixture of oxaloacetate [13]. The controls without mitochondria or enzyme preparations were always run in parallel. In order to minimize the spontaneous decarboxylation of oxaloacetate, all manipulations were carried out as rapidly as possible so that the whole operation was terminated within 1 h.

Other analytical and enzymatic procedures

Mitochondrial marker enzymes glutamate dehydrogenase (EC 1.4.1.2) and malate dehydrogenase were assayed as described by Bergmeyer [12]. The protein

content in mitochondria was determined by the biuret method [14], and in dilute enzyme preparations by the procedure of Lowry et al. [15] or by reading optical absorbance at 280 nm. In sucrose-containing fractions the protein was precipitated by trichloroacetic acid, washed, then treated by the biuret reagent.

Other analytical methods used are described in the text.

Chemicals

Oxaloacetic acid, sodium 2-oxoglutarate, sodium pyruvate, L-malic acid and dithioerythritol were purchased from Sigma or Boehringer. Alumina Cγ gel was from Sigma. Glutamate-oxaloacetate transaminase (EC 2.6.1.1) was from Boehringer. Crystalline lactate dehydrogenase from beef heart was from Biomed (Kraków, Poland). Malate dehydrogenase was purified from beef heart sarcosomes by the method of Dupourque and Kun [16], except that matrix from sonicated beef heart mitochondria was used as the starting material and only three steps of the purification procedure were performed, NADH was from Reanal (Budapest, Hungary). The uniformly labelled L-[^{14}C]aspartic acid (100 Ci/mole) was purchased from the Institute for Research Production and Uses of Radioisotopes, Prague, Czechoslovakia.

RESULTS

Conditions for the enzymatic decarboxylation of oxaloacetate

Oxaloacetate is a rather unstable compound and undergoes a spontaneous decarboxylation to pyruvate. Therefore, in order to study the enzymatic decarboxylation it was necessary to establish optimal conditions for the enzymatic process and to decrease the non-enzymatic one. It was observed (Table I) that Mg^{2+} greatly increased the spontaneous decarboxylation of oxaloacetate in sucrose and Tris buffer media. However, in phosphate buffer the activatory effect of Mg^{2+} was strongly decreased. The time course of the enzymatic and non-enzymatic decarboxylations is shown in Fig. 1. The non-enzymatic reaction is activated by Mg^{2+}, but when

TABLE I

NONENZYMATIC AND ENZYMATIC DECARBOXYLATION OF OXALOACETATE IN VARIOUS INCUBATION MEDIA

All samples contained 5 mM sodium arsenite, 4,4 mM Tris–oxaloacetate and, where indicated, 5 mM $MgCl_2$ and sonicated rat liver mitochondria corresponding to 20 mg protein, in total volume of 2.0 ml. pH of the buffers was 7.4; incubation time was 10 min at 30 °C.

Incubation medium	Mg^{2+}	Pyruvate formation (μmoles/10 min)	
		Nonenzymatic (without mitochondria)	Enzymatic
Sucrose, 250 mM	—	0.30	0.90
Sucrose, Tris–HCl 20 mM, phosphate 50 mM	—	0.30	0.90
Sucrose	+	2.12	1.06
Tris–HCl	+	2.04	0.85
Tris–HCl, phosphate	+	0.68	0.90

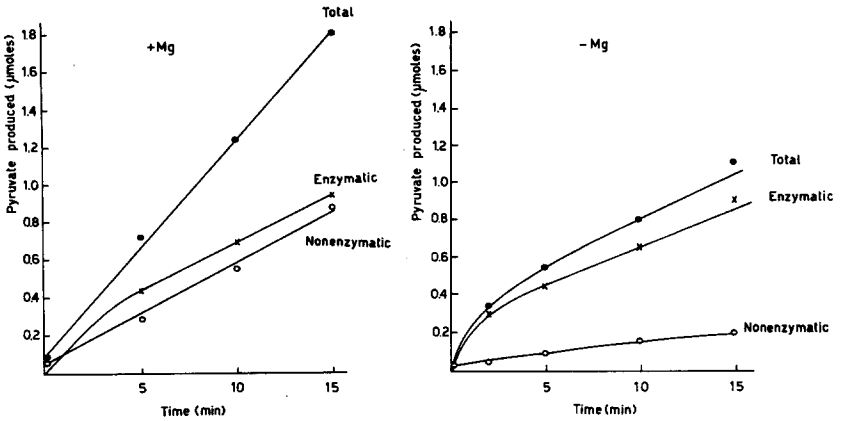

Fig. 1. Time course of the non-enzymatic and enzymatic decarboxylation of oxaloacetate in the presence and absence of Mg^{2+}. The incubation was performed in the standard medium with or without sonicated rat-liver mitochondria, 10 mg protein/ml. The samples were withdrawn at the times indicated. Pyruvate and oxaloacetate were measured as described in Materials and Methods.

it is subtracted from the total decarboxylation it appears that the enzymatic process is equal in the presence and absence of Mg^{2+}. This suggested that the enzymatic decarboxylation is magnesium independent. However, as this point was not quite clear from the beginning of this study (according to Corwin [3] this enzyme does require magnesium), we included Mg^{2+} in the experiments with crude preparations and cell fractionation studies. In these cases the controls with Mg^{2+} were always subtracted from the total activity.

Table II shows that the decarboxylase activity is increased by sonication of mitochondria or treatment with detergents, which indicates the latency of the enzyme. The treatment with detergents was less satisfactory, probably because of some inhibitory effect on the decarboxylase. Therefore, sonication was routinely used throughout in this study.

Sonicated mitochondria show a low activity at pH 5 and a broad optimum between pH 6.5 and 7.5 (Fig. 2). An analogous pH dependence curve was obtained for the purified enzyme, similar to that reported by Corwin [3].

TABLE II

LATENCY OF OXALOACETATE DECARBOXYLASE FROM RAT LIVER MITOCHONDRIA

The incubations were performed in the standard medium (see Materials and Methods) with the addition of 5 mM $MgCl_2$. Specific activity values are given as nmoles of pyruvate produced per min per mg protein. The values are means ±S.E. of the number of experiments shown in parentheses.

Treatment	Specific activity
Intact mitochondria	2.1±1.0 (10)
Sonication (2 min)	6.4±1.9 (10)
Lubrol (0.1 mg/mg protein)	4.6±1.7 (4)
Triton X-100 (0.2 %)	4.8 (1)

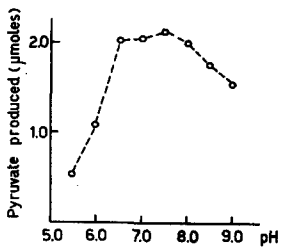

Fig. 2. pH dependence of oxaloacetate decarboxylase. Sonicated mitochondria (10 mg protein/ml) were incubated in the following 50 mM buffers: citrate-phosphate, pH 5.5–6.0; phosphate, pH 6.5–7.5; and Tris–HCl, pH 8.0–9.0. 5 mM $MgCl_2$ and 5 mM sodium arsenite were included in all incubation media.

In the course of this study it has been observed that the decarboxylating activity is strongly protected in the presence of reduced glutathione or dithioerythritol. Therefore, in order to avoid any loss of activity 0.5 mM dithioerythritol was added to tissue extracts during cell fractionation studies and in all steps of the purification procedure.

Subcellular localization of oxaloacetate decarboxylase in rat liver

It can be seen from Fig. 3 that oxaloacetate decarboxylase is present in the mitochondrial and cytoplasmic fractions at roughly equal activities. The distribution pattern of the mitochondrial marker enzyme glutamate dehydrogenase clearly indicates that the cytoplasmic oxaloacetate decarboxylase activity does not originate from disrupted mitochondria, since the contamination of the cytoplasmic fraction by glutamate dehydrogenase is negligible.

When mitochondria were further subfractionated with the use of digitonin [10] the activity was almost quantitatively recovered in mitoplasts (Table III, Expt 1). On the other hand, after sonic disruption of mitochondria (Expt 2) the activity was found in the supernatant. It can be concluded therefore that mitochondrial oxaloacetate decarboxylase is present in the matrix compartment.

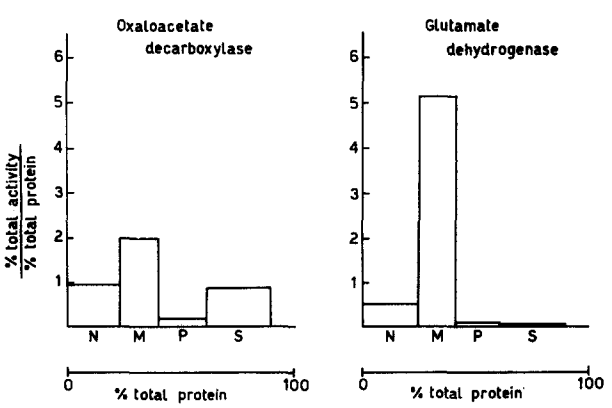

Fig. 3. Subcellular distribution of oxaloacetate decarboxylase in rat liver. The abbreviations for the subcellular fractions are as follows: N, crude nuclear fraction; M, mitochondria; P, microsomes; S, supernatant after 105 000 × *g*. For other experimental details see Materials and Methods.

TABLE III

INTRAMITOCHONDRIAL LOCALIZATION OF OXALOACETATE DECARBOXYLASE

The assays for oxaloacetate decarboxylase were performed in the presence of 5 mM $MgCl_2$. The total amount of mitochondrial protein was 88 mg and 276 mg in Expts 1 and 2 respectively. In Expt 1 the total activity of oxaloacetate decarboxylase was 470 munits and that of malate dehydrogenase 134 units. In Exp. 2 the total activity of oxaloacetate decarboxylase was 1020 munits and of glutamate dehydrogenase 270 units. The mitoplasts (Expt 1) were obtained by the digitonin method [10]. The membranes (Expt 2) were obtained by sonication of mitochondria and subsequent centrifugation at 105 000 $\times g$ during 1 h. For the measurements of the enzymatic activities see Materials and Methods. Abbreviations: OM, outer membranes; IS, intermembrane space; IM, inner membranes.

Expt	Fraction	Protein (%)	Oxaloacetate decarboxylase (%)	Malate dehydrogenase (%)	Glutamate dehydrogenase (%)
1	Total	100	100	100	
	Mitoplasts	73	95	92	
	Supernatant (OM+IS)	40	6	6	
	Recovery	113	101	78	
2	Total	100	100		100
	Membranes (OM+IM)	27	12		0.01
	Soluble (Matrix+IS)	71	84		106
	Recovery	98	96		106

Purification of oxaloacetate decarboxylase from rat liver mitochondria

The purification procedure of Corwin [3] was applied to sonicated mitochondria with some modifications. When the elution from alumina gel was carried out using 0.3 M phosphate buffer (Table IV, Expt 1) the highest specific activity obtained was 55 munits/mg protein. If, in addition to Corwin's procedure, chromatography on DEAE-Sephadex was applied subsequently (Fig. 4), the specific activity could be increased to 130 munits/mg protein. However, when 0.04 M phosphate buffer was used to elute the alumina gel, as in the original procedure of Corwin [3], the highest specific activity was 123 munits/mg protein (Expt 2) and could not be further increased by DEAE-Sephadex chromatography, since the enzyme appeared to be highly unstable at such a low protein concentration. The fractionation with $(NH_4)_2SO_4$ and heat treatment at 50 °C also inactivated the enzyme very strongly. On the other hand, the 100 000 $\times g$ supernatant of sonicated mitochondria can be kept frozen for several weeks without any appreciable loss of activity, provided that 0.5 mM dithioerythritol is present.

Properties of partially purified oxaloacetate decarboxylase and the mechanism of its action

The dependence of the activity upon concentration of oxaloacetate measured with the purified enzyme shows a typical saturation curve (Fig. 5) and the Lineweaver–Burk plot (Fig. 6) gives the apparent K_m of 0.55 mM. The K_m values of the same magnitude (0.42–0.59 mM) were also obtained with sonicated mitochondria and

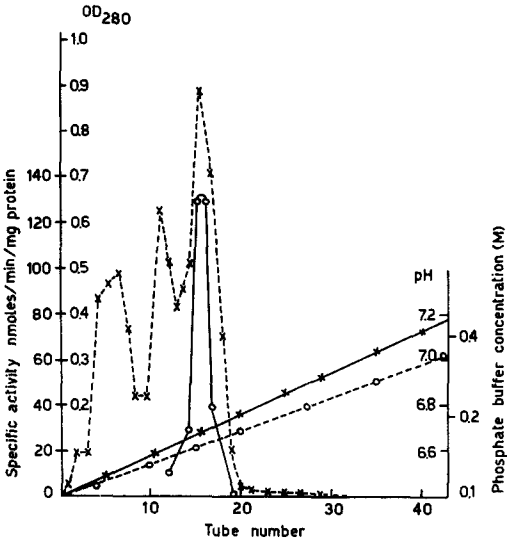

Fig. 4. Ion exchange chromatography of oxaloacetate decarboxylase on DEAE-Sephadex. The fraction eluted from alumina $C\gamma$ gel (60 mg protein, specific activity 50 munits/mg) was passed through the column of Sephadex G-25 equilibrated with 50 mM phosphate buffer, pH 6.4, The total protein fraction was collected and was immediately applied to a column (1.5 cm × 35 cm) of DEAE-Sephadex A-50 previously equilibrated with 0.1 M phosphate buffer, pH 6.4. The elution was performed with the linear gradient of phosphate buffer. The mixing chamber contained 150 ml of 0.1 M phosphate buffer, pH 6.4; 150 ml of 0.5 M sodium phosphate buffer, pH 7.4, was in the second chamber. All buffers contained 0.5 mM dithioerythritol. The volume of each fraction was 5.5 ml. The most active fraction was eluted at pH 6.65 with 0.18 M phosphate buffer. Enzyme activity (○–○) was tested by the spectrophotometric assay [12]. Protein content (×- - -×) was measured by reading the absorbance at 280 nm. pH gradient (×–×), phosphate gradient (○- - -○).

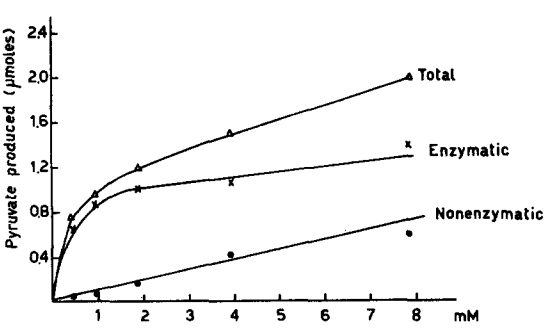

Fig. 5. The dependence of the rate of oxaloacetate decarboxylation on oxaloacetate concentration. The enzyme eluted from alumina $C\gamma$ gel was used. 5 mM $MgCl_2$ was included in the standard medium.

TABLE IV

PURIFICATION OF OXALOACETATE DECARBOXYLASE FROM RAT LIVER MITOCHONDRIA

Frozen mitochondria from 30 rat livers were thawed and suspended in 80 ml of 15 mM Tris–HCl, 5 mM phosphate buffer, pH 7.4, and 0.5 mM dithioerythritol, and sonicated. The suspension was centrifuged at 105 000 × *g* for 1 h and the pellet discarded. The clear supernatant fluid is designated as Sonic extract. This extract (50 ml) was acidified to pH 5.8 with 1 M HCl in ice cold bath with vigorous stirring. The suspension was centrifuged for 20 min at 20 000 × *g* and the pellet discarded. The supernatant adjusted to pH 6.2 and calcium phosphate gel, prepared according to Kunitz [17] was added (0.75 g of dry wt of the gel per 1 g protein). The suspension was stirred gently for 15 min, centrifuged at 20 000 × *g* for 15 min and the sedimented gel discarded. Alumina Cγ gel was added to the supernatant (1 g of the dry weight of the gel per 1 g protein) with constant stirring. After 15 min standing, the mixture was centrifuged and the gel containing adsorbed enzyme was stored at 0 °C. The supernatant was treated with alumina gel in the same way twice and the gel fractions were collected. The enzyme was eluted from alumina gel by the use of 10 ml portions of phosphate buffer, pH 7.4, with the addition of 0.5 mM dithioerythritol. The use of the buffer of low molarity (Expt 2) gave the enzyme of higher specific activity than the elution with concentrated buffer (Expt 1). For the conditions of DEAE-Sephadex chromatography see legend to Fig. 4.

Step	Protein (mg)	Total activity (munits)	Specific activity (munits/mg protein)	Yield (%)
Expt 1				
Sonicated mitochondria	2592	14550	5.6	100
Sonic extract	1632	15340	9.4	105
Supernatant pH 5.8	280	9500	34	66
Calcium phosphate gel supernatant	173	7650	45	52
Elutions from alumina Cγ gel (phosphate buffer 0.3 M):				
1st	65	3580	55	35
2nd	30	1110	37	
3rd	12	430	36	
Chromatography on DEAE-Sephadex: fractions with maximum activity	12	1560	130	
Expt 2 (a fragment)				
Calcium phosphate gel supernatant	295	16500	56	50
Elutions from alumina Cγ gel (phosphate buffer 0.04 M):				
1st	16	2000	123	16
2nd	12	1400	68	
3rd	7	300	43	

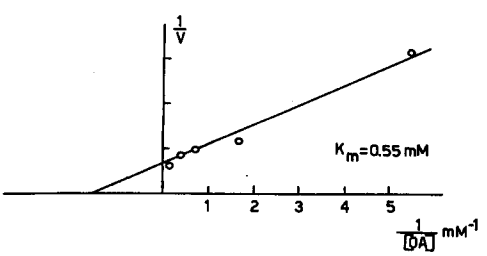

Fig. 6. The Lineweaver–Burk plot of oxaloacetate decarboxylase. The conditions are as in Fig. 5. OA, oxaloacetate.

TABLE V

EFFECT OF DIVALENT CATIONS, CHELATING AGENTS AND *p*-CHLOROMERCURIBENZOATE ON PURIFIED OXALOACETATE DECARBOXYLASE

The samples contained 3 mg protein in 2.0 ml of the standard medium. The enzyme eluted from alumina Cγ gel of specific activity 50 munits/mg protein was used.

Additions	Activity (% of the control)
None (control)	100
Mg^{2+} (0.5 mM)	100
Mg^{2+} (5 mM)	108
Mn^{2+} (1 mM)	98
Zn^{2+} (1.5 mM)	46
Ethyleneglycol-bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (5 mM)	92
EDTA (5 mM)	105
Thenoyltrifluoroacetone (0.5 mM)	100
8-Hydroxyquinoline (1 mM)	92
p-Chloromercuribenzoate (0.5 mM)	51

crude enzyme preparations. These are relatively high values; probably the true K_m is lower because it seems likely that the keto form of oxaloacetate is the proper substrate of the decarboxylase [18].

The effect of various divalent cations and chelating agents was tested and only Zn^{2+} was found inhibitory (Table V). A number of substances chelating Mg^{2+} and iron ions did not inhibit the enzyme either. On the other hand, *p*-chloromercuribenzoate was strongly inhibitory which, together with the observed protecting effect of reduced glutathione and dithioerythritol, points to the essential role of SH groups in this enzyme.

Several keto- and hydroxy-acids, like 2-oxoglutarate, acetoacetate, malate and citrate have been found inhibitory for oxaloacetate decarboxylase from cod fish muscle [4]. None of these compounds exerts any effect on rat liver mitochondrial decarboxylase. The enzyme is also insensitive to avidine.

The results showing an almost complete independence of the mitochondrial decarboxylase on Mg^{2+} and Mn^{2+} are in contradiction to the data of Corwin [3]. They reveal that this enzyme is different from the cod fish enzyme [4] and other known oxaloacetate decarboxylases from bacteria [19, 20] which absolutely require divalent metal ions for their activity.

Another possible mechanism of action of β-decarboxylases is that involving Schiff base formation between the substrate and ε-amino group of lysine residue of the enzyme. As predicted by this mechanism, interaction of the enzyme with ^{14}C-labelled substrate, followed by borohydride reduction, would lead to inactivation of the enzyme and fixation of the label in the acid-stable enzyme–substrate compound. Such a mechanism was shown by Fridovich and Westheimer [21] to apply to the action of crystalline acetoacetate decarboxylase from *Clostridium acetabulicum*.

In order to check whether this mechanism applies to the mitochondrial oxaloacetate decarboxylase, the purified enzyme was incubated with [^{14}C]oxaloacetate, precipitated with trichloroacetic acid and the radioactivity in the precipitate was

TABLE VI

BINDING OF [^{14}C]OXALOACETATE TO OXALOACETATE DECARBOXYLASE AFTER BOROHYDRIDE REDUCTION

20 nmoles of [U-^{14}C]L-aspartic acid (2 μCi) were incubated with 10 μmoles of 2-oxoglutarate and 10 μg of crystalline glutamate-oxaloacetate transaminase in 0.5 ml of 0.1 M phosphate buffer, pH 7.4, for 5 min at 30 °C. Thereafter, the samples were cooled to 0 °C and 4.1 mg oxaloacetate decarboxylase (enzyme eluted from alumina Cγ gel) was added, with the exception of Sample 5 to which 4 mg serum albumin was added instead. In Sample 4, [^{14}C]aspartate and the oxaloacetate generating system were omitted. KCN, 0.25 mM final concentration, was added to Sample 3. After 1 min, a freshly prepared sodium borohydride solution (0.5 M) was added in three 25 μl portions (except to Sample 1). pH was adjusted to neutral, after each addition of borohydride, by a few drops of diluted HCl. After 30 min, trichloroacetic acid (5 % final concentration) was added to all samples. The pellets were washed several times with 5 % trichloroacetic acid then dissolved in 0.2 ml concentrated formic acid and counted for radioactivity in a liquid scintillation counter. For the determination of enzymatic activity parallel samples were run to which unlabelled oxaloacetate was added (2-oxoglutarate and transaminase being omitted). The samples were not precipitated with trichloroacetic acid but were passed through Sephadex G-25 instead, in order to remove the excess of oxaloacetate and borohydride.

Sample No.	Additions	Omissions	Enzyme activity (munits)	Radioactivity bound (cpm)
1	None		143	414
2	$NaBH_4$ (40 mM)		30	4492
3	$NaBH_4$ + KCN		111	2225
4	$NaBH_4$	Oxaloacetate generating system	156	–
5	Serum albumin + $NaBH_4$	Oxaloacetate decarboxylase	–	322

counted. A parallel sample was not deproteinized, but was filtered through Sephadex G-25 and its enzymatic activity was measured. As shown in Table VI, a high amount of radioactivity was fixed by the enzyme preparation after reduction by borohydride. Parallel to this, there was a decrease of enzymatic activity. Cyanide present in the incubation medium protects the enzyme from inactivation by borohydride and also diminishes the fixation of radioactivity. This effect of cyanide has been explained by Fridovich and Westheimer [21] for the acetoacetate-splitting enzyme as being due to the formation of a weak additive complex with the Schiff base.

[^{14}C]Oxaloacetate used in these experiments was generated in the incubation mixture from 2-oxoglutarate and [^{14}C]aspartate in the presence of glutamate-oxaloacetate transaminase. This enzyme also produces a Schiff base, but the enzyme–substrate complex cannot be reduced by borohydride to a stable compound [22]. This was confirmed in a control run (Table VI, Sample 5) in which glutamate-oxaloacetate transaminase, preincubated with 2-oxoglutarate, 4 mg serum albumin and [^{14}C]aspartate, did not bind radioactivity after borohydride reduction. In order to correlate further the enzymatic activity with the extent of labelling, we compared two fractions eluted from the column, one with a high and another with a low specific activity. A strong positive correlation between enzymatic activity and the amount of fixed radioactivity was observed (Table VII).

These results strongly suggest that the mechanism of action of mitochondrial

TABLE VII

BINDING OF [^{14}C]OXALOACETATE TO THE FRACTIONS OF OXALOACETATE DECARBOXYLASE OF DIFFERENT ENZYMATIC ACTIVITIES

Fractions 1 and 2 from DEAE-Sephadex column contained 3.7 and 3.0 mg protein respectively. The treatment by borohydride and the measurement of enzymatic activity was as described in the legend to Table VI.

Fraction No.	Additions	Enzyme activity		Radioactivity bound		
		(munits)	(%)	Total (cpm)	Minus control (cpm)	(%)
1	None (control)	204	100*	1429		
	$NaBH_4$	Not measured		6403	4974	100**
2	None (control)	26	13	1245		
	$NaBH_4$	Not measured		2100	855	17

* Taken arbitrarily as 100 % enzymatic activity.
** Taken arbitrarily as 100 % bound radioactivity.

oxaloacetate decarboxylase is analogous to that established by Warren et al. [23] for acetoacetate decarboxylase from bacteria. The mitochondrial enzyme appeared, however, completely inactive towards acetoacetate as a substrate and it was not inhibited by acetoacetate. It was also insensitive to inhibition by phosphopyridoxal which, in combination with reduction by borohydride, was also used to identify reactive lysine residues of enzymes which per se have no requirement for this cofactor [24]. These observations point to the high specificity of the mitochondrial enzyme towards oxaloacetate.

TABLE VIII

THE OCCURRENCE OF OXALOACETATE DECARBOXYLASE IN MITOCHONDRIA FROM VARIOUS TISSUES AND ANIMALS

The activity of sonicated mitochondria was measured in standard conditions. The isolation of mitochondria is described in Materials and Methods. The number of experiments is given in parentheses.

	Activity of the enzyme (munits · $mg^{-1} \pm$S.E.)	
Rat		
Liver	6.4±1.9	(12)
Kidney	8.5±2.4	(5)
Brain	3.4	(1)
Heart	0	(6)
Skeletal muscle	0	(1)
Rabbit		
Liver	5.6	(2)
Heart	0	(6)
Guinea pig		
Liver	16.0	(2)
Heart	0	(2)

The occurrence of oxaloacetate decarboxylase in mitochondria from various tissues and animals

Among the tissues tested, the highest activity of oxaloacetate decarboxylase was found in liver and kidney mitochondria and a lower activity in the brain (Table VIII). No activity could be detected in heart and skeletal muscle mitochondria both in the absence and in the presence (not shown) of Mg^{2+}. Slater et al. [25] have suggested that in rabbit heart mitochondria, oxaloacetate can be decarboxylated to pyruvate in the presence of catalytic amounts of acetyl-CoA. We tested this possibility by incubating sonicated rat and rabbit heart mitochondria in the presence of oxaloacetate and a system supplying acetyl-CoA (acetylcarnitine and CoA). It appeared that no decarboxylation of oxaloacetate occurred under these conditions and all oxaloacetate which disappeared was converted to citrate. It has been observed [1, 26], however, that heart mitochondria can produce pyruvate when oxidizing malate. This activity was identified as being due to malic enzyme [6, 27] (malate decarboxylating dehydrogenase, EC 1.1.1.40). This enzyme in rat heart mitochondria is dependent on Mg^{2+} or Mn^{2+} and requires NADP [6, 26]. The NAD-dependent malic enzyme (EC 1.1.1.39) was also found in animal tissues [6, 26, 28].

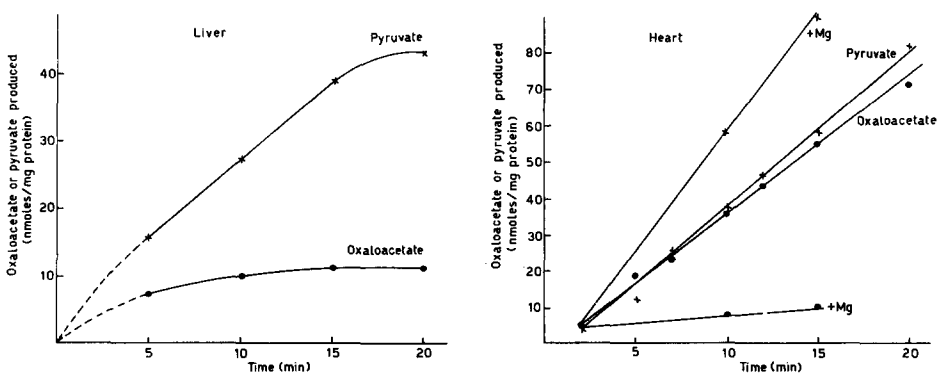

Fig. 7. Production of oxaloacetate and pyruvate by rat liver and heart mitochondria oxidizing malate. The incubation was carried out at 30 °C in Erlenmayer flasks shaken gently. The incubation medium contained 125 mM KCl, 5 mM phosphate buffer, 20 mM Tris–HCl buffer, pH 7.4, 5 mM sodium malate, 5 mM sodium arsenite, 1.3 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 5 mM $MgCl_2$ where indicated and liver mitochondria (7.4 mg protein) or heart mitochondria (5.7 mg protein) in the total volume of 2.0 ml. The samples were withdrawn at indicated times, deproteinized with perchloric acid, neutralized with $KHCO_3$, and oxaloacetate and pyruvate were assayed enzymatically. The amounts of oxaloacetate and pyruvate were not corrected for the non-enzymatic decarboxylation of oxaloacetate.

When rat liver and heart mitochondria oxidize malate in the presence of uncoupler and arsenite, the products are accumulated at different kinetics (Fig. 7). In liver mitochondria, both in the presence and absence of magnesium, the production of pyruvate is approximately linear up to 15 min and the level of oxaloacetate increases for only 10 min. Since the activity of malic enzyme in sonicated liver mitochondria was determined as 1.4 munits/mg protein, it can be concluded that this enzyme may account for a maximum of 50% of the pyruvate produced; the rest must be due to the action of oxaloacetate decarboxylase. In heart mitochondria in the ab-

sence of Mg^{2+} there is a linear accumulation of both pyruvate and oxaloacetate. The addition of Mg^{2+} results in a strong inhibition of oxaloacetate production and an increase in pyruvate accumulation. The interpretation of this experiment is that in liver mitochondria the decarboxylase prevents the accumulation of oxaloacetate above a certain level. In contrast to this, in heart mitochondria partly deficient in magnesium, malate dehydrogenase (EC 1.1.1.37) produces a large amount of oxaloacetate. Upon addition of Mg^{2+}, malic enzyme competes strongly with malic dehydrogenase for either the substrate or the nucleotide and only a small amount of oxaloacetate is accumulated. This experiment shows that oxaloacetate decarboxylase is functionally active in liver mitochondria when oxaloacetate is generated inside.

DISCUSSION

Enzymatic decarboxylation of oxaloacetate in mitochondria from several animal tissues seems to be well established on the basis of the results obtained in this study as well as those reported earlier [3]. However, the problem is still open as to whether the observed oxaloacetate decarboxylating activity is the proper and only ability of a specific enzyme or a kind of side activity of an enzyme which has a different function. Such enzymes are known to be present in plants, bacteria and animals [29]. For example, Kobes and Dekker [30] studied the occurrence and mechanism of action of 2-keto-4-hydroxyglutarate aldolase from bovine liver cytosol and found that this enzyme could decarboxylate oxaloacetate at a rate which was 50% of its aldolase activity. It seems possible that the activity found by us in rat liver soluble fraction may be ascribed to this enzyme. This point has to be cleared up by further investigation. However, no such enzyme is present, to the authors' knowledge, in mitochondria of liver, kidney and brain, and it seems plausible that the mitochondrial enzyme is a true oxaloacetate decarboxylase.

It is known that oxaloacetate can also be decarboxylated by malic enzyme under certain conditions [31]. However, mitochondrial oxaloacetate decarboxylase activity described in this paper was not due to malic enzyme because of a different pH dependence and its insensitivity to divalent cations.

We have not been able to confirm the activation of rat liver mitochondrial oxaloacetate decarboxylase by Mg^{2+} as reported by Corwin [3].

Although Schiff base formation needs to be finally confirmed with the highly purified enzyme, the evidence obtained so far strongly suggests this mechanism of action for mitochondrial oxaloacetate decarboxylase. It is thus evident that the mitochondrial enzyme is different from cod fish muscle [4] oxaloacetate decarboxylase as well as from bacterial decarboxylases which are metal dependent [19, 20] or avidine sensitive [32].

The role of oxaloacetate decarboxylase in mitochondria may be a matter of speculation. It is well known that oxaloacetate is an inhibitor of several mitochondrial enzymes, such as succinate dehydrogenase [33], malate dehydrogenase [16], glutamate-oxaloacetate transaminase [34] and phosphoenolpyruvate carboxykinase [35]. Therefore, the maintenance of oxaloacetate concentration at a relatively low level may be of vital importance for mitochondrial metabolism. Oxaloacetate is produced in the tricarboxylic acid cycle by the oxidation of malate and also, in gluconeogenic tissues, by pyruvate carboxylation. In connection with this, it is interesting to note

that oxaloacetate decarboxylase was found only in mitochondria possessing pyruvate carboxylase (Table VIII). It may be supposed, therefore, that the role of oxaloacetate decarboxylase is that of a safety valve preventing the accumulation of oxaloacetate above a certain level. This hypothesis is, however, difficult to reconcile with the high K_m of the decarboxylase (0.55 mM) as compared to the low concentration of intramitochondrial oxaloacetate (e.g. 10–100 μM for guinea pig liver mitochondria oxidizing malate, ref. 36). Nevertheless, it was shown in the present investigation that oxaloacetate originating from malate oxidation does undergo enzymatic decarboxylation in liver mitochondria at the rate of 3–4 nmoles/min/mg protein (Fig. 7). This is again difficult to reconcile with a relatively high rate of oxaloacetate transport across mitochondrial membrane. Gimpel et al. [37] determined V for the inward transport of oxaloacetate as 80–270 nmoles/min/mg protein. It was found, however, that the outward transport of oxaloacetate generated inside mitochondria is much slower [38]. It amounted to 5 nmoles/min/mg protein, and this may explain why in our conditions the decarboxylase could compete to a significant degree with the transport of oxaloacetate.

Enzymatic decarboxylation of oxaloacetate in liver mitochondria may also explain a spontaneous reactivation of succinate oxidation inhibited by added oxaloacetate studied in this laboratory [39]. The release of inhibition has been interpreted as being due to the removal of oxaloacetate by acetyl-CoA and endogenous fatty acids have been proposed as the source of this acetyl-CoA. Now it seems more likely that it was pyruvate formed from oxaloacetate that provided acetyl-CoA in those experiments.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Lech Wojtczak for the stimulating discussion and for the help in preparing the manuscript. The skilful technical assistance of Mrs Barbara Burcan is appreciated.

REFERENCES

1 Oestreicher, A. B., van den Bergh, S. G. and Slater, E. C. (1969) Biochim. Biophys. Acta 180, 45–55
2 Lopes-Cardozo, M. and van den Bergh, S. G. (1971) 7th FEBS Meet., Varna, Abstr., No. 601, p. 227, Bulgarian Biochem. Biophys. Soc., Sofia
3 Corwin, L. M. (1959) J. Biol. Chem. 234, 1338–1341
4 Schmitt, A., Bottke, I. and Siebert, G. (1966) Z. Physiol. Chem. 247, 18–34
5 Kosicki, G. W. and Westheimer, F. H. (1968) Biochemistry 7, 4310–4314
6 Wojtczak, A. B. and Wałajtys, E. (1973) 9th Int. Congr. Biochem., Stockholm, Abstracts, p. 350
7 Hogeboom, G. H. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. 1, pp. 16–19, Academic Press, New York
8 Chappell, J. B. and Hansford, R. G. (1969) in Subcellular Components (Birnie, G. D. and Fox, S. M., eds), pp. 43–56, Butterworths, London
9 Max, S. R., Garbus, J. and Wehman, H. J. (1972) Anal. Biochem. 46, 576–584
10 Schnaitman, C. and Greenawalt, J. W. (1968) J. Cell Biol. 38, 158–175
11 De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604–617
12 Bergmeyer, H. U. (1963) Methods of Enzymatic Analysis, pp. 253–259 and pp. 335–339, Academic Press, New York

13 Kosicki, G. W. (1968) Biochemistry 7, 4299–4302
14 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–766
15 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
16 Dupourque, D. and Kun, E. (1968) Eur. J. Biochem. 6, 151–155
17 Kunitz, M. (1952) J. Gen. Physiol. 35, 423–450
18 Pogson, C. I. and Wolfe, R. G. (1972) Biochem. Biophys. Res. Commun. 46, 1048–1054
19 Horton, A. A. and Kornberg, H. L. (1964) Biochim. Biophys. Acta 89, 381–383
20 Plaut, G. W. and Lardy, H. A. (1949) J. Biol. Chem. 180, 13–27
21 Fridovich, I. and Westheimer, F. H. (1962) J. Am. Chem. Soc. 84, 3208–3209
22 Gray, C. J. (1971) Enzyme Catalyzed Reactions, pp. 251–256, Van Nostrand Reinhold Co., London
23 Warren, S., Zerner, B. and Westheimer, F. H. (1966) Biochemistry 5, 817–823
24 Schnackerz, K. D. and Noltmann, E. A. (1971) Biochemistry 10, 4837–4843
25 Slater, E. C., Tamblyn-Hague, C. and Davis-van Thienen, W. (1965) Biochim. Biophys. Acta 96, 206–216
26 Davis, E. J., Lin, R. C. and Chao, D. L. (1972) in Energy Metabolism and Regulation of Metabolic Processes in Mitochondria (Mehlman, M. A. and Hnason, R. W., eds), pp. 211–238, Academic Press, New York
27 Nolte, J., Bridczka, D. and Pette, D. (1972) Biochim. Biophys. Acta 284, 497–507
28 Sauer, L. A. (1973) Biochem. Biophys. Res. Commun. 50, 524–531
29 Rutter, W. J. (1964) Fed. Proc. 23, 1248–1257
30 Kobes, R. D. and Dekker, E. E. (1971) Biochim. Biophys. Acta 250, 238–250
31 Ochoa, S. (1952) in The Enzymes (Sumner, J. B. and Myrbäck, K., eds), Vol. 2, pp. 929–1032, Academic Press, New York
32 Stern, J. R. (1967) Biochemistry 6, 3545–3551
33 Wojtczak, L., Wojtczak, A. B. and Ernster, L. (1969) Biochim. Biophys. Acta 191, 10–21
34 Fahien, L. A. and Strmecki, M. (1969) Arch. Biochem. Biophys. 130, 468–477
35 Ballard, F. J. (1970) Biochem. J. 120, 809–814
36 Garber, J. A. and Salganicoff, L. (1973) J. Biol. Chem. 248, 1520–1529
37 Gimpel, J. A., de Haan, E. J. and Tager, J. M. (1973) Biochim. Biophys. Acta 292, 582–291
38 Gimpel, J. A. (1973) Mitochondrial Processes Involving Oxaloacetate, Ph. D. Thesis, Amsterdam, Drukkerij Gerja, Waarland
39 Wojtczak, A. B. (1969) Biochim. Biophys. Acta 172, 52–65