Biochem. J. (1969) **114**, 513
Printed in Great Britain

Control of the Citric Acid Cycle by Glyoxylate

THE MECHANISM OF INHIBITION OF OXOGLUTARATE DEHYDROGENASE, ISOCITRATE DEHYDROGENASE AND ACONITATE HYDRATASE

BY A. ADINOLFI, R. MORATTI, S. OLEZZA AND A. RUFFO
Impresa Enzimologia, C.N.R., c/o Institute of Biological Chemistry, University of Pavia, 27100 Pavia, Italy

(*Received* 14 *March* 1969)

1. The effects of glyoxylate on partially purified preparations of aconitate hydratase, isocitrate dehydrogenase and oxoglutarate dehydrogenase were compared with those of oxalomalate and hydroxyoxoglutarate (obtained by condensation of glyoxylate with oxaloacetate and pyruvate respectively). 2. Glyoxylate (1mM) did not affect aconitate hydratase and isocitrate dehydrogenase, whereas oxalomalate (1mM) inhibited the enzyme activities completely. 3. Glyoxylate (0·025mM) inhibited oxoglutarate dehydrogenase irreversibly, whereas the same concentrations of oxalomalate and hydroxyoxoglutarate were ineffective. This inhibitory effect was prevented if oxoglutarate, pyruvate or oxaloacetate was mixed with the enzyme before the glyoxylate. 4. Incubation of oxoglutarate dehydrogenase with radioactive glyoxylate produced radioactive carbon dioxide; radioactivity was also recovered in the portion of the enzyme identified with thiamin pyrophosphate. 5. The behaviour of glyoxylate in producing multiple inhibitions of the citric acid cycle, either by direct interaction with oxoglutarate dehydrogenase, or by means of its condensation compounds which inhibit aconitate hydratase and isocitrate dehydrogenase, is discussed.

Previous results showed that glyoxylate, under physiological conditions of temperature and pH, reacted with two metabolites of the citric acid cycle, oxaloacetate and pyruvate, yielding respectively a C_6 tricarboxylic acid, identified as oxalomalate (Ruffo, Testa, Adinolfi & Pelizza, 1962), and a C_5 dicarboxylic acid, identified as 3-hydroxy-2-oxoglutarate (Ruffo, Testa, Adinolfi, Pelizza & Moratti, 1967). Oxalomalate appeared to be a specific inhibitor of aconitate hydratase (EC 4.2.1.3) and of isocitrate ($NADP^+$) dehydrogenase (EC 1.1.1.42), but inhibited oxoglutarate dehydrogenase (EC 1.2.4.2) to a much smaller extent. Adinolfi, Olezza & Ruffo (1967) suggested that glyoxylate alone inhibited the activity of oxoglutarate dehydrogenase to a greater extent than did either oxalomalate or 3-hydroxy-2-oxoglutarate.

In the present work we compared the effect of glyoxylate on partially purified preparations of oxoglutarate dehydrogenase, isocitrate dehydrogenase and aconitate hydratase with that produced by oxalomalate and hydroxyoxoglutarate. The main finding is that glyoxylate inhibited oxoglutarate dehydrogenase irreversibly, probably by binding to the TPP* of the enzyme, but did not affect the other two enzymes. Oxalomalate and hydroxyoxoglutarate inhibited the three enzymes to different extents, but the mechanism was competitive in each case.

* Abbreviation: TPP, thiamin pyrophosphate.

MATERIALS AND METHODS

Materials. NAD^+ and $NADP^+$ were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., CoA was obtained from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, or from the Sigma Chemical Co., thiamin phosphate and TPP were obtained from Fluka A.-G., Buchs, Switzerland, thiamin was obtained from E. Merck A.-G., Darmstadt, Germany, and Amberlite IR-120 from British Drug Houses Ltd., Poole, Dorset. Trisodium DL-isocitrate was a Sigma preparation (93–98% pure); *cis*-aconitic acid was obtained from Fluka A.-G., trisodium citrate dihydrate was obtained from E. Merck A.-G., potassium oxoglutarate, sodium pyruvate, sodium glyoxylate and oxaloacetic acid were obtained from Sigma or from Fluka A.-G. [1-^{14}C]- and [2-^{14}C]-Glyoxylate were obtained from the Radiochemical Centre, Amersham, Bucks., in the form of sodium salts of specific radioactivity respectively 8·44mc/m-mole and 4·6mc/m-mole. 2,5-Diphenyloxazole, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and Cab-O-Sil were obtained from Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A. Tris was a Sigma product. All the other reagents were from C. Erba, Milan, Italy.

Preparations of enzymes and assay methods. Aconitate

hydratase was prepared from pig heart (Anfinsen, 1955) and its activity was determined with citrate as substrate (Ruffo *et al.* 1962, 1967).

Isocitrate dehydrogenase ($NADP^+$) was a commercial preparation obtained from Boehringer und Soehne G.m.b.H. containing 1·8 units/mg. The activity was determined as described by Ruffo *et al.* (1967), except that we decreased the concentration of the enzyme and increased the concentration of $NADP^+$ (Fig. 1).

Oxoglutarate dehydrogenase (specific activity 1·4–1·6 units/mg.) was purified from pig heart (Sanadi, Littlefield & Bock, 1952). The enzyme activity was determined after preincubation (2 mg. of protein/ml.) in 0·04 M-potassium phosphate buffer, pH 7·4, for 10 min. at 25° in the presence and in the absence of the inhibitors, with either 2,6-dichlorophenol-indophenol or NAD^+ as electron acceptor.

In the first case the assay described by Sanadi *et al.* (1952) was modified as follows: 0·1 ml. of the preincubated enzyme solution was pipetted into a Beckman spectrophotometer cuvette containing, in a final volume of 2·9 ml.: 100 μmoles of potassium phosphate buffer, pH 7·4, 10 μmoles of $MgCl_2$, 5 μmoles of potassium oxoglutarate and 0·1 ml. of 0·2% (w/v) 2,6-dichlorophenol-indophenol. The change in extinction was determined at 600 nm., the readings being taken at 30 sec. intervals for 3 min.

In the second case, samples (0·02 ml.) of the preincubated enzyme solution were assayed as described by Hift, Ouellet, Littlefield & Sanadi (1953), except that cysteine and phosphorylating enzyme were omitted and the concentration of CoA was increased to 0·5 μmole/3 ml. The extinction at 340 nm. was measured every 20 sec. for 3 min.

Preparation and incubation of the inhibitors. Sodium oxalomalate trihydrate was prepared as described by Ruffo *et al.* (1962). The barium salt of 3-hydroxy-2-oxoglutaric acid was prepared as reported (method no. 1) by Ruffo *et al.* (1967). The solutions they used, as well as glyoxylate solution, were prepared just before use as described by Ruffo *et al.* (1967) and either added to aconitate hydratase immediately before the assay of the activity, or preincubated with the oxoglutarate dehydrogenase preparation for 10 min. at 25° as described above. The results of these experiments are shown in Figs. 1–6. Isocitrate dehydrogenase assays were started by addition of enzyme (no preincubation with inhibitor).

Glyoxylate decarboxylation. The decarboxylation of glyoxylate was determined by measuring radioactive CO_2 production in Warburg vessels by incubating increasing concentrations of oxoglutarate dehydrogenase with glyoxylate labelled respectively at C-1 or C-2. The incubation was carried out in Warburg flasks with two side arms, one containing 0·26 μmole (1 μc) of [1-^{14}C]- or [2-^{14}C]-glyoxylate and the other 0·2 ml. of 2 M-HCl.

Appropriate samples of the enzyme in 0·04 M-potassium phosphate buffer, pH 7·4, were added to the main chamber. The final volume was adjusted to 3 ml. The central well contained 0·2 ml. of 2 M-NaOH absorbed on a filter-paper strip. The incubation (30 min. at 25° in air) was started by

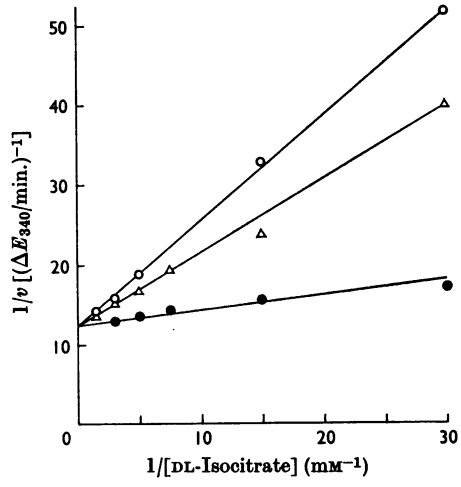

Fig. 1. Competitive inhibition of isocitrate dehydrogenase by oxalomalate and hydroxyoxoglutarate. Incubation mixtures (3 ml. of 0·025 M-tris–HCl buffer, pH 7·4, 0·6 mM-$MnCl_2$, 0·33 mM-$NADP^+$ and DL-isocitrate as indicated) were prepared. The reaction was started by adding 0·01 ml. of enzyme solution (1 mg. of protein/ml.). ●, Control; △, 1·5 μmoles of hydroxyoxoglutarate present; ○, 0·15 μmole of oxalomalate present.

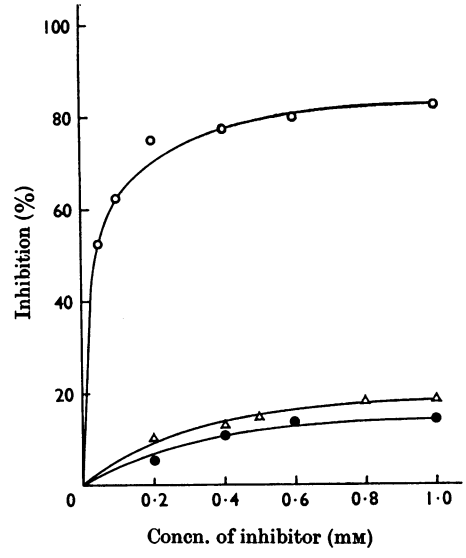

Fig. 2. Inhibition of oxoglutarate dehydrogenase by glyoxylate, hydroxyoxoglutarate and oxalomalate. The enzyme (2 mg./ml.) was preincubated in 0·04 M-potassium phosphate buffer, pH 7·4 at 25°, in the absence and in the presence of inhibitors at the concentrations indicated. After 10 min. samples (0·02 ml.) were added to cuvettes containing: 100 μmoles of potassium phosphate buffer, pH 7·4, 1 μmole of NAD^+, 10 μmoles of $MgCl_2$, 5 μmoles of oxoglutarate, 0·5 μmole of CoA and water to 3 ml. The readings were taken at 340 nm. for 3 min. and ΔE was measured for the interval 0–20 sec. ○, Effect of glyoxylate; ●, effect of oxalomalate; △, effect of hydroxyoxoglutarate.

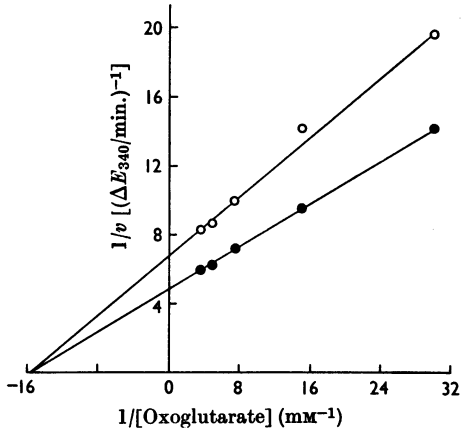

Fig. 3. Inhibition of oxoglutarate dehydrogenase by glyoxylate. Enzyme (2mg./ml.) was preincubated for 10min. at 25° in the presence and in the absence of glyoxylate (0·025mM). Samples (0·02ml.) were then assayed as described in Fig. 2, except that the concentration of oxoglutarate was as indicated. ●, Control; ○, 0·025mM-glyoxylate.

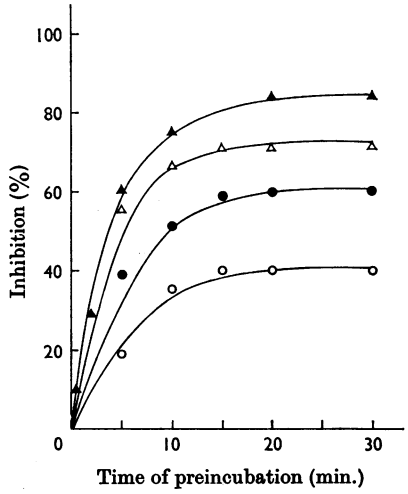

Fig. 4. Effect of time of preincubation with glyoxylate on the inhibition of oxoglutarate dehydrogenase. The enzyme (2mg./ml.) was preincubated in 0·04M-potassium phosphate buffer, pH7·4, at 25° in the absence and in the presence of different concentrations of glyoxylate for the times indicated. Samples (0·02ml.) were then assayed as described in Fig. 2. Concentration of glyoxylate: ○, 0·025mM; ●, 0·05mM; △, 0·1mM; ▲, 0·2mM.

the addition of glyoxylate to the enzyme. At the end of the incubation the HCl was added to the main chamber and the incubation was continued for 10min. The paper strip was

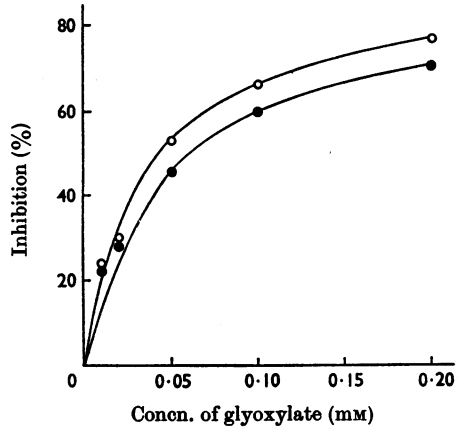

Fig. 5. Comparison of the rate of reduction of NAD$^+$ and 2,6-dichlorophenol-indophenol by oxoglutarate dehydrogenase in the presence of glyoxylate. The enzyme was preincubated with glyoxylate at the concentrations indicated and the reduction of NAD$^+$ was determined as reported in Fig. 2, with 0·02ml. samples. The reduction of 2,6-dichlorophenol-indophenol was determined at 600nm., by adding 0·1ml. of the preincubated enzyme to a cuvette containing: 100μmoles of potassium phosphate buffer, pH7·4, 10μmoles of $MgCl_2$, 5μmoles of oxoglutarate, 0·1ml. of 0·2% (w/v) 2,6-dichlorophenol-indophenol and water to 2·9ml. The readings were taken for 3min.; ΔE between 30 and 60sec. was taken as a measure of the enzyme activity. ○, NAD$^+$; ●, 2,6-dichlorophenol-indophenol.

collected, dried at room temperature and shaken for 10min. in a bottle containing 10ml. of a suspension of the following composition: 10% (w/v) 2,5-diphenyloxazole, 0·1% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 4% (w/v) Cab-O-Sil. The bottle was then placed in the scintillation counter (Nuclear–Chicago model 550) for measurement of the radioactivity.

Identification of a glyoxylate derivative of TPP. The incubation mixture and the method of deproteinization were similar to those used by Kohlhaw, Deus & Holzer (1965) for the identification of 2-hydroxymethyl-TPP, except that oxoglutarate dehydrogenase replaced pyruvate oxidase and the final volume for incubation was decreased to one-tenth. The composition of the sample (final vol. 2ml.) was: 30μmoles of potassium phosphate buffer, pH6·0, 10μmoles of $MgCl_2$, 2μmoles of TPP, 2·5μmoles of glyoxylate, 0·26μmole (1μc) of [2-^{14}C]glyoxylate and enzyme (17mg. of protein). An identical sample in which the enzyme had been boiled for 10min. was used as control. Both the samples were incubated under H_2 for 30min. at 37°, and deproteinized by adding 18ml. of boiling methanol. After centrifuging, the supernatant was evaporated to dryness as described by Kohlhaw *et al.* (1965) and the residue, dissolved in 12ml. of water, was centrifuged again at 35000*g* for 30min. at 0°. The supernatant was again evaporated to dryness, and the final residue redissolved in 0·3ml. of water. Then 10μl. of the solution was spotted at the centre of a strip of cellulose acetate (Cellogel; Chemetron Chimica,

Milan, Italy) previously wetted with 0·1 M-potassium phosphate buffer, pH 7·0. Another strip was prepared with 0·2 μmole each of TPP, thiamin phosphate and thiamin at a common origin. The strips were placed in a conventional electrophoretic apparatus, with the same buffer, and 150 v was applied for 105 min. at 0–4°. The strips were removed and examined under u.v. light, and the spots corresponding to the standards for thiamin, thiamin phosphate and TPP were cut out, suspended in test tubes containing 1·5 ml. of water and eluted for 2 hr. at room temperature. Samples (0·2 ml.) of each eluate were then mixed with 1 ml. of anhydrous ethanol and added to 10 ml. of the scintillation suspension described above for detection of radioactivity. The results are reported in Table 1.

RESULTS

Effect of the inhibitors on aconitate hydratase and isocitrate dehydrogenase. We repeated the experiments described by Ruffo *et al.* (1967) with oxalomalate and hydroxyoxoglutarate and compared the results with those of another experiment with concentrations of glyoxylate increasing from 0·1 to 1·6 mM, incubated under the same experimental conditions. The results showed that glyoxylate did not affect the activity of these enzymes, and confirmed our previous results with oxalomalate and hydroxyoxoglutarate.

With regard to the mechanism of inhibition, the results plotted by the procedure of Lineweaver & Burk (1934) confirmed the competitive inhibition on aconitate hydratase by oxalomalate and hydroxyoxoglutarate. Isocitrate dehydrogenase was also inhibited competitively with respect to isocitrate (Fig. 1).

Effect of the inhibitors on oxoglutarate dehydrogenase. The enzyme was preincubated for 10 min. at 25° in three separate experiments with oxalomalate, hydroxyoxoglutarate and glyoxylate, since it appeared (Ruffo *et al.* 1967) that the enzyme was less sensitive to inhibition. The results (Fig. 2) showed that the inhibition due to glyoxylate was about four times that due to oxalomalate and hydroxyoxoglutarate at a concentration of 1 mM and about eight times as great at a concentration of 0·2 mM.

With regard to the mechanism of inhibition we confirmed our previous results (Ruffo *et al.* 1967) that oxalomalate and hydroxyoxoglutarate inhibited competitively with oxoglutarate; however, the results reported in Fig. 3 showed that the much more powerful inhibition by glyoxylate did not affect the K_m for oxoglutarate. Fig. 4 shows that this is not due to the inhibition being non-competitive in the classical sense, but is a consequence of a time-dependent inactivation of the enzyme by glyoxylate. The results of Fig. 5 show comparable inhibition by glyoxylate of the reduction of both NAD^+ and 2,6-dichlorophenol-indophenol; this suggests that the lipoyl dehydrogenase component

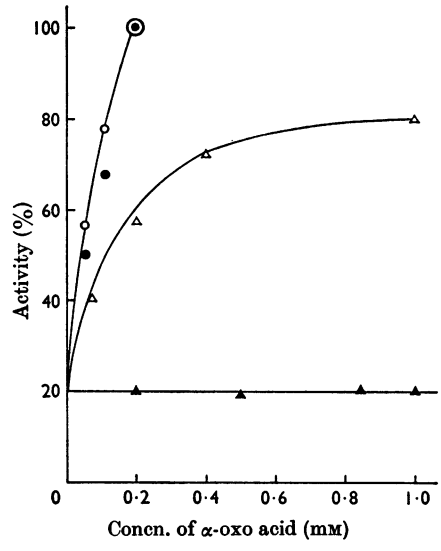

Fig. 6. Protection of oxoglutarate dehydrogenase from glyoxylate inhibition. The enzyme was preincubated with 0·2 mM-glyoxylate in the presence and in the absence of oxoglutarate, oxaloacetate or pyruvate at the concentrations indicated. Samples (0·02 ml.) were taken and the activity was determined as reported in Fig. 2. ○, Effect of oxoglutarate; ●, effect of oxaloacetate; △, effect of pyruvate; ▲, enzyme preincubated with glyoxylate alone and oxoglutarate (or oxaloacetate or pyruvate) added immediately before the determination of the activity.

of the oxoglutarate dehydrogenase was not directly involved in the inhibition, for this part of the enzyme complex is not necessary for dye reduction (Sanadi *et al.* 1952).

In further experiments we investigated whether the substrate, and structural analogues like oxaloacetate and pyruvate, reversed the inhibition of oxoglutarate dehydrogenase.

The results of an experiment in which the enzyme was preincubated with glyoxylate, in the presence or absence of oxoglutarate, oxaloacetate and pyruvate, are reported in Fig. 6. It appears from these that the addition of either the substrate or oxaloacetate protected the enzyme from the inhibition caused by 0·2 mM-glyoxylate. Pyruvate also protected the enzyme, but only partially and at concentrations higher than that of glyoxylate. Moreover, if these substances were added to the enzyme after the preincubation with glyoxylate, no protection was obtained. TPP caused only a partial protection at a concentration 300 times that of the glyoxylate.

Decarboxylation of glyoxylate by oxoglutarate dehydrogenase. The results reported above suggested that glyoxylate inhibited the portion of oxoglutarate

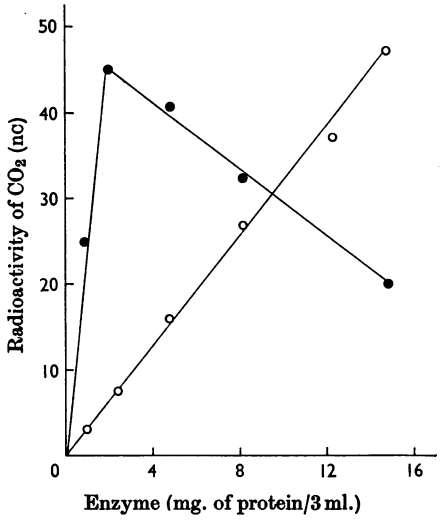

Fig. 7. Decarboxylation of glyoxylate by oxoglutarate dehydrogenase. Each Warburg vessel contained in the main chamber 3ml. of 0·04M-potassium phosphate buffer, pH 7·4, enzyme as indicated and 1μmole of oxoglutarate (when present); in the first side arm was 0·26μmole (1μc) of [1-^{14}C]glyoxylate; in the second side arm was 0·2ml. of 2M-HCl; in the centre well was 0·2ml. of 2M-NaOH absorbed on filter-paper strips. The incubation at 25° for 30min. was started by mixing the glyoxylate solution with the contents of the main chamber and was stopped by addition of HCl. After 10min. the vessels were removed and the paper strips collected, dried and the radioactivity was determined as described in the text. ○, Glyoxylate; ●, glyoxylate and oxoglutarate.

dehydrogenase concerned with the decarboxylating activity. In this case glyoxylate should be decarboxylated by oxoglutarate dehydrogenase. This would correspond with the results of Kohlhaw *et al.* (1965), who found decarboxylation of glyoxylate and formation of a condensation compound (identified as 2-hydroxymethyl-TPP) during incubation with pyruvate dehydrogenase.

We incubated [1-^{14}C]glyoxylate with increasing concentrations of oxoglutarate dehydrogenase in the presence and absence of oxoglutarate, and determined the radioactivity in the carbon dioxide formed. The results (Fig. 7) showed that the production of labelled carbon dioxide was proportional to the enzyme concentration, and confirmed the synergistic effect of oxoglutarate on the decarboxylation of glyoxylate (Koch & Stokstad, 1966; Stewart & Quayle, 1967), although only at low concentrations of the enzyme. On increasing the concentration of enzyme a decrease of the decarboxylation was observed, which with 15mg. of enzyme amounted to about 50% inhibition with respect to the sample without oxoglutarate. When the incubation with the enzyme was repeated with [2-^{14}C]glyoxylate as substrate, no radioactive carbon dioxide was detectable.

Table 1. *Recovery of the radioactivity of glyoxylate in the TPP derivatives of oxoglutarate dehydrogenase*

Two samples of enzyme (one of which had been boiled for 10min.), containing 17mg. of protein, were incubated with 1μc ($1·5 \times 10^6$ c.p.m.) of [2-^{14}C]glyoxylate, deproteinized and dried as described in the text. The residue was dissolved in 0·3ml. of water and samples were taken for electrophoretic separation and determination of radioactivity. The results are given in c.p.m. for the soluble residue corresponding to 17mg. of original protein.

	Fresh enzyme	Boiled enzyme	Difference
TPP	47250	4440	42810
Thiamin phosphate	46590	4710	41880
Thiamin	30600	0	30600
Total	124440	9150	115290
Recovery (% of the total radioactivity)	8·0	0·6	7·4

Identification of the TPP-glyoxylate derivatives. We incubated [2-^{14}C]glyoxylate with oxoglutarate dehydrogenase and after deproteinization followed the technique of Kohlhaw *et al.* (1965) for isolating hydroxymethyl-TPP, until obtaining the residue from which they had isolated hydroxymethyl-TPP by column chromatography. We identified this compound by electrophoretic separation on cellulose acetate strips, with TPP, thiamin phosphate and thiamin as markers.

The spots corresponding to those of the markers were cut out, eluted with water and their radioactivity was determined.

The results reported in Table 1 show that a significant percentage of the radioactivity of glyoxylate was found in the positions corresponding to TPP, thiamin phosphate and thiamin. The two last-named compounds were probably derived from the hydrolysis of TPP during either the incubation with the enzyme or the preparation of the deproteinized extract. The small amount of radioactivity found in the spots from incubations with boiled enzyme may be explained either by physical adsorption or a possible chemical reaction between glyoxylate and TPP linked to the denatured enzyme.

DISCUSSION

The results obtained with oxalomalate and hydroxyoxoglutarate as inhibitors showed that the inhibition of isocitrate dehydrogenase was competitive, as previously found for aconitate hydratase (Ruffo *et al.* 1962). These inhibitors presumably act

owing to their structural resemblance to citrate and isocitrate. The results with glyoxylate showed that at the concentrations in which oxalomalate and hydroxyoxoglutarate produced complete inhibition glyoxylate did not produce any effect on these enzymes. However, glyoxylate at low concentrations inhibited oxoglutarate dehydrogenase whereas oxalomalate and hydroxyoxoglutarate had a much smaller effect. This difference in behaviour has been explained in terms of the different mechanism of the inhibition by glyoxylate. The inhibition of oxoglutarate dehydrogenase by glyoxylate is time-dependent and irreversible; either oxoglutarate, or the other α-oxo acids tested, protected the enzyme from the inhibition, but did not reverse it. Moreover the experiments with radioactive glyoxylate showed that it was decarboxylated by the enzyme and, at the same time, a radioactive derivative of TPP was formed. The results, taken in conjunction with those of Kohlhaw *et al.* (1965), suggested that the latter was hydroxymethyl-TPP. Thus it seems that glyoxylate inhibits oxoglutarate dehydrogenase by binding to the TPP of the enzyme and there being decarboxylated.

We conclude that glyoxylate can affect the citric acid cycle at three points, by directly inhibiting oxoglutarate dehydrogenase, and by indirectly, through its condensation derivatives, inhibiting aconitate hydratase and isocitrate dehydrogenase.

REFERENCES

Adinolfi, A., Olezza, S. & Ruffo, A. (1967). *Biochem. J.* **104**, 50P.

Anfinsen, C. B. (1955). In *Methods in Enzymology*, vol. 1, p. 695. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Hift, E. H., Ouellet, L., Littlefield, G. W. & Sanadi, D. R. (1953). *J. biol. Chem.* **204**, 565.

Koch, J. & Stokstad, E. L. R. (1966). *Biochem. biophys. Res. Commun.* **23**, 585.

Kohlhaw, G., Deus, B. & Holzer, H. (1965). *J. biol. Chem.* **240**, 2135.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Ruffo, A., Testa, E., Adinolfi, A. & Pelizza, G. (1962). *Biochem. J.* **85**, 588.

Ruffo, A., Testa, E., Adinolfi, A., Pelizza, G. & Moratti, R. (1967). *Biochem. J.* **103**, 19.

Sanadi, D. R., Littlefield, J. W. & Bock, R. M. (1952). *J. biol. Chem.* **197**, 851.

Stewart, P. R. & Quayle, J. R. (1967). *Biochem. J.* **102**, 885.