Inhibition of Citrate Oxidation by Glyoxylate in Rat-Liver Homogenates

By F. D'ABRAMO, MARTA ROMANO and A. RUFFO
Laboratory of Biochemistry, Institute of Tumours, Pascale Foundation, Naples

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Glyoxylic acid is known to inhibit the oxygen uptake of various respiring-tissue suspensions. The cause of this inhibition was attributed by Kleinzeller (1943) to a specific inhibition of the decarboxylative reactions, as it appeared in his experiments that the inhibition of decarboxylation of pyruvate by macerated yeast juice, and other tissue suspensions, increased with increasing concentration of glyoxylate. Since in our Laboratory, in the course of a study on the synthesis of glycine from glyoxylic acid (D'Abramo & Tomasos, 1955, 1956), it was found that the addition of different concentrations of glyoxylate to tissue suspensions produced varying inhibition of oxygen uptake, we investigated the mechanism of the inhibition produced by glyoxylate by studying its effect on the oxidation of components of the tricarboxylic acid cycle.

For this purpose, small amounts of glyoxylate were added to rat-liver homogenates incubated in the presence of citrate, *cis*-aconitate, *iso*citrate, α-oxoglutarate, succinate, fumarate, oxaloacetate, malate or pyruvate. The oxygen uptake was followed manometrically and citrate was determined at the end of the incubation period. The results showed that whereas 0·001 M-glyoxylate did not affect the endogenous respiration, it produced a small inhibition of the rate of oxygen uptake in the presence of almost all the citric acid cycle intermediates tested, and caused a small parallel increase of the citrate content of the tissue suspension. Both the inhibition of the oxidation, and the accumulation of citrate, reached very high values only when oxaloacetate was the substrate.

EXPERIMENTAL

Substrates. The following substrates were tested as acids neutralized to pH 7 at the start of the experiment: citric (Merck, Darmstadt, Germany); DL-*iso*citric (Sigma Chemical Co., St Louis, Mo., U.S.A.); α-oxoglutaric (General Biochemical Inc., Ohio, U.S.A.); succinic, pyruvic and fumaric (Eastman Kodak Organic Chemicals, Rochester, U.S.A.); malic (Bayer, Leverkusen, Germany); oxaloacetic, *cis*-aconitic and glyoxylic (Light and Co. Ltd. Colnbrook, Bucks). The purity of glyoxylate was tested by the chromatographic analysis according to Cavallini & Frontali (1954), which showed the two characteristic spots of glyoxylate. No oxalate was contained in our preparation, as shown by the absence of turbidity after the addition of a few drops of a saturated $CaCl_2$ solution.

Homogenates and incubation mixtures. Rat-liver homogenates were prepared by homogenizing (in an ice-cold glass Potter homogenizer) small pieces of liver with a phosphate–saline solution made from 27 ml. of the Krebs's phosphate–saline (Krebs, 1950), free from Ca^{2+} ions; 3 ml. of 0·1 M-phosphate buffer, pH 7·4, and 1 ml. of 0·1 M-$MgCl_2$. A volume (3 ml.) of this suspension, containing approx. 0·4 g. of homogenized tissue, was pipetted into Warburg vessels (about 20 ml. capacity) at 0°, containing 0·01 M-NaF and different concentrations (generally 0·002 M, corresponding to 8 μmoles/flask) of substrate, in the presence or absence of 0·001 M-glyoxylic acid (4 μmoles) neutralized immediately before use. The final volume of the incubation mixture was always 4 ml., giving a dilution of the homogenate of 1:10. Fluoride was added to the reaction mixture because preliminary experiments showed that the rate of citrate accumulation increased in the presence of this substance. The gas phase was O_2 and the time of incubation was 60 min. at 38°, unless otherwise indicated. The enzymic activity was stopped by adding either 0·5 ml. of 30% (w/v) trichloroacetic acid, or 2 ml. of 5% (w/v) tungstic acid, prepared immediately before use. The oxygen uptake was measured manometrically in the Warburg apparatus, CO_2 being absorbed on filter paper moistened with NaOH and placed in the centre well. Citrate was determined (*a*) after trichloroacetic acid deproteinization by the method of Pucher & Shermann (1936) as modified by Ruffo & D'Abramo (1953), or (*b*) after tungstate deproteinization by the method of Ettinger, Goldbaum & Smith (1952).

RESULTS

Effect of glyoxylate on the oxidation of the citric acid cycle intermediates

The effect of small concentrations (0·001 M) of glyoxylate added to many substrates of the citric acid cycle is reported in Table 1. Whereas addition of the low concentration of glyoxylate to the tissue suspension had little effect on the oxygen uptake (Table 1, Expt. 1), there was a small but constant inhibition of the oxygen uptake with the various substrates of the tricarboxylic acid cycle (Table 1, Expts, 2–5). At the same time a small increase in production of citrate was found in all the vessels at the end of the incubation period. Both these effects increased when oxaloacetate was the substrate (Table 1, Expt. 4). In this case it appeared that almost all the oxaloacetate added was recovered as citrate at the end of the incubation.

Effect of oxaloacetate and glyoxylate concentration on citrate accumulation

Since the greatest effect was observed when oxaloacetate was the substrate, an attempt was made in the following experiments to explain the mode of action of glyoxylate. The first point investigated was the effect of the addition of a small constant amount of glyoxylate together with increasing concentrations of oxaloacetate (Table 2).

The results of Expt. 1 (Table 2) demonstrated that the percentage inhibition of oxygen uptake was approximately constant, whereas the amount of extra citrate found at the end of the incubation

Table 1. *Effect of glyoxylate on oxygen uptake and citrate accumulation in rat-liver homogenates*

Rat-liver homogenate, 1:10; glyoxylate, 0·001 M; other substrates, 0·002 M; NaF, 0·01 M; pH 7·4; incubation, 60 min.; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Expt. no.	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate Found (μmoles)	Citrate Increase (μmoles)
1	None	18·7	—	0	—
	Glyoxylate	19·7	—	0	—
	Citrate	28·1	—	0·92	—
	Citrate + glyoxylate	26·2	6·7	1·56	0·64
2	*cis*-Aconitate	31·3	—	1·20	—
	cis-Aconitate + glyoxylate	30·6	2·2	1·70	0·50
	DL-*iso*Citrate	14·7	—	0·80	—
	DL-*iso*Citrate + glyoxylate	13·1	11·0	1·60	0·80
3	α-Oxoglutarate	28·0	—	1·12	—
	α-Oxoglutarate + glyoxylate	26·2	6·4	1·50	0·38
	Succinate	27·5	—	1·26	—
	Succinate + glyoxylate	25·1	8·7	3·00	1·74
4	Oxaloacetate	24·2	—	0·90	—
	Oxaloacetate + glyoxylate	13·5	44·0	10·20	9·30
	Pyruvate	28·2	—	1·90	—
	Pyruvate + glyoxylate	25·1	11·0	2·90	1·00
5	Fumarate	30·1	—	1·95	—
	Fumarate + glyoxylate	28·0	7·0	3·10	1·15
	Malate	26·4	—	2·44	—
	Malate + glyoxylate	24·0	8·6	3·04	0·60

Table 2. *Citrate accumulation at different concentrations of oxaloacetate*

Rat-liver homogenate, 1:10; glyoxylate, 0·001 M; oxaloacetate as indicated; NaF, 0·01 M; pH 7·4; incubation, 60 min.; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Expt. no.	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate Found (μmoles)	Citrate Increase (μmoles)
1	None	19·5	—	0·86	—
	Glyoxylate	19·6	—	0·85	—
	Oxaloacetate (0·001 M)	21·3	—	0·77	—
	Oxaloacetate + glyoxylate	10·1	51	4·65	3·88
	Oxaloacetate (0·002 M)	22·5	—	1·05	—
	Oxaloacetate + glyoxylate	11·5	51	9·10	8·05
	Oxaloacetate (0·003 M)	23·0	—	2·30	—
	Oxaloacetate + glyoxylate	12·8	46	10·90	8·60
	Oxaloacetate (0·004 M)	24·6	—	8·50	—
	Oxaloacetate + glyoxylate	13·0	48	15·60	7·10
2	None	19·9	—	0·60	—
	Glyoxylate	20·5	—	0·89	0·29
	Oxaloacetate (0·005 M)	29·5	—	7·62	—
	Oxaloacetate + glyoxylate	14·8	49	21·10	13·48
	Oxaloacetate (0·01 M)	30·1	—	10·50	—
	Oxaloacetate + glyoxylate	15·2	50	26·60	16·10

Table 3. *Accumulation of citrate at different concentrations of glyoxylate*

Rat-liver homogenate, 1:10; oxaloacetate, 0·002 M; glyoxylate as indicated; NaF, 0·01 M; pH 7·4; incubation, 60 min.; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Expt. no.	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate	
				Found (μmoles)	Increase (μmoles)
1	None	19·7	—	0·61	—
	Oxaloacetate	21·0	—	0·92	0·31
	Glyoxylate (0·001 M)	20·1	0	0·92	—
	Oxaloacetate + glyoxylate	13·4	36	8·56	7·64
2	Glyoxylate (0·002 M)	12·3	40	2·40	—
	Oxaloacetate + glyoxylate	12·4	41	8·40	6·00
3	Glyoxylate (0·004 M)	9·8	52	1·80	—
	Oxaloacetate + glyoxylate	11·2	48	6·80	5·00

increased with oxaloacetate concentration up to 0·002 M and then remained constant. Moreover, in the experiments with glyoxylate (0·001 M) and increasing concentrations of oxaloacetate, the formation of citrate appeared to be roughly proportional to the oxaloacetate added. Expt. 2, with higher concentrations of oxaloacetate, confirmed the results on the inhibition of the oxygen uptake, but also showed that the citrate which accumulated was no longer proportional to the concentration of oxaloacetate added. Thus it appeared that the optimum concentration of oxaloacetate for testing the effect of glyoxylate on the accumulation of citrate was 0·002 M, where the synthesis of citrate without the inhibitor was practically nil and consequently the effect of the inhibitor was more evident.

The second point investigated was the effect of adding increasing amounts of glyoxylate to a fixed concentration (0·002 M) of oxaloacetate. The results (Table 3) showed that the amount of citrate found at the end of the incubation was not increased by raising the concentration of glyoxylate from 0·001 to 0·004 M, in contrast with the increase in the preceding experiments after the increase in oxaloacetate concentration (Table 2). The amount of citrate found was also here (Table 3) approximately equivalent to the oxaloacetate added. At the same time Expt. 1 (Table 3) confirmed that 0·001 M-glyoxylate did not affect the endogenous oxygen uptake of the liver, but produced a marked inhibition of the oxidation of oxaloacetate and a very marked accumulation of citrate. Moreover, in the control experiments (glyoxylate and liver without oxaloacetate) there was an increased production of citrate with an increase of glyoxylate concentration from 0·001 to 0·004 M. This citrate accumulation was probably due to the inhibition by glyoxylate of the oxidation of oxaloacetate either present in the liver or formed in the course of incubation. The parallel inhibition of oxygen uptake and citrate accumulation (Table 3, Expts. 2 and 3), which did not occur at the lowest glyoxylate concentration (Expt. 1), seems to confirm this possibility. The inhibition of oxygen uptake in these last experiments (2 and 3) was calculated by assuming a value of 19·5 μl./min. as basal rate (average of four control experiments reported in Tables 1–3).

Inhibition by glyoxylate of citrate oxidation

The results in Table 3, showing that the accumulation of citrate did not increase with increasing concentration of glyoxylate, suggested that the accumulation of citrate was due to an inhibition of its oxidation by glyoxylate rather than by an extra synthesis of citrate from oxaloacetate and glyoxylate. The experiments reported in Table 4, showing that the addition of 0·002 M-glyoxylate to a reaction mixture containing citrate completely prevented disappearance of citrate after the first 15 min., seem to confirm this possibility. It will be noticed that with citrate plus glyoxylate (0·002 M) at 15 and 30 min. the uptake of oxygen was in excess of that expected by the disappearance of citrate. This is also shown in the experiments reported in Table 6, and is probably due to endogenous substrates or to some glyoxylate being oxidized. However, 0·001 M-glyoxylate did not produce an accumulation of citrate, probably because, after part of the glyoxylate had been oxidized, the amount left was too low to cause inhibition.

These results, and the preceding ones showing that oxaloacetate was necessary for the maximal effect to be obtained, lead us to suppose that the inhibition of citrate oxidation may possibly be produced by a new inhibitor formed by a condensation between glyoxylate and oxaloacetate. Oxaloacetate, in the experiments of Tables 4 and 6, could have been formed from citrate during its initial oxidation. In this case the addition of oxaloacetate, together with glyoxylate, should produce a larger inhibition of the oxidation of citrate than that given by glyoxylate alone. Table 5 compares the

Table 4. *Effect of glyoxylate on citrate oxidation*

Rat-liver homogenate, 1:10; citrate, 0·002M; glyoxylate as indicated; NaF, 0·01M; pH 7·4; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Time (min.)	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate Found (μmoles)	Citrate Decrease (μmoles)
0	Citrate + glyoxylate (0·002M)	—	—	8·5	—
15	Citrate	34·8	—	3·0	5·5
15	Citrate + glyoxylate (0·001M)	35·0	0	3·1	5·4
15	Citrate + glyoxylate (0·002M)	34·3	2	7·3	1·2
30	Citrate	32·6	—	1·2	7·3
30	Citrate + glyoxylate (0·001M)	32·1	1	1·3	7·2
30	Citrate + glyoxylate (0·002M)	28·0	14	7·5	1·0
60	Citrate	29·6	—	0·1	8·4
60	Citrate + glyoxylate (0·001M)	30·1	0	0·4	8·1
60	Citrate + glyoxylate (0·002M)	17·1	43	7·3	1·2

Table 5. *Effect of addition of oxaloacetate plus glyoxylate on citrate oxidation*

Rat-liver homogenate, 1:10; citrate, 0·002M; oxaloacetate, 0·002M; glyoxylate, 0·001M; NaF, 0·01M; pH 7·4; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Time (min.)	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate Found (μmoles)	Citrate Δ (μmoles)
0	Citrate + oxaloacetate + glyoxylate	—	—	7·9	—
15	Citrate + oxaloacetate	38·1	—	4·5	−3·4
15	Citrate + glyoxylate	37·7	1	4·5	−3·4
15	Citrate + oxaloacetate + glyoxylate	24·6	36	13·5	+5·6
30	Citrate + oxaloacetate	34·7	—	3·9	−4·0
30	Citrate + glyoxylate	31·6	9	3·9	−4·0
30	Citrate + oxaloacetate + glyoxylate	20·0	42	17·2	+9·3

Table 6. *Effect of glyoxylate on the earlier stages of citrate oxidation*

Rat-liver homogenate, 1:10; citrate, 0·002M; glyoxylate, 0·002M; NaF, 0·01M; pH 7·4; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Time (min.)	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate Found (μmoles)	Citrate Decrease (μmoles)
0	Citrate + glyoxylate	—	—	8·85	—
5	Citrate	—	—	7·51	1·34
5	Citrate + glyoxylate	—	—	7·51	1·34
10	Citrate	—	—	6·00	2·85
10	Citrate + glyoxylate	—	—	6·20	2·60
15	Citrate	29·2	—	4·40	4·45
15	Citrate + glyoxylate	27·8	5	6·51	2·34
20	Citrate	30·0	—	4·10	4·75
20	Citrate + glyoxylate	28·2	6	8·35	0·50

inhibition of citrate oxidation, caused by glyoxylate (0·001M) and oxaloacetate (0·002M) with that obtained with glyoxylate alone. The glyoxylate or glyoxylate and oxaloacetate was added from the side arm to the main chamber at the beginning of the incubation. Under these conditions, the inhibition of the citrate oxidation was much larger than that observed in the preceding experiment at the corresponding time, and greater formation of citrate occurred; after incubation for 30 min. this roughly corresponded with the transformation of all the added oxaloacetate into citrate.

Formation of an inhibitor during the incubation of glyoxylate and oxaloacetate

The previous results lead us to suppose that both the inhibition of the oxygen uptake and the accumulation of citrate could be produced by a

Table 7. *Effect of oxaloacetate plus glyoxylate on citrate oxidation at the earlier stages of incubation*

Rat-liver homogenate, 1:10; citrate, 0·002 M; oxaloacetate, 0·001 M; glyoxylate, 0·001 M; NaF, 0·01 M; pH 7·4; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Time (min.)	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate	
				Found (μmoles)	Δ (μmoles)
0	Citrate + oxaloacetate + glyoxylate	—	—	8·4	—
5	Citrate + oxaloacetate	—	—	5·5	−2·9
5	Citrate + oxaloacetate + glyoxylate	—	—	10·6	+2·2
10	Citrate + oxaloacetate	41·4	—	5·0	−3·4
10	Citrate + oxaloacetate + glyoxylate	26·0	37	12·4	+4·0
15	Citrate + oxaloacetate	41·0	—	4·4	−4·0
15	Citrate + oxaloacetate + glyoxylate	25·8	34	12·5	+4·1
20	Citrate + oxaloacetate	43·2	—	4·0	−4·4
20	Citrate + oxaloacetate + glyoxylate	22·2	49	12·4	+4·0

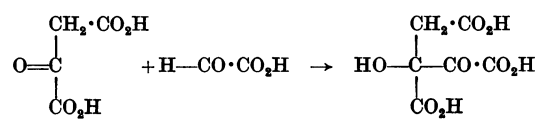
substance formed during the incubation of glyoxylate with oxaloacetate. In this case, after the addition of glyoxylate alone there would be an initial lag period before the inhibition of the oxidation of citrate occurred, since for the inhibition the formation of oxaloacetate from citrate would be necessary. This idea is in agreement with the results in Table 6, where citrate (0·002 M) was incubated for 5, 10, 15 or 20 min. in the presence or absence of glyoxylate (0·002 M). They show that glyoxylate did not interfere with citrate oxidation during incubation for 5 or 10 min., but inhibition started after 15 min. and only after 20 min. was it almost complete. This gradual appearance of inhibition did not occur in the next experiment (Table 7). Here the inhibition occurred immediately because glyoxylate (0·001 M final concentration in vessel) and oxaloacetate (0·001 M final concentration in vessel) were added together from the side arm at the beginning of the incubation. A very high inhibition of citrate oxidation occurred even after incubation for 5 min., the maximum value being already reached after 10 min. and remaining constant until 20 min. Moreover, it appeared that the citrate formed exceeded the value of citrate initially present in the flask (8 μmoles) by an amount corresponding approximately to the oxaloacetate added (4 μmoles).

DISCUSSION

The results presented in this paper confirm that glyoxylate is an inhibitor of tissue oxidations, and strongly indicate that one of the main points of action of this substance is the oxidation of citrate.

The finding which showed that the depression of the uptake of oxygen and the accumulation of citrate reached maximal rates only when glyoxylate and oxaloacetate were incubated together (Table 1), leads us to suppose that oxaloacetate under these conditions formed citrate, which was not further oxidized. However, the formation of citrate did not seem to be caused by the condensation of glyoxylate plus oxaloacetate, as might be supposed from the results of Campbell, Smith & Eagles (1953), Olson (1954) and Saz & Hillary (1956), who found that crude extracts of several micro-organisms synthesize citrate from glyoxylate plus succinate. Nor does the so-called 'glyoxylate bypass', recently discovered by Kornberg & Krebs (1957) in cell-free extracts of *Pseudomonas*, explain our observations. The observations that the formation of citrate (*a*) was always correlated with the inhibition of the oxygen uptake and (*b*) depended on increasing oxaloacetate concentration (Table 2), whereas it was practically independent of increase in the concentration of glyoxylate (Table 3), are against these possibilities. It seems, on the contrary, that both the depression of oxygen uptake and the formation of citrate can be explained by supposing that oxaloacetate may react in liver cells in two different ways: (*a*) with acetylcoenzyme A to form citrate; (*b*) with a small amount of glyoxylate to give rise to an inhibitor of citrate oxidation.

The formation of an inhibitor is in accord with the experiments with citrate added before the incubation (Tables 4–7). They showed that the addition of glyoxylate alone strongly inhibits the disappearance of citrate (Tables 4 and 6), and the addition of glyoxylate plus oxaloacetate, besides increasing the inhibition, produces also extra formation of citrate (Tables 5 and 7) approximately corresponding to the oxaloacetate added.



Oxalomalic acid

This inhibitory compound could well be a new tricarboxylic acid containing six carbon atoms, such as oxalomalic acid. It seems very likely that such a compound, even at very low concentrations, could compete with the enzymic systems related to citrate oxidation.

The existence of the inhibitory effect of glyoxylate on the tricarboxylic acid cycle appears to be of great interest if we consider that glyoxylate has recently been shown to take part in many metabolic transformations involving carbohydrates, fats and protein derivatives. The fact that small amounts of glyoxylate in the presence of oxaloacetate will inhibit citrate oxidation strongly indicates that in the cells under physiological conditions, glyoxylate, however formed, must be promptly removed to avoid the inhibitory effects described above. On the other hand, glyoxylate could inhibit the normal rate of oxidations and might be connected with the widely observed oxidative impairment of cancer tissues (see Warburg, 1956; Weinhouse, Warburg, Burk & Schadē, 1956; Ruffo, Capobianco & Adinolfi, 1956*a*, *b*), which has not yet been adequately explained.

SUMMARY

1. The effect of low concentrations (0·001 M) of glyoxylate on the oxidative rate of the main intermediates of the tricarboxylic acid cycle was investigated in respiring rat-liver homogenates.

2. The addition of glyoxylate had little effect on the endogenous oxygen uptake of the homogenate, but produced a small inhibition of the oxidative rate with citrate, D-*iso*citrate, *cis*-aconitate, α-oxoglutarate, succinate, fumarate, malate and pyruvate, and a small increase in the accumulation of citrate. A very marked inhibition of the uptake of oxygen, and a large accumulation of citrate, occurred when oxaloacetate was the substrate.

3. The oxidation of citrate was inhibited either by glyoxylate alone, after incubation for 20 min., or by glyoxylate plus oxaloacetate, after incubation for 5 min. Both these results indicate that oxaloacetate was necessary for obtaining maximal rates on inhibition.

4. The dependence of citrate accumulation on oxaloacetate and glyoxylate suggests that the two substances react together to form an inhibitor of citrate metabolism. This would account for both citrate accumulation and the depressed oxygen uptake.

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The Relationship between Catalase and Haemoglobin in Human Blood

By H. MILLER
Portsmouth and Isle of Wight Area Pathological Service, Royal Hospital, Portsmouth

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There is insufficient evidence so far to indicate the biological function of catalase, but there is indication that its presence in the living cell is protective against the toxic effects of hydrogen peroxide which may be produced as a by-product during the various oxidative enzymic reactions that occur in living organisms. (Keilin & Hartree, 1945).

Catalase, which like haemoglobin is a haematin–protein complex, is found principally in the red-blood cells and liver cells and is produced in the marrow and liver cells (Theorell, 1951). Its presence in plasma is probably due to haemolysis (Perlmann & Lipmann, 1947).

Interest in a possible role for hydrogen peroxide in the catabolism of haemoglobin was initiated by Bingold (1935), who found that haemoglobin could