Biochem. J. (1967) 102, 885

The Synergistic Decarboxylation of Glyoxylate and 2-Oxoglutarate by an Enzyme System from Pig-Liver Mitochondria

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(*Received* 19 *July* 1966)

1. An enzyme system that catalyses a synergistic decarboxylation of glyoxylate and 2-oxoglutarate has been purified from pig-liver mitochondria. 2. The purified system is specific for glyoxylate and 2-oxoglutarate as substrates, although in earlier stages of purification glycine and L-glutamate are also active. 3. The reaction is inhibited strongly by EDTA and *N*-ethylmaleimide. Substrate analogues, present at concentrations equimolar with respect to the substrates, are not effective as inhibitors. 4. The reaction proceeds in the absence of added cofactors. Magnesium chloride, mercaptoethanol and sucrose stimulate the reaction, and stabilize the activity of the enzyme. 5. The pH optimum of the reaction is 7·0. The K_m values of glyoxylate and 2-oxoglutarate, at saturating concentration of the corresponding co-substrate, are 16 mM and 3·6 mM respectively. 6. Isotopic work with specifically labelled [^{14}C]glyoxylate and 2-oxo[^{14}C]-glutarate suggests that the enzyme system catalyses an initial condensation of glyoxylate and 2-oxoglutarate that results in, or leads to, release of C-1 of both substrates as carbon dioxide. C-2 of glyoxylate and C-5 of 2-oxoglutarate do not appear as carbon dioxide. 7. The stoicheiometry of the reaction is complex. During the initial stages of the reaction, more carbon dioxide is recovered from 2-oxoglutarate than from glyoxylate. Subsequently, there is a disproportionate increase with time of carbon dioxide evolution from the carboxyl group of glyoxylate. The excess of decarboxylation of glyoxylate over 2-oxogluturate is further increased by treatment of reaction products with acid.

Although many enzymic transformations of glyoxylate have been reported in mammalian tissue, many aspects of its role in mammalian intermediary metabolism remain obscure. Several reactions for its formation have been reported: transamination between glycine and 2-oxoglutarate in liver (Cammarata & Cohen, 1950); oxidation of glycine by a glycine oxidase found in liver or kidney (Ratner, Nocito & Green, 1944); reversible cleavage of 4-hydroxy-2-oxoglutaric acid to pyruvate and glyoxylate in liver (Dekker & Maitra, 1962; Kuratomi & Fukunaga, 1960, 1963). Similarly, several reactions for its further metabolism or breakdown in mammalian tissue have been reported: amination or transamination to glycine (Weinhouse & Friedmann, 1951; Nakada & Weinhouse, 1953*a*,*b*; Fleming & Crosbie, 1960; Nakada, 1964); condensation of pyruvate and glyoxylate to give 4-hydroxy-2-oxoglutaric acid (see above); non-enzymic condensation with oxaloacetate (Ruffo, Adinolfi, Budillon & Capobianco, 1962) or acetoacetate (Ellington, Mellanby & Williamson, 1964); oxidation to oxalate (Weinhouse & Friedmann, 1951; Nakada & Weinhouse, 1953*a*; Crawhall & Watts, 1962; Ratner *et al.* 1944); oxidative decarboxylation to formate and carbon dioxide by uncharacterized enzyme systems (Weinhouse & Friedmann, 1951; Nakada & Weinhouse, 1953*a*; Nakada, Friedmann & Weinhouse, 1955; Ratner *et al.* 1944). In addition to these transformations, two types of decarboxylation of glyoxylate have been described. Nakada & Sund (1958) reported that washed homogenates of many rat tissues, including liver, converted glyoxylate into formate and carbon dioxide and that this reaction was stimulated by L-glutamate. A partially purified enzyme catalysing the oxidative decarboxylation of glyoxylate was obtained from rat-liver mitochondria. The enzyme required NAD, TPP,‡ manganese chloride and L-glutamate for maximum rates. The reaction products were reported to be *N*-formyl-L-glutamate and carbon

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‡ Abbreviation: TPP, thiamine pyrophosphate.

dioxide. It was suggested that the *N*-formyl-L-glutamate hydrolysed to formate and glutamate. The second type of decarboxylation of glyoxylate was discovered by Crawhall & Watts (1962) with human- and rat-liver mitochondria. They, too, observed a decarboxylation of glyoxylate that was stimulated by L-glutamate. However, the latter workers were unable to detect *N*-formyl-L-glutamate either as an intermediate or product of the reaction. Further, they found that 2-oxoglutarate was more effective in promoting the decarboxylation than was L-glutamate. When ^{14}C-labelled substrates were used it was found that both C-1 of glutamate and C-1 of glyoxylate contributed largely to the evolved carbon dioxide. The nature of this synergistic decarboxylation of glyoxylate and 2-oxoglutarate was not known.

The object of the work now described was to re-examine the decarboxylation of glyoxylate by liver mitochondria, and to purify the enzyme system involved. For convenience, pig liver was used as the source of mitochondria. An enzyme system has been purified that catalyses a decarboxylation of glyoxylate and 2-oxoglutarate in the presence of each other, and some isotopic studies have been made of the mechanism of the reaction. Part of the work reported here has been reported in preliminary form (Stewart & Quayle, 1966).

MATERIALS AND METHODS

All chemicals, unless noted, were A.R. grade. Water was distilled twice in all-glass apparatus. Glyoxylic acid was obtained from British Drug Houses Ltd., Poole, Dorset. L-Lactate–NAD oxidoreductase (EC 1.1.1.28) (lactate dehydrogenase), L-glutamate–NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3) (glutamate dehydrogenase), 2-oxoglutarate, NAD and NADP were supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Glyoxylic acid was 99% pure when assayed with lactate dehydrogenase and NAD (Meister, 1957). 2-Oxoglutarate was assayed at 100% purity with $NADH_2$ and glutamate dehydrogenase (Bergmeyer & Bernt, 1963). Labelled substrates were obtained from The Radiochemical Centre, Amersham, Bucks. At intervals during these studies, samples of the radioactive substrates were chromatographed on paper and on columns as described below, in order to remove small amounts of impurity that formed during storage.

2-Oxo[1-^{14}C]glutarate was prepared and separated from DL-[1-^{14}C]glutamate by the method described by Haslam & Krebs (1963).

Chromatography and radioautography. The two-dimensional chromatography system described by Large, Peel & Quayle (1961) was used. Columns (3·0ml. bed vol., 1·0cm. diam.) of Dowex 1 (X4; formate form; 200–400 mesh) were also used. Glyoxylate was eluted from these columns with 10vol. of 0·10N-formic acid, and 2-oxoglutarate with 10vol. of 1·0N-formic acid. Radioautography was carried out by using Kodirex X-ray film (Kodak), with exposure times of 2–10 days.

Measurement of protein. Protein was assayed by the method of Warburg & Christian (1941).

Preparation of mitochondria from pig liver. Mitochondria were prepared as follows. About 500g. of liver from a recently killed pig was passed through a Latapie mincer at room temperature. Subsequent operations were carried out at 2°. Mince (1vol.) was added to 4vol. of freshly-prepared cold 0·25M-sucrose, pH7·4, mixed and homogenized in a stainless-steel Potter–Elvehjem homogenizer. The homogenate was centrifuged at 600*g* for 10min. The sediment was resuspended in 1·5vol. of sucrose solution and centrifuged at 340*g* for 10min. Supernatants from these two centrifugings were pooled and centrifuged at 1300*g* for 30min. The resulting supernatant was discarded and the sediment resuspended in 1·5vol. of sucrose solution and centrifuged at 1300*g* for 10min. The supernatant and fluffy layer were carefully poured off and the sediment was resuspended in 1·5vol. of sucrose and the centrifuging repeated. The sediment that resulted was divided into eight portions and stored at −20° until required for preparation of the enzyme. The mitochondria stored in this way retained activity over several months.

Enzyme purification. Enzyme was prepared from mitochondria as follows. One portion of mitochondria was thawed and suspended in 4ml. of 0·02M-potassium phosphate, pH7·0. This suspension was passed through a Hughes (1951) press at −15°. The resulting extract was suspended in 8ml. of 0·02M-potassium phosphate, pH7·0, and centrifuged at 25000*g* for 10min. The supernatant was decanted and brought to 30% saturation with 0·4vol. of cold saturated $(NH_4)_2SO_4$ solution, pH7·4, and left for 5min. at 0°. A precipitate sometimes formed at this stage, and was removed by centrifuging at 25000*g* for 5min. and discarded. The solution was then adjusted to 55% saturation with additional $(NH_4)_2SO_4$ solution (0·5vol.) and left for 5min. at 0°. The precipitate, which contained all the enzyme activity, was recovered by centrifuging at 25000*g* for 10min. and dissolved in the minimum volume of 0·01M-potassium phosphate, pH7·0. Preparations at this stage could be stored at −20° with only slight loss of activity over 5 days.

This solution was applied to a column (50cm. × 1·6cm.) of Sephadex G-200 (bead form), equilibrated with 0·01M-potassium phosphate, pH7·0. Elution of enzyme was carried out with the same solution at a flow rate of about 10ml./hr. until all the protein had been eluted (Fig. 1*a*). After enzymic assay of fractions (5ml.), active material was pooled and applied to a column (1·6cm. diam.) of calcium phosphate gel (Swingle & Tiselius, 1951) and cellulose, mixed in the proportion of 3mg. dry wt. of gel and 7mg. dry wt. of cellulose per mg. of protein. The column was equilibrated with 4vol. of 0·01M-potassium phosphate, pH7·0, the enzyme solution applied, the column washed with another 4vol. of the same buffer, and elution carried out with 0·02M-potassium phosphate, pH7·7, until no further protein appeared in the eluate. Active material was then eluted with 0·05M-potassium phosphate, pH7·7 (Fig. 1*b*). Fractions (5ml.) were collected at a rate of 20ml./hr. and assayed.

At this stage, enzymic activity became unstable (half-life approx. 12hr. at 2°) but could be stabilized by the addition of sucrose, mercaptoethanol and $MgCl_2$ to final concentrations of 0·9M, 0·02M and 0·02M respectively. The activity then had a half-life of 10 days or more at 2°.

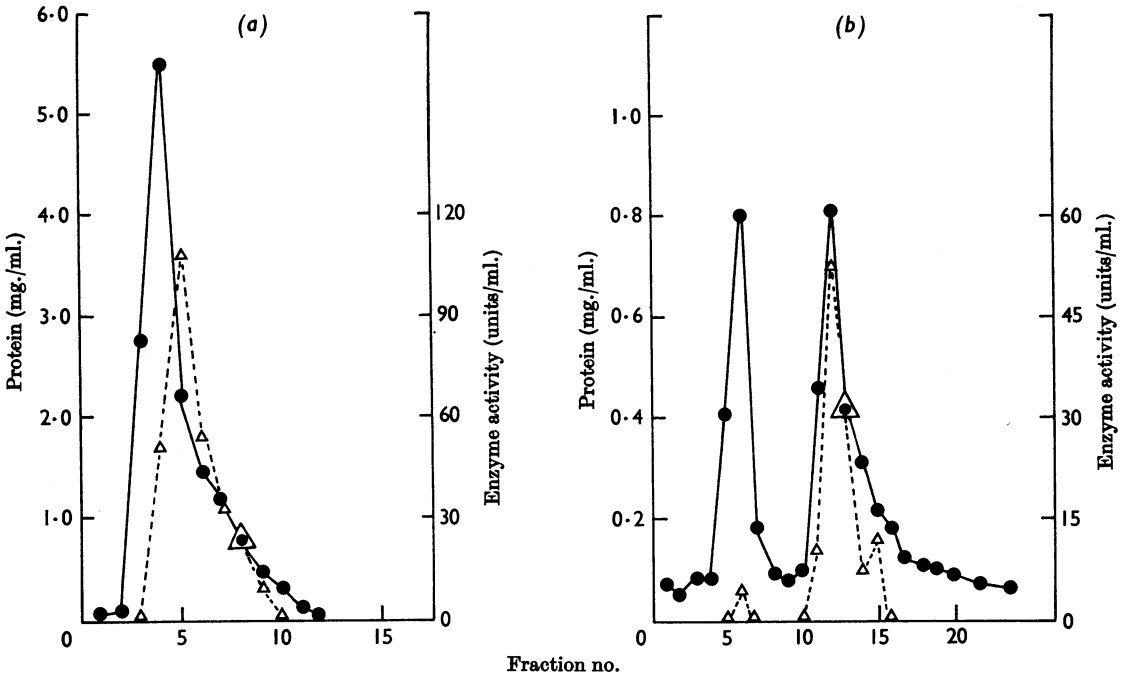


Fig. 1. Chromatography of enzyme preparations on columns of Sephadex G-200 and calcium phosphate–cellulose. (*a*) Sephadex G-200: 112mg. of protein (from precipitation with ammonium sulphate) of specific activity 13·5units/mg. was applied to a column equilibrated with 0·01M-potassium phosphate, pH7·0, and eluted with same buffer as described in the Materials and Methods section. (*b*) Calcium phosphate–cellulose: 170mg. of protein (from Sephadex G-200 column) of specific activity 30units/mg. was applied to a column equilibrated with 0·01M-potassium phosphate, pH7·0 and eluted as described in the Materials and Methods section. ●, Protein; △, enzyme activity.

Table 1. *Purification of glyoxylate–2-oxoglutarate decarboxylase from pig liver*

Step	Fraction	Vol. (ml.)	Activity (units/ml.)	Protein (mg./ml.)	Sp. activity (units/mg. of protein)	Total units	Yield (%)
1	Homogenate from 100g. wet wt. of liver	450	17	20	0·84	7500	100
2	Mitochondria isolated from homogenate, and suspended in buffer	16	160	50	3·2	2600	29
3	Supernatant after disruption of mitochondria	8	250	30	8·3	2000	27
4	Fraction precipitated by ammonium sulphate (30–50% satn.)	1·5	700	47	15	1050	14
5	Selected combined fractions from Sephadex G-200 column	10	100	4·5	22	1000	13
6	Selected combined fractions from calcium phosphate–cellulose column	31	30	0·8	37	930	12
7	Fraction obtained from 6, activated with sucrose, mercaptoethanol and $MgCl_2$	38	55	0·65	85	2100	28

Besides stabilizing the enzyme, this treatment also resulted in twofold activation. It was possible to increase the specific activity of the enzyme by passing the non-stabilized preparation eluted from the calcium phosphate–cellulose column through a column of DEAE-cellulose (1·7cm.× 17cm.) equilibrated with 0·02M-potassium phosphate, pH7·0. The active material passed unretarded through the column, while some inactive protein was bound. This

step can give a further two- or three-fold improvement in specific activity, at the cost of a further loss of stability.

The overall purification procedure is summarized in Table 1.

For later experiments involving decarboxylation of limiting amounts of non-radioactive glyoxylate and 2-oxoglutarate, the enzyme system used was one that had been purified only as far as precipitation by $(NH_4)_2SO_4$ at 40–50% of saturation.

Manometric assay of enzyme activity. The activity of the enzyme was assayed by manometric determination of CO_2 production. Incubations were carried out in Warburg micro-flasks (7–10ml. vol.) with two side arms at 37° in a N_2 atmosphere. To start the reaction, enzyme solution was tipped from one side arm into the main compartment. The main compartment contained 40μmoles each of sodium glyoxylate and sodium 2-oxoglutarate, adjusted to pH7·0, and 50μmoles of potassium phosphate, pH7·0. The total volume of the complete reaction mixture was 0·95ml. After 60min. of shaking, 0·10ml. of 7N-H_2SO_4 was added from the second side arm and CO_2 output determined after a further 60min. of shaking. One unit of enzyme activity is defined as that amount of enzyme catalysing the production of 1mμmole of CO_2/min. under the conditions of assay. Controls were performed by incubating a Warburg flask without tipping the enzyme in from the first side arm. At the end of the reaction time, both enzyme and acid were tipped in together. The volume of CO_2 thus liberated was subtracted from the CO_2 yield in the test flasks.

There was a linear relationship between the decarboxylation of glyoxylate and 2-oxoglutarate and the amount of enzyme added. Under the conditions used, progress of the decarboxylation was linear with time for about 20min. and thereafter fell off slowly.

Radioactive assay of enzyme activity. The Warburg

Table 2. *Location of decarboxylation activity in the mitochondria of pig liver*

Crude particulate fraction was prepared by mincing 100g. wet wt. of pig liver, and homogenizing in 200ml. of a solution containing 31m-moles of KCl, 0·5m-mole of $MgSO_4$ and 4m-moles of sodium phosphate at pH7·4. The sediment formed by centrifuging at 6000*g* for 10min. was washed twice with 860ml. of the buffer solution and the final sediment resuspended in 500ml. of buffer. Reaction mixtures contained 1·0ml. of liver preparation, and other additions as noted, in a final volume of 1·2ml. Otherwise, assays were carried out as described in the Materials and Methods section. Mitochondria were prepared as described in the Materials and Methods section. They were suspended in 0·25M-sucrose, pH7·4, so that 1·0ml. of suspension contained mitochondria prepared from 3g. of liver. Reaction mixtures contained 0·5ml. of mitochondrial suspension, 0·50ml. of 0·25M-sucrose, pH7·4, and other additions as noted, in a final volume of 1·14ml. Otherwise assays were carried out as described in the Materials and Methods section. Mitochondrial extract (containing 15mg. of protein/ml.) was prepared by passing mitochondria (from 25g. of liver), suspended in 10ml. of 20mM-potassium phosphate, pH7·0, through a Ribi cell fractionator (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) at 25000lb./in.^{2} and 5°. The extract was centrifuged at 28000*g* for 20min., the supernatant removed and the sediment resuspended in 20mM-potassium phosphate, pH7·0 (equal to volume of supernatant). Reaction mixtures contained 1·0ml. of mitochondrial extract, or 1·0ml. of supernatant, or 1·0ml. of resuspended sediment, plus substrate as noted in a final volume of 1·1ml. Otherwise, assays were carried out as described in the Materials and Methods section.

	Additions	CO_2 evolved (μl./hr.)
I. Crude particulate fraction	None	124
	Glyoxylate (10μmoles)	161
	L-Glutamate (10μmoles)	104
	2-Oxoglutarate (10μmoles)	101
	Glyoxylate (10μmoles)+L-glutamate (10μmoles)	242
	Glyoxylate (10μmoles)+2-oxoglutarate (10μmoles)	158
II. Mitochondria	None	9
	Glyoxylate (7μmoles)	19
	L-Glutamate (7μmoles)	5
	2-Oxoglutarate (7μmoles)	22
	Glyoxylate (7μmoles)+L-glutamate (7μmoles)	60
	Glyoxylate (7μmoles)+2-oxoglutarate (7μmoles)	256
III. Mitochondrial extract	Whole extract+glyoxylate (5μmoles) +2-oxoglutarate (5μmoles)	123
	Supernatant+glyoxylate (5μmoles) +2-oxoglutarate (5μmoles)	110
	Sediment+glyoxylate (5μmoles) +2-oxoglutarate (5μmoles)	15

micro-flasks and reaction mixture were the same as those described above for the manometric assay. In addition, evolved CO_2 was collected in 0·05ml. of 5N-NaOH on filter paper in the centre well of the flask. After reaction, the filter papers were removed and placed in 5·0ml. of phosphor solution prepared by dissolving 60g. of naphthalene, 4g. of 2,5-diphenyloxazole, 0·2g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (both obtained from Nuclear Enterprises, Edinburgh), 100ml. of methanol and 20ml. of ethylene glycol (extra-pure; British Drug Houses Ltd.) in 1l. of dioxan. The centre well was washed out twice with 0·2ml. samples of water, and these washings were added to the phosphor. Standards containing labelled glyoxylate or 2-oxoglutarate were prepared and assayed under exactly the same conditions. The scintillation flasks were assayed as a routine 24hr. after preparation. Under these conditions, counting efficiency was about 30%. Scintillation spectrometry was carried out in a Nuclear-Chicago 6801 system.

Table 3. *Decarboxylation of glyoxylate and 2-oxoglutarate by an ammonium sulphate fraction of mitochondrial extract*

Expt. 1. Mitochondria from 200g. of liver prepared and disrupted in a Hughes press as described in the text were extracted with 35ml. of 20mM-potassium phosphate, pH7·0, and the protein was precipitated between 40 and 50% of $(NH_4)_2SO_4$ saturation. The precipitate was dissolved in 5ml. of the same phosphate buffer. Reaction mixtures contained 0·25ml. of enzyme preparation (40mg. of protein/ml.), 20μmoles of potassium phosphate buffer, pH7·0, and additions as noted, to a final volume of 1·0ml. Otherwise, assay was carried out as described in the Materials and Methods section. Expts. 2 and 3. These were conducted with different enzyme preparations, containing 30 and 35mg. of protein/ml. respectively. Otherwise, procedure was identical with that of Expt. 1.

Expt. no.	Additions	CO_2 evolved (μl./hr.)
1	None	4
	Glyoxylate (40μmoles)	2
	2-Oxoglutarate (40μmoles)	29
	Glyoxylate (40μmoles)+2-oxoglutarate (40μmoles)	215
	Glyoxylate (40μmoles)+2-oxoglutarate (40μmoles) (enzyme boiled 5min.)	17
2	Glyoxylate (40μmoles)+2-oxoglutarate (40μmoles)	175
	Glyoxylate (40μmoles)+2-oxoglutarate (40μmoles)+NAD (5μmoles)+NADP (5μmoles)+TPP (1μmole)+CoA (5μmoles)+ATP (5μmoles)+$MgCl_2$ (5μmoles)	181
3	Glyoxylate (40μmoles)+2-oxoglutarate (40μmoles)	197
	Glyoxylate (40μmoles)+L-glutamate (40μmoles)	11

RESULTS

Intracellular location of the enzymes. In Table 2, the synergistic decarboxylation of glyoxylate and 2-oxoglutarate in pig-liver homogenate, mitochondria and disrupted mitochondria is shown. In crude homogenates it is difficult to detect any synergism owing to high blank values, and also L-glutamate stimulates decarboxylation more than 2-oxoglutarate. However, with mitochondria, glyoxylate and 2-oxoglutarate show a strong mutual enhancement of decarboxylation: L-glutamate is far less effective than is 2-oxoglutarate.

Within mitochondria, the enzyme system responsible for the decarboxylation is concentrated in the soluble fraction rather than the particulate.

Partial purification of enzyme from disrupted mitochondria. Most of the enzyme is precipitated from the soluble fraction of disrupted mitochondria within 40–50% of saturation with ammonium sulphate. As a result of this purification step, the ability of L-glutamate to substitute for 2-oxoglutarate is further decreased (Table 3). No evidence for a cofactor requirement was found.

Passage of enzyme through a column of Sephadex G-50 (1·5cm. × 10cm.), equilibrated with sodium phosphate buffer (0·02M, pH7·0 or 8·5) did not appreciably affect its activity. Added TPP was

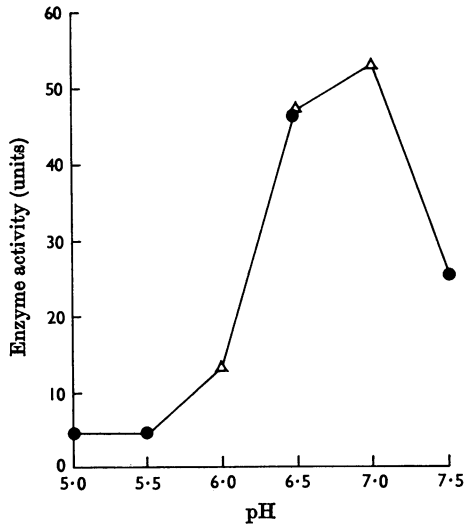


Fig. 2. Effect of pH on carbon dioxide production. Reaction mixtures contained 1·4mg. of protein, prepared by ammonium sulphate precipitation as described in Table 3. Reaction conditions were as described in the Materials and Methods section, except that sodium acetate (50μmoles) was substituted for potassium phosphate where indicated. △, Potassium phosphate; ●, sodium acetate.

without effect on the activity of the enzyme eluted from such columns.

Effect of pH *on enzyme activity.* When the synergistic decarboxylation of glyoxylate and 2-oxoglutarate by the enzyme preparation was determined at different pH values, a peak of activity was seen at pH7·0 (Fig. 2). There was no obvious effect of acetate buffer on the reaction as compared with phosphate. The activity at pH 7·0 was not altered when tris–hydrochloric acid was substituted for potassium phosphate.

Effect of aerobic conditions on the decarboxylation reaction. The substitution of air for nitrogen in the gas space above the incubation mixture decreased the production of carbon dioxide. Thus, in a typical experiment with nitrogen present, 96 μl. of carbon dioxide was evolved in 1 hr., whereas under air 65 μl. of gas was produced. Under air with alkali present in the centre well, a net uptake of 4 μl. of gas was observed. This experiment shows that all the gas produced during the reaction is carbon dioxide.

Specificity of the decarboxylation reaction. The purified enzyme system did not catalyse any significant decarboxylation when glyoxylate was replaced in the standard assay mixture by equimolar amounts of glycollate, glycolaldehyde, malonate or pyruvate. Replacement of glyoxylate by glycine resulted in a 93% fall in activity. Replacement of 2-oxoglutarate by malonate, pyruvate, succinate or glutarate resulted in a complete loss of decarboxylase activity. Substitution of L-glutamate for 2-oxoglutarate gave variable results, but always the activity was decreased by more than 80%. Oxaloacetate was tested as a substitute for 2-oxoglutarate, but the rapid spontaneous decarboxylation of the former prevented any meaningful measurements being made.

Inhibition and stimulation of the decarboxylation reaction. The same substrate analogues, described above, were tested as possible inhibitors of the reaction by assay of glyoxylate and 2-oxoglutarate decarboxylation in their presence. None showed more than slight inhibition when added at the same concentration as substrates (Table 4).

NAD and NADP were without appreciable effect on the decarboxylation of glyoxylate and 2-oxoglutarate and were not reduced when tested spectrophotometrically at 340 mμ. TPP gave a slight stimulation. This compares with an earlier stage in the purification of the enzyme where TPP showed no effect.

The effect of *N*-ethylmaleimide, EDTA, avidin, magnesium chloride, mercaptoethanol and sucrose on the decarboxylation was tested. *N*-Ethylmaleimide and EDTA inhibited the reaction, the inhibition being more effective when they were preincubated with the enzyme. Avidin did not inhibit the reaction; rather it showed an activating effect. Magnesium chloride, mercaptoethanol and sucrose stimulated the reaction. Their cumulative effect, however, was less than additive. It was also noted (as mentioned above) that the presence of these compounds stabilized the enzyme preparation.

Kinetics of the reaction. The relationship between

Table 4. *Effect of potential inhibitors or activators on the decarboxylation reaction*

The routine assay procedure outlined in the Materials and Methods section was used; 40 μmoles each of glyoxylate and 2-oxoglutarate were present in each flask, with additions as noted. Reaction mixtures contained sufficient purified enzyme preparation (specific activity 70–120 units/mg.) to give a net yield of 20–40 μl. of CO_2 in control assays in the absence of any added inhibitors or activators. Where preincubation with enzyme is indicated, the material was added to the enzyme solution in one side arm before the assay was begun. In other cases the activator or inhibitor was added with the substrates to the main compartment of the Warburg flask.

Additions	CO_2 production (% of control assay)
None	100
Glycollate (40 μmoles)	100
Glycolaldehyde (40 μmoles)	93
Glycine (40 μmoles)	98
L-Glutamate (40 μmoles)	95
Pyruvate (40 μmoles)	109
	100
Malonate (40 μmoles)	85
	89
Succinate (40 μmoles)	100
	93
Glutarate (40 μmoles)	87
	85
NAD (4 μmoles)	105
NADP (4 μmoles)	91
TPP (4 μmoles)	116
	127
N-Ethylmaleimide (2 μmoles)	76
	87
N-Ethylmaleimide (10 μmoles)	36
	44
N-Ethylmaleimide (10 μmoles) preincubated with enzyme	18
	18
EDTA (10 μmoles)	56
	44
EDTA (10 μmoles) preincubated with enzyme	0
	0
Avidin (0·6 mg.) preincubated with enzyme	151
	145
Magnesium chloride (5 μmoles)	175
	181
Mercaptoethanol (10 μmoles)	144
Sucrose (0·3 m-mole) preincubated with enzyme for 2 hr. at 2°	220
Sucrose (0·3 m-mole) preincubated with enzyme for 16 hr. at 2°	420

the rate of decarboxylation and the substrate concentration was determined by holding the concentration of one substrate constant at a saturating level (32 mM), and varying the concentration of the other substrate. A plot of the results obtained is shown in Fig. 3. 2-Oxoglutarate at concentrations greater than 16 mM displayed substrate inhibition under these conditions. Glyoxylate consistently gave results that yielded a sigmoidal plot. Replotted in the form of Lineweaver–Burk reciprocal plots, the K_m values under these conditions were: 2-oxoglutarate, 3·6 mM; glyoxylate, 16 mM.

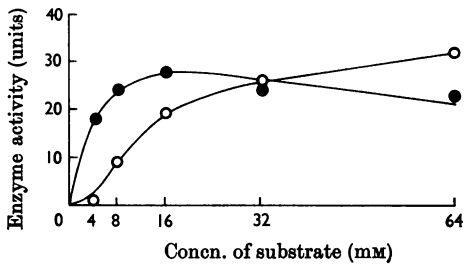


Fig. 3. Effect of substrate concentration on carbon dioxide formation. Reaction mixtures contained 0·33 mg. of protein (specific activity, 72 units/mg.), prepared by the complete purification procedure. Reaction conditions were as described in the Materials and Methods section, except that the amount of one substrate was varied, and the final volume was 1·25 ml. Saturating substrate was present at a final concentration of 32 mM. ●, 2-Oxoglutarate concentration varied; ○, glyoxylate concentration varied.

Decarboxylation of limiting amounts of glyoxylate and 2-oxoglutarate. When the partially purified enzyme was incubated with limiting amounts of either glyoxylate or 2-oxoglutarate until carbon dioxide evolution had virtually stopped, rather variable values for μmoles of carbon dioxide evolved/μmole of substrate added were obtained (Table 5).

These values were taken from six experiments with two different enzyme preparations. On average, slightly more than 1 μmole of carbon dioxide was evolved from either 1 μmole of glyoxylate or 2-oxoglutarate in the presence of 2 μmoles of the other substrate.

Origin of the carbon dioxide formed during reaction. C-1 of both glyoxylate and 2-oxoglutarate

Table 5. *Yield of carbon dioxide from limiting amounts of glyoxylate and 2-oxoglutarate*

The complete system, placed in a micro-Warburg flask, consisted of: in the main compartment, 20 μmoles of sodium phosphate buffer (pH 7·0), sodium glyoxylate and sodium 2-oxoglutarate as indicated (both solutions previously adjusted to pH 7·0); in the first side arm, 0·2 ml. of enzyme extract (40–50%-saturated ammonium sulphate fraction); in the second side arm, 0·1 ml. of trichloroacetic acid (50%, w/v). The total volume was 1·0 ml. The mixture was incubated at 37°, under N_2, for 1–2 hr. until reaction was essentially finished. Acid was then tipped from the second side arm, and the total CO_2 released was read after a further 20 min. In all cases, initially bound CO_2 in the reaction mixtures was determined by tipping in acid at the same time as the enzyme, and this was subtracted from that measured at the end of the experiment. There was negligible reaction if either glyoxylate or 2-oxoglutarate was incubated singly. The results are taken from six separate experiments performed with two different enzyme preparations.

Amount of substrates added (μmoles)		Yield of CO_2 (μmoles)	Mean
Glyoxylate	2-Oxoglutarate		
2	2	1·7	2·3
		1·8	
		3·0	
		2·5	
		2·5	
1	1	0·62	0·62
1	2	2·2	1·4
		0·78	
		1·4	
		1·2	
		1·6	
		1·3	
2	1	1·5	1·1
		0·8	
		0·9	
		1·2	

is recovered in carbon dioxide formed during reaction of both substrates with active enzyme. C-2 of glyoxylate and C-5 of 2-oxoglutarate appear to a negligible extent in the carbon dioxide formed. This is shown by the results presented in Tables 6 and 7. There was no significant decarboxylation of single substrates by active enzyme, nor was there reaction of substrates in the presence of boiled enzyme.

Comparison of carboxyl groups of glyoxylate and 2-oxoglutarate as sources of carbon dioxide. It had been noted in early experiments that when [1-^{14}C]-glyoxylate or 2-oxo[1-^{14}C]glutarate was incubated with active enzyme, and the reaction terminated with sulphuric acid, more $^{14}CO_2$ was recovered from glyoxylate than from 2-oxoglutarate. This observation was explored more fully by carrying out reactions of varying incubation times with enzyme and acid. The results of typical experiments are shown in Table 8. In the first three experiments of Table 8 the time of incubation with enzyme was varied, and acid was not used to stop the reaction. Under these conditions there is initially a greater yield of carbon dioxide from 2-oxoglutarate, but as the reaction proceeds proportionately more carbon dioxide is yielded from glyoxylate. The yield of carbon dioxide from 2-oxoglutarate is proportional to reaction time; the yield from glyoxylate increases disproportionately. Hence the ratio (R) of glyoxylate decarboxylated to 2-oxoglutarate decarboxylated rises with reaction time.

The same experiments were repeated, but in addition the reaction was stopped, and bound carbon dioxide displaced, by treatment with dilute sulphuric acid for 60 min. The effect is greatly to increase the yield of carbon dioxide from glyoxylate, and leave unaffected the yield from 2-oxoglutarate. Again, there is a disproportionate increase in yield of carbon dioxide from glyoxylate, so that R rises with incubation time.

In Table 9 results are shown of experiments in which incubation time with enzyme has been maintained constant while time of subsequent incubation with acid was varied. An increase in R is again seen with increase in the time of incubation with acid, and likewise the increase in R is due to a disproportionate increase in yield of carbon dioxide from glyoxylate. The yield from 2-oxoglutarate remains approximately constant, apparently unaffected by exposure to acid.

Various combinations of incubation conditions have been tried in which glyoxylate and 2-oxoglutarate have together been incubated with active enzyme or boiled enzyme, in the absence of enzyme, and in the presence of sulphuric acid (Table 10). It is clear from these experiments that the evolution

Table 6. *Decarboxylation of* [^{14}C]*glyoxylate in the presence of 2-oxoglutarate*

The main compartment of each micro-Warburg flask contained 50 μmoles of potassium phosphate buffer, pH 7·0, and additions as indicated: the first side arm contained 0·95 mg. of purified enzyme (specific activity 50 units/mg.); the second side arm contained 0·10 ml. of 7 N-H_2SO_4; the centre well contained 0·05 ml. of 5 N-NaOH. The total volume of the incubation mixture was 0·95 ml. Incubation was for 133 min. at 37° under N_2, the acid was then tipped in and incubation continued for 45 min. The centre-well contents were withdrawn and analysed for $Na_2{}^{14}CO_3$ as described in the Materials and Methods section.

Additions	$^{14}CO_2$ evolved (μmoles)
Sodium [1-^{14}C]glyoxylate (20 μmoles, $3·8 \times 10^4$ counts/min.) + sodium 2-oxoglutarate (20 μmoles)	2·60* 2·73*
Sodium [1-^{14}C]glyoxylate (20 μmoles, $3·8 \times 10^4$ counts/min.)	0·054
Sodium [1-^{14}C]glyoxylate (20 μmoles, $3·8 \times 10^4$ counts/min.) + sodium 2-oxoglutarate (20 μmoles), enzyme boiled	0·028
Sodium [2-^{14}C]glyoxylate (20 μmoles, $2·9 \times 10^5$ counts/min.) + sodium 2-oxoglutarate (20 μmoles)	0·003* 0·003*
Sodium [2-^{14}C]glyoxylate (20 μmoles, $2·9 \times 10^5$ counts/min.)	0·003
Sodium [2-^{14}C]glyoxylate (20 μmoles, $2·9 \times 10^5$ counts/min.) + sodium 2-oxoglutarate (20 μmoles), enzyme boiled	0·002

* Results of duplicate assays.

Table 7. *Decarboxylation of 2-oxo*[^{14}C]*glutarate in the presence of glyoxylate*

Experimental conditions were as in Table 6, except that 0·65 mg. of enzyme was used and incubation was for 230 min. Each assay was performed in duplicate.

Additions	$^{14}CO_2$ evolved (μmoles)
Sodium 2-oxo[1-^{14}C]glutarate (10 μmoles, $1·92 \times 10^5$ counts/min.) + sodium glyoxylate (40 μmoles)	1·55 1·55
Sodium 2-oxo[1-^{14}C]glutarate (10 μmoles, $1·92 \times 10^5$ counts/min.)	0·062 0·058
Sodium 2-oxo[1-^{14}C]glutarate (10 μmoles, $1·92 \times 10^5$ counts/min.) + sodium glyoxylate (40 μmoles), enzyme boiled	0·022 0·021
Sodium 2-oxo[5-^{14}C]glutarate (10 μmoles, $2·07 \times 10^5$ counts/min.) + sodium glyoxylate (40 μmoles)	0·002 0·003
Sodium 2-oxo[5-^{14}C]glutarate (10 μmoles, $2·07 \times 10^5$ counts/min.)	0·001 0·002
Sodium 2-oxo[5-^{14}C]glutarate (10 μmoles, $2·07 \times 10^5$ counts/min.) + sodium glyoxylate (40 μmoles), enzyme boiled	0·001 0·001

Table 8. *Effect of time of incubation with enzyme and acid on production of carbon dioxide from* [1-^{14}C]*glyoxylate and* 2-*oxo*[1-^{14}C]*glutarate*

The main compartment of each micro-Warburg vessel contained 50μmoles of potassium phosphate buffer, pH7·0, sodium glyoxylate (20μmoles), sodium 2-oxoglutarate (20μmoles) and 2×10^5 counts/min. of either sodium [1-^{14}C]glyoxylate or sodium 2-oxo[1-^{14}C]glutarate as appropriate; the first side arm contained 0·4mg. of purified enzyme (122units/mg.); the second side arm contained 0·10ml. of 7N-H_2SO_4 in incubations of Expt. 2; the centre well contained 0·05ml. of 5N-NaOH. The total volume of the incubation mixture was 0·95ml. Incubation was at 37° under N_2. The contents of the centre wells were analysed as in Table 2. In Expt. 2 the reaction was terminated by tipping the acid in, followed by incubation for a further 60min. before the centre well contents were analysed.

	Duration of incubation (min.)		CO_2 formed (μmoles)		
Expt.	With enzyme	With acid	From [1-^{14}C]-glyoxylate	From 2-oxo-[1-^{14}C]glutarate	$^{14}CO_2$ from glyoxylate / $^{14}CO_2$ from 2-oxoglutarate
1	30	0	0·066	0·17	0·39
	60	0	0·30	0·33	0·91
	120	0	0·81	0·61	1·3
2	30	60	0·51	0·15	3·4
	60	60	1·5	0·31	4·8
	120	60	3·4	0·60	5·7

Table 9. *Effect of incubation with acid on yield of carbon dioxide from* [1-^{14}C]*glyoxylate and* 2-*oxo*[1-^{14}C]*glutarate*

The main compartment of each Warburg micro-flask contained 50μmoles of potassium phosphate buffer, pH7·0, sodium glyoxylate (20μmoles), sodium 2-oxoglutarate (20μmoles) and $1\cdot6\times10^5$ counts/min. of either sodium [1-^{14}C]glyoxylate or $1\cdot08\times10^5$ counts/min. of sodium 2-oxo[1-^{14}C]glutarate as appropriate; the first side arm contained 0·18mg. of purified enzyme (70units/mg.); the second side arm contained 0·10ml. of 7N-H_2SO_4 (except in the case of zero time of incubation with acid); the centre well contained 0·05ml. of 5N-NaOH. The total volume of the incubation mixture was 0·95ml. Incubation was at 37°, under N_2, for 60min. with enzyme, then with acid as indicated. The contents of the centre wells were analysed as in Table 6.

Duration of incubation with acid (min.)	CO_2 formed (μmole)		
	From [1-^{14}C]-glyoxylate	From 2-oxo-[1-^{14}C]glutarate	$^{14}CO_2$ from glyoxylate / $^{14}CO_2$ from 2-oxoglutarate
0	0·08	0·09	0·89
5	0·20	0·11	1·8
12	0·34	0·11	3·1
28	0·41	0·10	4·1
60	0·48	0·11	4·4
80	0·52	0·11	4·7
111	0·70	0·12	5·8

of excess of carbon dioxide arising from the carboxyl group of glyoxylate, as compared with that from the carboxyl group of 2-oxoglutarate, depends on the incubation of the two substrates together, in the presence of active enzyme, followed by acid treatment. Variation of the ratio of glyoxylate concentration to 2-oxoglutarate concentration from 10 to 0·1 resulted in values of 4·7 and 3·2 for R. This difference in R is probably not significant, since the reaction velocity at low concentrations becomes difficult to measure with accuracy.

It is known that hydrogen peroxide can oxidatively decarboxylate glyoxylate, and hence any hydrogen peroxide that might be formed in the reaction mixture due to oxidase activity might result in release of carbon dioxide from C-1 of glyoxylate. It seemed unlikely that any significant quantities of hydrogen peroxide would be formed during the incubation, since the incubation was performed under nitrogen; nevertheless, the possibility was examined by testing the effect of catalase (from C. F. Boehringer und Soehne, Mannheim, Germany) on the release of carbon dioxide from reaction mixtures. It was found that addition of 0·5mg. of catalase to reaction mixtures (containing

Table 10. *Dependence of enhanced decarboxylation of glyoxylate on the prior incubation with active enzyme*

The main compartment of each micro-Warburg vessel contained 50 μmoles of phosphate buffer, pH 7·0, 20 μmoles of sodium glyoxylate, 20 μmoles of sodium 2-oxoglutarate and $1{\cdot}6 \times 10^5$ counts/min. of sodium [1-^{14}C]glyoxylate or $1{\cdot}08 \times 10^5$ counts/min. of sodium 2-oxo[1-^{14}C]glutarate as indicated; the first side arm contained, where indicated, 0·14 mg. of purified enzyme (4·3 units/mg.) either active or boiled; the second side arm contained 0·10 ml. of 7 N-H_2SO_4. The total volume of the incubation mixture was 0·95 ml. Incubation was at 37° under N_2.

Labelled substrate present	Time of incubation (min.) Active enzyme	Boiled enzyme	Absence of enzyme	Acid	$^{14}CO_2$ evolved (μmole)
[1-^{14}C]Glyoxylate	60	—	—	60	0·52
	—	60	—	60	0·018
	—	—	60	60	0·018
	—	—	—	60	0·009
2-Oxo[1-^{14}C]glutarate	60	—	—	60	0·11
	—	60	—	60	0·015
	—	—	60	60	0·013
	—	—	—	60	0·013

active enzyme) identical with those described in Table 10 did not result in lowering of the value of *R*. This shows that glyoxylate oxidation by hydrogen peroxide cannot be the explanation for the disproportionate evolution of carbon dioxide from C-1 of glyoxylate.

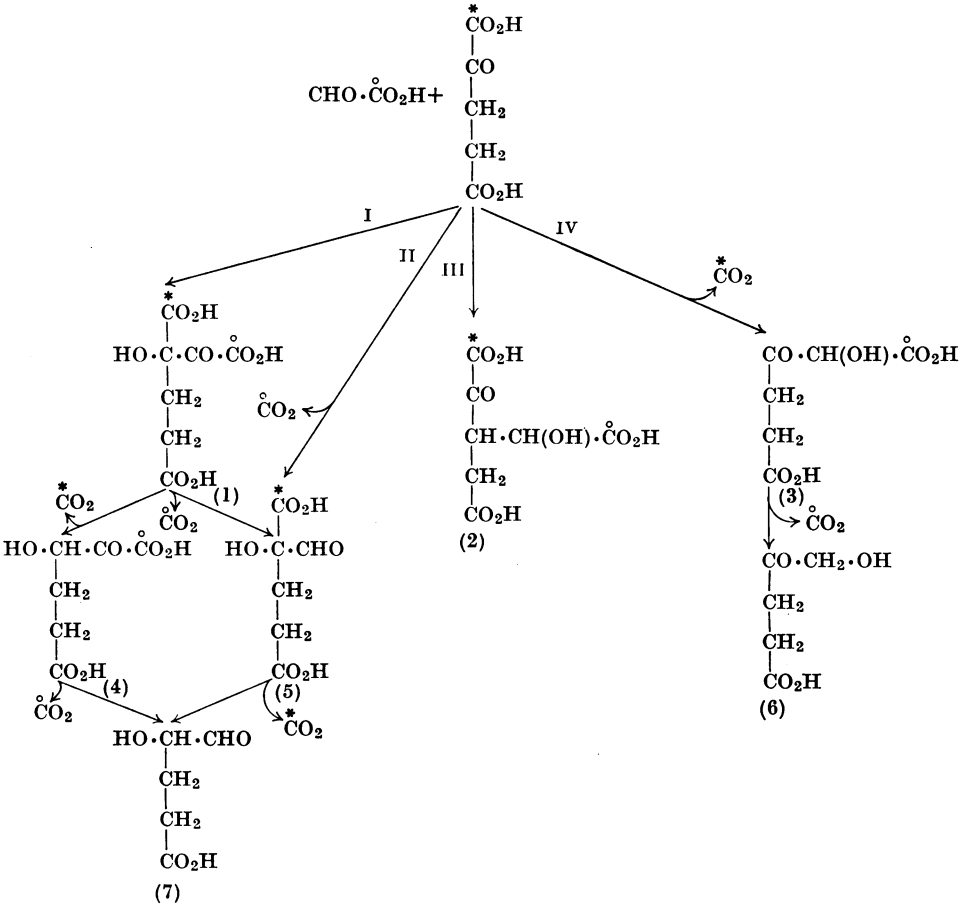
Formation of radioactive products from [^{14}C]*glyoxylate and 2-oxo*[^{14}C]*glutarate.* The enzyme preparation was incubated with labelled substrates under the same conditions as used for the manometric assay of enzyme activity except that the reaction was stopped by addition of 4 vol. of ethanol in place of sulphuric acid. The precipitated protein was removed by centrifuging and samples of the resulting supernatant were analysed by two-dimensional chromatography. No radioactive products were detected from incubation mixtures containing either [1-^{14}C]glyoxylate and 2-oxoglutarate or glyoxylate and 2-oxo[1-^{14}C]glutarate. When [2-^{14}C]glyoxylate and 2-oxo[5-^{14}C]glutarate were used as the respective tracers, analysis of the products showed the presence of two major radioactive products in the general area of the chromatogram characteristic of succinate. Their chromatographic behaviour indicated that the same pair of radioactive compounds was produced from either [2-^{14}C]glyoxylate or 2-oxo[5-^{14}C]-glutarate. These products have not been identified chemically.

DISCUSSION

The present work shows that a system is present in pig-liver mitochondria that catalyses the synergistic decarboxylation of glyoxylate and 2-oxoglutarate. The results are thus in accord with the similar conclusions of Crawhall & Watts (1962) obtained with intact human- and rat-liver mitochondria, and the enzyme system whose purification and properties are reported above may well account for their observations. Since glutamate may be converted into 2-oxoglutarate by transamination with glyoxylate, the low activity of glutamate in the decarboxylating system may well be due to its acting as a precursor of 2-oxoglutarate. The alternative explanation, that it might be acting as a substrate in its own right in a system similar to that reported by Nakada & Sund (1958), cannot be excluded without further experiment, but if such a system is present then it is present in very low activity as compared with the glyoxylate–2-oxoglutarate-decarboxylating system.

The results reported in this paper indicate that the enzyme system isolated from pig-liver mitochondria effects a condensation of glyoxylate and 2-oxoglutarate that also results in, or leads to, decarboxylation of the α-carboxyl groups of both acids. The findings in support of this are: (i) Decarboxylation is dependent on the simultaneous presence of both substrates. (ii) Carbon dioxide evolved from [1-^{14}C]glyoxylate and 2-oxoglutarate, or 2-oxo[1-^{14}C]glutarate and glyoxylate, is radioactive; whereas that from [2-^{14}C]glyoxylate and 2-oxoglutarate, or 2-oxo[5-^{14}C]glutarate and glyoxylate, is virtually non-radioactive. (iii) Non-volatile radioactive reaction products appear after incubation of [2-^{14}C]glyoxylate or 2-oxo[5-^{14}C]-glutarate with non-radioactive co-substrates, whereas no radioactive products could be detected when [1-^{14}C]glyoxylate or 2-oxo[1-^{14}C]glutarate was used as tracer.

Four main possibilities for the condensation between glyoxylate and 2-oxoglutarate may be



Scheme 1. Possible mechanisms for the decarboxylation of glyoxylate and 2-oxoglutarate.

envisaged; these reactions are designated I, II, III or IV in Scheme 1.

Reaction I represents an acyloin condensation leading to a hydroxy-keto tricarboxylic acid (1). Successive decarboxylations of the carboxyl groups β and α to the keto group would lead to hydroxylaevulic acid (7) via the intermediate 3-hydroxy-2-oxoadipate (4). If the decarboxylations were carried out in the reverse order, i.e. the α-carboxyl followed by the β-carboxyl, then the same product (7) would result as before but the intermediate would be different, i.e. compound (5). Since the initial reaction I is an acyloin condensation, it might be expected to involve TPP as a coenzyme (Holzer, 1961) and glyoxyl-TPP, $HO_2C \cdot CH(OH) \cdot TPP$, as an intermediate. However, this intermediate decarboxylates to hydroxymethyl-TPP, $CH_2(OH) \cdot TPP$, and is so unstable that it has not been isolated (Kohlaw, Deus & Holzer, 1965). Acyloin condensation of this with 2-oxoglutarate would then lead by route II directly to compound (5) and thence to compound (7). Route III represents an aldol condensation leading to compound (2). Route IV represents an acyloin condensation between glyoxylate and an intermediate such as active succinic acid semialdehyde, $HO_2C \cdot CH_2 \cdot CH_2 \cdot CH(OH) \cdot TPP$, derived from the reaction between 2-oxoglutarate and TPP. The product (3) being an α-hydroxy-β-oxo acid should readily decarboxylate to compound (6).

The structure of compound (2) does not provide such a ready explanation for the loss of the carboxyl groups derived from the C-1 position of both glyoxylate and 2-oxoglutarate as does any of the routes I, II or IV. For reasons outlined above, reaction II is considered more likely than reaction I. Since the present work shows that the synergistic decarboxylation of glyoxylate and 2-oxoglutarate leads to loss of the α-carboxyl group from the two substrates, or derivatives therefrom, compound (6)

or (7) appears to be the most likely possibility for the main product of the reaction. The reaction is carried out by a purified enzyme system in the absence of added cofactors, under nitrogen, and hence oxidative reactions are unlikely, unless accompanied by compensating reductive reactions.

Studies of the total yields of carbon dioxide arising from incubation of limiting amounts of either glyoxylate or 2-oxoglutarate with excess of the other co-substrate gave rather variable results (Table 5). Formation of compounds (6) or (7) would demand twice the number of moles of carbon dioxide released/mole of limiting substrate. The average values observed fell short of this (Table 5), but the high K_m values for the substrates in this system make completion of the reaction difficult.

If the observed synergistic decarboxylation reaction is represented by formation of either compound (6) or (7) then the ratio of $^{14}CO_2$ evolved from [1-^{14}C]glyoxylate to $^{14}CO_2$ evolved from 2-oxo[1-^{14}C]glutarate should be unity. Incomplete reaction would be characterized by a ratio greater than unity for route II and less than unity for route IV. The results of the experiments designed to test this prediction were, however, complex. The ratio rose from 0·39 to 1·33 during a typical 2 hr. incubation when no acid was used to stop the reaction; these results are thus in reasonable accord with either route IV, or route I followed by breakdown of compound (1) to compound (7) via compound (4). However, when the reaction was terminated with acid a disproportionate release of $^{14}CO_2$ from the [1-^{14}C]glyoxylate took place, raising the values of the ratio beyond 5. This effect of the acid was dependent on the prior incubation of the substrate in the presence of active (i.e. unboiled) enzyme. It is clear that some breakdown of enzymically formed product is occurring in the presence of acid but its nature is not known at present. One possibility, although somewhat unlikely, is that compound (6) or compound (7) (or a keto compound derived from them) might condense non-enzymically by acyloin condensation with a further molecule of glyoxylate to give a β-keto acid which then decarboxylated, leaving another keto group for the same process to be repeated. This would result in the ratio rising in favour of carbon dioxide arising from glyoxylate. However, chemical work is now needed to establish the nature of the reaction products. Such chemical work may well be somewhat complicated by the possibilities of dehydration, isomerization and lactonization.

It has already been noted that our results are in essential accord with the relevant findings of Crawhall & Watts (1962) in their investigation of the decarboxylation of glyoxylate and 2-oxoglutarate by intact rat- or human-liver mitochondria. Franke & Jilge (1961) observed a synergistic decarboxylation of glyoxylate and 2-oxoglutarate by extracts of *Aspergillus niger* in which 1 mole of carbon dioxide was liberated from the condensation of 1 mole of glyoxylate and 1 mole of 2-oxoglutarate. Treatment of the product of the condensation with 2,4-dinitrophenylhydrazine gave a compound that was tentatively identified as the osazone of 4-hydroxy-5-oxovaleric acid. On this basis they suggested that the structure of the product of the condensation reaction was that of compound (4), which decarboxylated during treatment with 2,4-dinitrophenylhydrazine.

Recently Okuyama, Tsuiki & Kikuchi (1965) reported work on 2-oxoglutarate-dependent oxidation of glyoxylate by *Rhodopseudomonas spheroides*. These authors present evidence for: (1) condensation of glyoxylate and 2-oxoglutarate to form 3-hydroxy-2-oxoadipate (compound 4 of Scheme 1); (2) oxidative decarboxylation of 3-hydroxy-2-oxoadipate to form 2-hydroxyglutarate; (3) dehydrogenation of 2-hydroxyglutarate to regenerate 2-oxoglutarate. It is postulated that these reactions may form a 2-oxoglutarate cycle for the total oxidation of glyoxylate to carbon dioxide, and isotopic data are cited in support of this. Okuyama *et al.* (1965) state that they have found (in unpublished work) the same intermediates in rat-liver mitochondria.

We have no evidence for significant occurrence of such a cyclic system in the partially purified enzyme system (40–50%-saturated ammonium sulphate fraction) from pig liver. Decarboxylation of 40 μmoles each of glyoxylate and 2-oxoglutarate, under nitrogen, evolved 7·8 μmoles of carbon dioxide; addition of cofactors, which included 5 μmoles of NAD, only increased this carbon dioxide evolution to 8 μmoles. It may also be noted that Crawhall & Watts (1962) found no evidence for extensive mitochondrial conversion of [2-^{14}C]glyoxylate into $^{14}CO_2$.

After submission of this manuscript the work of Koch & Stokstad (1966) became available to us. These authors describe the purification of an enzyme from rat-liver mitochondria that catalyses a condensation between 2-oxoglutarate and glyoxylate with elimination of carbon dioxide (i.e. reaction IV in Scheme 1 of this paper). They offer evidence that the product of the condensation is 2-hydroxy-3-oxoadipate (compound 3), and that this compound spontaneously decarboxylates in acid solution to give compound (6) (Scheme 1). Many features of the work described by Koch & Stokstad (1966) correlate with that described by us. The enzyme purification has in both cases resulted in similar yields of an enzyme of similar specific activity. In both cases the presence of TPP in the incubation mixture causes some stimula-

tion of activity. Our inability to find a labelled product from [1-^{14}C]glyoxylate could be ascribed to spontaneous decarboxylation of compound (3) to compound (6) taking place in the acidic solvent systems that were used for the chromatography. It may be noted that, in contrast, Koch & Stokstad (1966) trapped the intermediate compound as a phenylhydrazone before chromatography. The results of their work do not, apparently, help to explain the enhanced evolution of carbon dioxide from the carboxyl group of glyoxylate, as compared with that from α-oxoglutarate, which we observed under our experimental conditions.

We thank Miss M. C. Davidson for technical assistance and the Medical Research Council for financial support.

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