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INHIBITION OF THE KREBS CYCLE BY GLYOXYLIC ACID

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Abstract—Evidence is presented that glyoxylic acid inhibits the Krebs cycle activity of pea mitochondria. The extent and nature of the inhibition is compared with the inhibition produced by 4-hydroxy-2-oxoglutarate. It is argued that inhibition by glyoxylate is direct and does not require prior condensation with oxaloacetate or pyruvate. The loci of inhibition by glyoxylate have been identified as pyruvic oxidase and tentatively as α-oxoglutaric oxidase and succinic oxidase. The physiological significance of these results is briefly discussed.

INTRODUCTION

ZELITCH and Barber[1] have reported that the oxidation of certain Krebs cycle acids by leaf mitochondria was inhibited by glycollate and glyoxylate. They suggested that glyoxylate and oxaloacetate may condense to produce an inhibitor of citrate oxidation.

Glyoxylic acid undergoes a non-enzymic reaction with oxaloacetate to produce oxalomalate which subsequently decarboxylates to give 4-hydroxy-2-oxoglutarate.[2,3] The latter compound is also formed by the enzyme-catalysed-condensation of pyruvate and glyoxylate.[2,4,5] The possible role of oxalomalate and 4-hydroxy-2-oxoglutarate as inhibitors of the Krebs cycle has been investigated in animal tissues by Ruffo and co-workers[2,6,7] and in plants by Laties and co-workers.[3,8] Three sites of inhibition by oxalomalate and hydroxyoxoglutarate have been suggested: aconitase, isocitric dehydrogenase and α-ketoglutaric dehydrogenase.

Earlier work of Kleinzeller[9] suggested that the inhibition of respiration in animal tissues produced by glyoxylate could be due to the blocking of pyruvate oxidation. More recently Ruffo[10] has suggested that glyoxylate may directly inhibit a number of Krebs cycle enzymes. Davies and Corbett[11] have shown that glyoxylate is a substrate of pyruvic decarboxylase but the formaldehyde formed in the decarboxylation does not readily leave the enzyme surface and consequently glyoxylate is a potent inhibitor of pyruvic decarboxylase. If glyoxylate is an inhibitor of pyruvic oxidase, it could inhibit the Krebs cycle without prior condensation with pyruvate or oxaloacetate. Such inhibition is of particular importance in plant biochemistry, because it could conceivably explain the inhibitory effect of light on the respiration of green leaves.

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[2] A. RUFFO, E. TESTA, A. ADINOLFI and G. PELIZZA, *Biochem. J.* **85**, 588 (1962).
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[8] G. G. LATIES, *Phytochem.* **6**, 181 (1967).
[9] A. KLEINZELLER, *Biochem. J.* **37**, 674 (1943).
[10] A. RUFFO, *Bull. Soc. Chim. Biol.* **49**, 461 (1967).
[11] D. D. DAVIES and J. R. CORBETT, submitted for publication (1968).

In this paper we present evidence that glyoxylate inhibits the Krebs cycle in pea mitochondria. The loci of inhibition have been identified as pyruvic oxidase and probably α-oxoglutaric oxidase and succinic oxidase. The extent and nature of the inhibition is compared with the inhibition produced by 4-hydroxy-2-oxoglutarate.

RESULTS

(1) *Effect of Glyoxylate and 4-hydroxy-2-Oxoglutarate on the Decarboxylation of Pyruvate-1-^{14}C*

Experiments with purified pyruvic decarboxylase from wheat germ have shown that glyoxylate behaves as a competitive inhibitor of pyruvic decarboxylation.[11] A crude preparation of pyruvic oxidase was obtained from pea mitochondria and shown to decarboxylate pyruvate-1-^{14}C. The results presented in Table 1 show that glyoxylate is a strong inhibitor and 4-hydroxy-oxoglutarate a relatively weak inhibitor of pyruvic decarboxylation. Using intact pea mitochondria to catalyse the complete reactions of the Krebs cycle,[12] glyoxylate and to a lesser extent hydroxyoxoglutarate inhibit the decarboxylation of pyruvate-1-^{14}C in the presence and absence of malate (Table 2).

TABLE 1. EFFECT OF GLYOXYLATE AND 4-HYDROXY-2-OXOGLUTARATE ON THE DECARBOXYLATION OF PYRUVATE-1-^{14}C BY PYRUVIC OXIDASE

Conditions	$^{14}CO_2$ cps	% Inhibition
Pyruvate-1-^{14}C	370	0
Pyruvate-1-^{14}C + glyoxylate (1 μmole)	60	84
Pyruvate-1-^{14}C + glyoxylate (0·1 μmole)	65	83
Pyruvate-1-^{14}C + glyoxylate (0·01 μmole)	90	75
Pyruvate-1-^{14}C + hydroxyoxoglutarate (1 μmole)	280	24
Pyruvate-1-^{14}C + hydroxyoxoglutarate (0·1 μmole)	320	14

Flasks contained: potassium phosphate buffer (pH 7·4, 100 μmoles) thiamine pyrophosphate (1 μmole), $MgCl_2$ (1 μmole) catalase (0·5 mg), sodium pyruvate-1-^{14}C (0·02 μmole, 3200 cps), enzyme (0·1 ml) with additions as indicated above, in a total volume of 2 ml. Temp. 30°. Incubation 1 hr.

TABLE 2. EFFECT OF GLYOXYLATE AND 4-HYDROXY-2-OXOGLUTARATE ON THE DECARBOXYLATION OF PYRUVATE-1-^{14}C BY PEA MITOCHONDRIA

Conditions	$^{14}CO_2$ cps	% Inhibition
Pyruvate-1-^{14}C	1870	0
Pyruvate-1-^{14}C + glyoxylate (1 μmole)	420	80
Pyruvate-1-^{14}C + hydroxyoxoglutarate (1 μmole)	960	49
Pyruvate-1-^{14}C + malate (1 μmole)	2120	0
Pyruvate-1-^{14}C + malate + glyoxylate (1 μmole)	670	68
Pyruvate-1-^{14}C + malate + hydroxyoxoglutarate (1 μmole)	1650	22

Flasks contained: mitochondrial suspension (1 ml), potassium phosphate buffer (pH 7·4, 100 μmoles), sucrose (500 μmoles), ATP (10 μmoles), $MgCl_2$ (5 μmoles), catalase (0·5 mg), sodium pyruvate-1-^{14}C (0·1 μmole, 3200 cps) with additions as above in a total volume of 2 ml. Temp. 30° Incubation 1 hr.

[12] D. D. DAVIES, *J. Exptl Botany* **4**, 173 (1953).

(2) *Effect of Glyoxylate and 4-Hydroxy-2-Oxoglutarate on the Release of ^{14}CO from Pyruvate-2-^{14}C*

Glyoxylate and hydroxyoxoglutarate were found to inhibit the decarboxylation of pyruvate-2-^{14}C by pea mitochondria and the results are presented in Table 3. The distribution of radioactivity among the organic acids was determined and the results are presented in Table 4. These results suggest that glyoxylate strongly inhibits the decarboxylation of pyruvate, The slight accumulation of radioactivity in α-oxoglutarate and succinate suggests the possibility that glyoxylate inhibits α-oxoglutaric oxidase and succinic oxidase. Hydroxyoxoglutarate produces a large accumulation of citrate suggesting an inhibition of isocitric dehydrogenase or aconitase and in addition the slight accumulation of radioactivity in α-oxoglutarate and succinate suggests that the respective oxidase enzymes are inhibited.

TABLE 3. FORMATION OF $^{14}CO_2$ FROM PYRUVATE-2-^{14}C BY PEA MITOCHONDRIA IN THE PRESENCE AND ABSENCE OF GLYOXYLATE, 4-HYDROXY-2-OXOGLUTARATE AND MALATE

Conditions	$^{14}CO_2$ cps	% Inhibition
Pyruvic acid-2-^{14}C	1400	—
Pyruvic acid-2-^{14}C + glyoxylate (1 μmole)	57	96
Pyruvic acid-2-^{14}C + hydroxyoxoglutarate (1 μmole)	90	93
Pyruvic acid-2-^{14}C + malate (1 μmole)	2800	—
Pyruvic acid-2-^{14}C + malate + glyoxylate (1 μmole)	165	94
Pyruvic acid-2-^{14}C + malate + hydroxyoxoglutarate (1 μmole)	1100	61

Conditions as in Table 2 except pyruvate-2-^{14}C (5500 cps, 0·1 μmole).

(3) *The Effect of Glyoxylate and 4-Hydroxy-2-Oxoglutarate on the Decarboxylation of Citric Acid-1,5-^{14}C*

If the decarboxylation of citric acid-1,5-^{14}C, proceeds via the Krebs cycle, the release of more than 50 per cent of the radioactivity requires more than one turn of the cycle. Accordingly the release of $^{14}CO_2$ from citric acid-1,5-^{14}C was studied in the presence and absence of pyruvate. The results presented in Table 5 show that glyoxylate has a slight inhibitory effect and the locus of inhibition is α-oxoglutarate oxidase. The inhibition produced by hydroxyoxoglutarate is consistent with the strong inhibition of aconitase and/or isocitric dehydrogenase.

(4) *Effect of Glyoxylate, Pyruvate and 4-Hydroxy-2-Oxoglutarate on the Decarboxylation of α-Oxoglutarate*

Assuming that α-oxoglutarate is oxidased via the Krebs cycle the production of $^{14}CO_2$ from α-oxoglutarate-5-^{14}C by mitochondria will require the presence of pyruvate or acetyl CoA. Accordingly the effect of glyoxylate and hydroxyoxoglutarate on the decarboxylation of α-oxoglutarate-5-^{14}C was determined in the presence and absence of pyruvate (Table 6). The effect of glyoxylate on the production of $^{14}CO_2$ from α-oxoglutarate-1-^{14}C was examined and the results are also presented in Table 6.

The results with α-oxoglutarate-5-^{14}C as substrate are consistent with inhibition by glyoxylate of pyruvic oxidase and a α-oxoglutarate oxidase. However, the stimulation in the release of $^{14}CO_2$ from α-oxoglutarate-1-^{14}C produced by glyoxylate is at first sight

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN KREBS CYCLE ACIDS FOLLOWING THE INCUBATION OF PYRUVATE-2-^{14}C WITH PEA MITOCHONDRIA IN THE PRESENCE AND ABSENCE OF MALATE, GLYOXYLATE AND 4-HYDROXY-2-OXOGLUTARATE

Conditions	Citrate		α-Oxo-glutarate		Succinate		Fumarate		Melate		Pyruvate		Unknown	
	A	B%	A	B%	A	B%	A	B%	A	B%	A	B%	A	B%
Pyruvate-2-^{14}C	72	5	132	10	27	2	119	8	913	63	165	11	16	1
Pyruvate-2-^{14}C + glyoxylate (1 μmole)	120	3	265	7	107	3	61	2	248	6	3000	78	32	1
Pyruvate-2-^{14}C + hydroxyoxoglutarate (1 μmole)	2380	65	405	11	65	2	32	1	264	7	455	13	29	1
Pyruvate-2-^{14}C + malate (1 μmole)	80	9	0	0	57	7	57	7	435	51	150	18	66	8
Pyruvate-2-^{14}C + malate + glyoxylate (1 μmole)	106	2	185	3	155	3	148	3	290	5	4400	83	30	1
Pyruvate-2-^{14}C + malate + hydroxyoxoglutarate (1 μmole)	1280	39	420	13	210	6	91	3	730	22	480	15	54	2

Conditions as in Table 2, except pyruvate-2-^{14}C 15,500 c. Radioactivity in a particular acid is given under column A as counts per second and under column B as a percentage of the total radioactivity found in the organic acids extracted by ether and keto acids measured by isolating their 2,4-dinitrophenylhydrazones.

TABLE 5. EFFECT OF PYRUVATE, GLYOXYLATE AND 4-HYDROXY-2-OXOGLUTARATE ON THE DECARBOXYLATION OF CITRIC ACID-1,5-^{14}C BY PEA MITOCHONDRIA

Conditions	$^{14}CO_2$ (cps)	Citrate		α-Oxo-glutarate		Succinate		Fumarate		Malate	
		A	B%	A	B%	A	B%	A	B%	A	B%
Citric acid-1,5-^{14}C	891	82	14	97	19	49	9	25	5	275	53
Citric acid-1,5-^{14}C + pyruvate (1 μmole)	1060	30	6	145	31	26	6	34	7	235	50
Citric acid-1,5-^{14}C + glyoxylate (1 μmole)	960	51	7	220	28	70	9	70	0	365	47
Citric acid-1,5-^{14}C + hydroxyoxoglutarate (1 μmole)	514	550	53	140	13	61	6	27	2	270	26

Conditions as in Table 2 except citrate-1,5-^{14}C (0·1 μmole; 2000 cps). Radioactivity in a particular acid is given under column A as counts per second and under column B as a percentage of the total radioactivity found in the organic acids extracted by ether.

inconsistent with inhibition of α-oxoglutaric oxidase. The synergistic decarboxylation of glyoxylate and oxoglutarate by an enzyme system from pig-liver mitochondria has been reported[13] and a similar synergistic reaction has been observed with purified pyruvic decarboxylase from wheat germ.[14]

TABLE 6. EFFECT OF GLYOXYLATE AND 4-HYDROXY-2-OXOGLUTARATE ON THE DECARBOXYLATION OF α-OXOGLUTARATE-5-^{14}C AND α-OXOGLUTARATE-1-^{14}C

Conditions	$^{14}CO_2$	% Inhibition or stimulation
α-Oxoglutarate-5-^{14}C	2330	—
α-Oxoglutarate-5-^{14}C + glyoxylate (1 μmole)	635	73
α-Oxoglytarate-5-^{14}C + hydroxyoxoglutarate (1 μmole)	1640	30
α-Oxoglutarate-5-^{14}C + pyruvate (1 μmole)	4730	—
α-Oxoglutarate-5-^{14}C + pyruvate + glyoxylate (1 μmole)	2870	39
α-Oxoglutarate-5-^{14}C + pyruvate + hydroxyoxoglutarate (1 μmole)	1940	59
α-Oxoglutarate-1-^{14}C	32	—
α-Oxoglutarate-1-^{14}C + glyoxylate (10 μmoles)	80	+150
α-Oxoglutarate-1-^{14}C + glyoxylate (1 μmole)	96	+200
α-Oxoglutarate-1-^{14}C + gluoxylate (0·1 μmole)	108	+238

Conditions as in Table 2 except α-oxoglutarate-5-^{14}C (6000 cps) and oxoglutarate-1-^{14}C (400 cps).

(5) *Effect of Glyoxylate and 4-Hydroxy-2-Oxoglutarate on the Release of $^{14}CO_2$ from Fumarate-2,3-^{14}C*

The release of $^{14}CO_2$ from fumarate-2,3-^{14}C was measured in the presence of pyruvate. The results presented in Table 7 are consistent with hydroxyoxoglutarate having its main inhibitory effect on the utilization of citrate. The inhibition produced by glyoxylate appears to be exerted at, at least two points—pyruvic oxidase and succinic oxidase.

DISCUSSION

It has been proposed by Ruffo *et al.*[7] that the ability of glyoxylate to control reaction rates in the Krebs cycle must be in some degree due to its condensation with oxaloacetate and pyruvate to form enzyme inhibitors. Whilst accepting this as a general proposition we note that the incubation of mitochondria with glyoxylate and radioactive Krebs cycle acids did not lead to the production of labelled hydroxyoxoglutarate. Alternatively it could be argued that the inhibition produced by hydroxyoxoglutarate is due to its breakdown to glyoxylate. However, the two inhibitors produce quite different labelling patterns when added to mitochondria catalysing the reactions of the Krebs cycle with labelled substrates. The inhibitions observed during the present investigation are illustrated in the diagram on p. 1107.

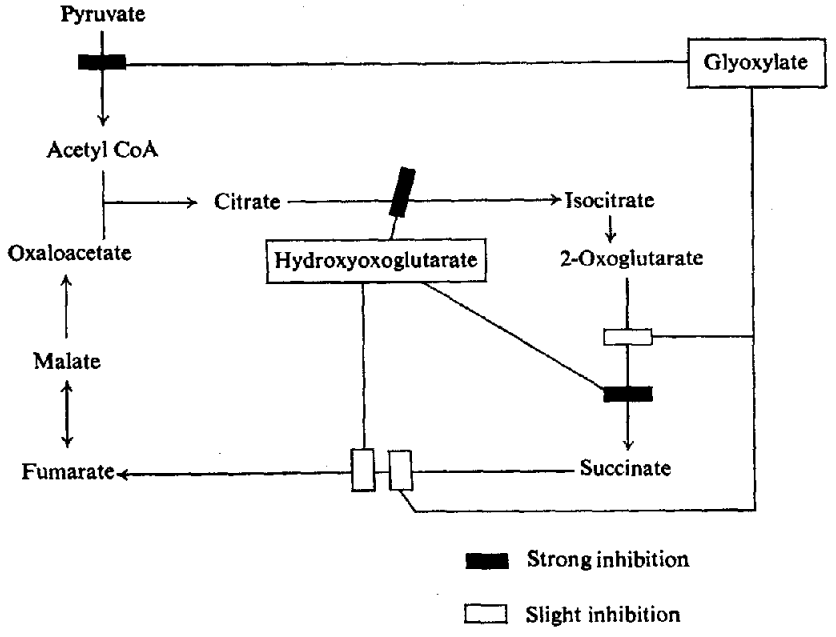
When pyruvate is the source of acetyl CoA for the Krebs cycle it appears likely that glyoxylate will be a more effective inhibitor than hydroxyoxoglutarate. However, when compounds other than pyruvate are the source of acetyl CoA, it appears likely that hydroxyoxoglutarate will be a more effective inhibitor than glyoxylate.

[13] P. R. STEWARD and J. R. QUAYLE, *Biochem. J.* **102**, 885 (1967).
[14] D. D. DAVIES and P. KENWORTHY, in preparation (1968).

TABLE 7. EFFECT OF GLYOXYLATE AND 4-HYDROXY-2-OXOGLUTARATE ON THE DECARBOXYLATION OF FUMARIC ACID-2,3-^{14}C BY PEA MITOCHONDRIA

Conditions	$^{14}CO_2$ (cps)	Citrate		Oxo-glutarate		Succinate		Fumarate		Malate		Oxalo-acetate		Pyruvate	
		A	B%	A	B%	A	B%	A	B%	A	B%	A	B%	A	B%
Fumarate-2,3-^{14}C + pyruvate (1 μmole)	746	140	42	22	6	22	6	29	8	86	26	9	3	32	9
Fumarate-2,3-^{14}C + pyruvate (1 μmole) + glyoxylate (1 μmole)	470	180	27	54	8	78	12	44	7	200	30	15	2	98	14
Fumarate-2,3-^{14}C + pyruvate (1 μmole) + hydroxyoxoglutarate (1 μmole)	187	870	75	38	3	36	3	44	4	101	9	traces		74	6

Conditions as in Table 2 except fumaric acid-2,3-^{14}C (3000 cps). Radioactivity in a particular acid is given under column A as counts per second and under column B as a percentage of the total radioactivity found in the organic acids extracted by ether and keto acids measured by isolating their 2,4-dinitrophenylhydrazones.



It is possible, that whilst the glyoxylate cycle is functioning, glyoxylate blocks the entry of pyruvate into the Krebs cycle and so directs metabolism in the direction of gluconeogenesis. A similar argument has been proposed in relation to the inhibition of pyruvic oxidase by palmitic acid.[15]

The physiological significance of these inhibitions is not clear. It is possible that the formation of glyoxylate during photosynthesis may block pyruvic oxidase and so produce light inhibition of respiration. On the other hand, the synthesis of acetyl CoA in chloroplasts has been suggested[16] to occur by the following reactions:

$$CO_2 \rightarrow \text{glycollate} \rightarrow \text{glyoxylate} \rightarrow \text{glycine} \rightarrow \text{serine} \rightarrow \text{pyruvate} \rightarrow \text{acetyl CoA}$$

In terms of the inhibitions reported here we would expect glyoxylate to be an inhibitor of acetyl CoA formation rather than a precursor.

The apparent inhibition of α-oxoglutaric oxidase by pyruvate and glyoxylate deserves comment. Analysis of this effect is complicated by the fact that pyruvate and glyoxylate stimulate the decarboxylation of α-oxoglutarate. This problem is currently under investigation and it appears probable that the mechanism is similar to the stimulation of glyoxylate decarboxylation by pyruvate.[11]

EXPERIMENTAL

Isolation of Mitochondria

Pea seeds (variety Alaska) were grown on vermiculite in the dark at 25°. Epicotyls were harvested when 10–15 cm long. Epicotyls (150 g) were ground in a mortar with a solution (300 ml) containing potassium phosphate buffer (pH 7·4, 0·1 M), sucrose (0·5 M) and EDTA (2 mM). The homogenate was strained through muslin and centrifuged for 10 min at 100 × *g*. The supernatant was centrifuged for 20 min at 20,000 × *g* and

[15] G. Jagow, B. Westermann and O. Wieland, *European J. Biochem.* 3, 512 (1968).
[16] S. P. J. Shah, L. J. Rogers and T. W. Goodwin, *Biochem. J.* **105**, 13P (1967).

the pellet collected. The pellet was washed twice by resuspending in the above solution and centrifuging for 20 min at 20,000 × *g*. The final pellet was suspended in 1 ml of a solution containing potassium phosphate buffer (pH 7·4, 20 mM), sucrose (0·5 M) and EDTA (2 mM). During the preparation, the temperature was maintained close to 0°.

Preparation of Pyruvic Oxidase

Pyruvic oxidase was solubilized by extracting the mitochondrial suspension in 1 % sodium cholate for 1 hr, and then centrifuging for 20 min at 100,000 × *g*. The supernatant was collected and the pyruvic oxidase concentrated by bringing the solution to 70% saturation with $(NH_4)_2SO_4$. The precipitate was collected by centrifuging 10 min at 20,000 × *g* and dissolved in potassium phosphate buffer (pH 7·4, 0·1 M).

Measurement of $^{14}CO_2$

Incubations were carried out in Warburg flasks containing 0·2 ml of KOH (20% w/v) in the centre well. At the end of the incubation 0·5 ml of a saturated solution of $KHSO_4$ was tipped from the side arm and the flask left to shake for 30 min to release $^{14}CO_2$ from solution. The KOH was carefully transferred by a Pasteur pipette to a 10 ml conical centrifuge tube and the centre well was washed out three times with water and the washing added to the tube. Barium acetate (0·2 ml 10% w/v) was added to the alkali followed by 5 ml of ethanol (60% v/v). The precipitate of barium carbonate-^{14}C was centrifuged down, washed by resuspension in more 60% ethanol and spun down again. The barium carbonate was plated out on an aluminium planchet and dried under an i.r. lamp. Counting was done on a Geiger counter. All figures have been corrected for background and coincidence.

Separation and Estimation of Organic Acids

Anhydrous Na_2SO_4 was added to the acidified incubation mixture to produce a paste which was then transferred to a cellulose thimble and extracted with ether in a Soxhlet for 25 hr. The ether extract was concentrated and the organic acids separated by paper chromatography in the solvents butanol–formic acid–water (60:12:90) and ethanol–ammonia–water (80:5:15). Radioactive spots were located by autoradiography and radioactivity measured by plating on a planchet of a Geiger counter. The 2,4-dinitrophenylhydrazones of the oxo-acids were prepared from a sample of the acidified reaction mixture. After standing overnight, the 2,4-dinitrophenylhydrazones were extracted with ether and separated by paper chromatography in tert.-amyl alcohol in rule H_2O (5:1) using paper previously washed in phosphate buffer (pH 6·5, 0·5 M). Radioactivity was determined as described for organic acids.

Preparations

α-Oxoglutarate-U-^{14}C was prepared according to the method of Haslam and Krebs.[17] 4-Hydroxy-2-oxoglutarate was prepared according to Laties[8] except that ethanol–ammonia–water (80:5:15) was used for chromatography instead of the acid solvent used by Laties. We have previously reported [18] that prolonged contact with an acid solvent leads to the formation of malate from hydroxyoxoglutarate.

[17] R. J. HASLAM and H. A. KREBS, *Biochem. J.* **86**, 432 (1963).
[18] G. RIBEREAU-GAYON and D. D. DAVIES, *C.R. Acad. Sci. Paris* **265**, 711 (1967).