muramic acid (C-7, C-8 and C-9) in preference to the pyranose ring (C-1 to C-6) when the incubation medium contained non-radioactive glucose. With the C_3 compounds the identity of the carbon atoms was maintained, i.e. C-1 $\rightarrow$ C-7; C-2 $\rightarrow$ C-8; C-3 $\rightarrow$ C-9.

4. Experiments with the above-named tracers and non-radioactive competitors suggest that phosphoenolpyruvate is the most likely immediate precursor of the side chain of muramic acid. It is not possible, however, completely to exclude any of the compounds involved in glucose degradation by the glycolytic pathway between triose phosphate and phosphoenolpyruvate.

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Biochem. J. (1962) **85**, 588

Control of the Citric Acid Cycle by Glyoxylate

1. A NEW INHIBITOR OF ACONITASE FORMED BY THE CONDENSATION OF GLYOXYLATE WITH OXALOACETATE

BY A. RUFFO, E. TESTA, ANNA ADINOLFI AND GIUSEPPINA PELIZZA

Department of Biological Chemistry, University of Perugia, and Department of Organic Synthesis, Lepetit, S.p.A., Milan, Italy

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Ruffo, Romano & Adinolfi (1959) showed that aconitase was inhibited when incubated in the presence of small amounts of glyoxylate and oxaloacetate. Since chromatographic and radioautographic evidence was given (Ruffo, Romano, Adinolfi & Verga, 1960) of the formation during the incubation of a new compound, probably a C_6 tricarboxylic acid, the condensation between sodium glyoxylate and sodium oxaloacetate was investigated under physiological conditions of pH and temperature, and is reported in the present paper.

The product of the reaction was isolated, its structure determined, and its mode of action on aconitase was studied.

EXPERIMENTAL

Condensation between sodium glyoxylate and sodium oxaloacetate. Oxaloacetic acid (1.32 g.) and sodium glyoxylate (1.14 g.) were dissolved in 50 ml. of water, and the pH was adjusted to 7.4 with about 20 ml. of N -NaOH. The solution was incubated for 3 hr. at 40° and then freeze-dried; the disappearance of the aldehyde group was determined

according to Kramer, Klein & Baseline (1959). The slightly hygroscopic compound obtained was dried for 24 hr. *in vacuo* over P_2O_5 and microanalysed (C, H, H_2O). Sodium was determined in the ash by potentiometric titration with 0.1N- $HClO_4$. The hydroxylic functions were determined by acetylation with acetic anhydride and pyridine, the mixture being heated for 8 hr. on a boiling-water bath. The combined carboxyl (as sodium salt) and hydroxy groups were determined by acetylation with acetyl chloride and pyridine, the mixture being heated for 1 hr. on a boiling-water bath. The presence of a keto group was detected according to Feigl (1957) by reaction with sodium nitroprusside in alkaline medium. The absence of any aldehyde group was shown by the reaction with decolorized fuchsin. The rate of the condensation of sodium oxaloacetate and sodium glyoxylate at pH 7.4 was followed by determining the disappearance of the aldehyde group of glyoxylate, at various times and in different conditions, according to the colorimetric method of Kramer *et al.* (1959). The colour was uninfluenced by the keto groups, either of the oxaloacetic acid or of pyruvic acid, which could be formed by decarboxylation of oxaloacetate. Similar experiments were also done in the presence of 0.02M- $MgCl_2$, at different temperatures and pH.

2,4-Dinitrophenylhydrazone of the condensation compound. 2,4-Dinitrophenylhydrazine (1.5 g.) was dissolved in 20 ml. of 50% (w/v) H_2SO_4 and diluted to 100 ml. with aq. 25% (v/v) ethanol. A sample (1 g.) of the freeze-dried new compound was dissolved in 70 ml. of this solution. Immediately CO_2 was evolved, and could be determined by acidimetric titration. After 30 min., the separated phenylhydrazone was collected, washed with 25% ethanol and dried at 60° *in vacuo*. The melting point (uncorrected) was determined on a Kofler apparatus. Microanalysis data are given in the Results section. A portion (500 mg.) of the dried compound was shaken with 2 ml. of aq. N- NH_3 - $CHCl_3$ (1:1, v/v); 0.05 ml. of the aqueous phase was spotted on Schleicher and Schüll (Dassel, Germany) filter paper no. 2043b and chromatographed for 14 hr. in butanol saturated with aq. 3% (w/v) NH_3 according to Cavallini & Mondovi (1957). The infrared spectrum of the 2,4-dinitrophenylhydrazone was determined in a Nujol (liquid paraffin) mull with a Perkin-Elmer model 12C spectrophotometer fitted with a NaCl prism.

Condensation of formaldehyde with sodium oxaloacetate. Oxaloacetic acid (0.5 g.) was dissolved in 5 ml. of water, and about 7.6 ml. of N-NaOH was added to adjust the pH to 7.4. This solution was mixed with 6.27 ml. of 1.8% (v/v) formaldehyde soln. (corresponding to 113 mg. of formaldehyde) and warmed at 40° for 3 hr. The disappearance of formaldehyde was determined according to Kramer *et al.* (1959) and the solution was then freeze-dried. The compound obtained was dried for 12 hr. *in vacuo* over P_2O_5 before being analysed. The analytical data are given in the Results section. To form the 2,4-dinitrophenylhydrazone 200 mg. of this compound was dissolved in 20 ml. of a solution of 2,4-dinitrophenylhydrazine prepared as indicated above. Again CO_2 was evolved and was quantitatively determined. An oily compound separated and solidified after the addition of solid NaCl. The filtered compound was crystallized from methanol and then analysed as indicated above.

Preparation and activity of crude and purified aconitase. Crude aconitase was prepared from rat liver according to

Johnson (1939), and purified aconitase from pig heart according to Anfinsen (1955). The activity was assayed as reported by Ruffo *et al.* (1959), usually with *cis*-aconitate as substrate. Further experiments were also done with citrate as substrate. In this case the enzymic solution was mixed in a quartz cuvette of the Beckman spectrophotometer with 0.05M-phosphate buffer, pH 7.4, containing citrate as suggested by Anfinsen (1955); the formation of *cis*-aconitate was determined by reading the increment of the extinction at 240 m μ .

Incubation of the new compounds with aconitase. Crude aconitase was incubated for 1 hr. at 38° with 10 μ moles of *cis*-aconitate as indicated above, with or without various concentrations of the new compounds. The same experiment was repeated with purified aconitase, the period of incubation being shortened to 15 min. To establish whether the inhibition was of the competitive type, purified aconitase was incubated with various concentrations of *cis*-aconitate with or without the same concentration of the condensation compound of sodium oxaloacetate with sodium glyoxylate. The reaction was stopped after 15 min. by the addition of 3 ml. of 5% (w/v) tungstic acid; citric acid was determined on the filtrates according to Ettinger, Goldbaum & Smith (1952). The results obtained were plotted according to Lineweaver & Burk (1934). Similar experiments were carried out with citrate as substrate. In this case the enzyme solution was added to a Beckman quartz cuvette containing 0.05M-phosphate buffer and increasing concentrations of citrate warmed at 20° (see Fig. 4). After mixing, the extinction at 240 m μ was read each 30 sec. and the time-interval between 30 and 60 sec. was chosen for comparative readings. The change of $E_{1cm.}/mg.$ of enzyme/min. was plotted according to Lineweaver & Burk (1934).

RESULTS

Preparation of trisodium α -hydroxy- β -oxalosuccinate by the condensation of sodium glyoxylate with sodium oxaloacetate. Formaldehyde reacts in its hydrate form with acid C-H groups, yielding a new C-C link (formylation) (Fournau, Benoit & Firmenich, 1930; Schöpf & Thierfelder, 1935), and this reaction may be catalysed by alkali-metal ions. Accordingly, glyoxylate may react with the acidic CH_2 group of oxaloacetate, giving a condensation compound. The rate of the reaction in our experiments was followed by determining the disappearance of the aldehyde group of glyoxylic acid at different temperatures of incubation and pH values, and in the presence or in the absence of Mg^{2+} ions. The results (Fig. 1) showed that the reaction did not occur in an acid medium, was rather slow at pH 7.4, but increased sensibly either by the addition of Mg^{2+} ions or by increasing the temperature to 40°. At 40° in the absence of Mg^{2+} ions the condensation was complete after 3 hr. The compound, when freeze-dried and finally dried, appeared as a white, slightly hygroscopic substance (Found: C, 21.9; H, 2.7; OH, 5.4; Na, 21.6; H_2O (by the Fischer method), 16.6. Calc. for

above, but with formaldehyde instead of glyoxylate. The results showed that, after incubation at pH 7.4 for 3 hr. at 40°, formaldehyde disappeared completely. The compound (V) obtained was freeze-dried (Found: C, 26.3; H, 2.3; Na, 20.6; OH, 7.9; H₂O (by the Fischer method), 8.4. Calc. for C₅H₄Na₂O₆·H₂O: C, 26.8; H, 2.2; Na, 20.5; OH, 7.6; H₂O, 8.1 %). Moreover, two carboxyl groups, a keto group and a non-tertiary hydroxy group were found, and no aldehyde group was present even after acidification, which produced immediate release of 1 mol.prop. of carbon dioxide/mol.prop. of the compound. The compound (V) reacted easily with 2,4-dinitrophenylhydrazine, lost carbon dioxide and gave a 2,4-dinitrophenylhydrazone (VI) of m.p. 189–192° (Found: N, 19.03. Calc. for C₁₀H₁₀N₄O₇: N, 18.78 %). Also in the compound (VI), two groups which may be acetylated (–OH and >NH) were detected. The infrared spectrum in a Nujol mull showed typical bands at 3340 cm.^{–1} (OH stretching), 3160 cm.^{–1} (NH stretching), 2600 cm.^{–1} (OH of carboxyl group), 1690 cm.^{–1} (CO stretching), 1620 and 1595 cm.^{–1} typical bands of phenylhydrazone derivatives), 1510 cm.^{–1} (asymmetrical NO₂ stretching) and 1350 cm.^{–1} (symmetrical NO₂ stretching). All these data showed that the condensation compound, formed by the reaction of sodium oxaloacetate with formate, was the monohydrate of disodium α-hydroxymethyl-β-oxosuccinate (V). Compound (V) reacted with 2,4-dinitrophenylhydrazine, liberating carbon dioxide and giving the corresponding phenylhydrazone of the γ-hydroxy-α-oxobutyric acid (equation 3).

Since no aldehyde group was detected after acidification of (V), it is clear that the carbon dioxide was evolved from the carboxyl group in the β-position to the keto group of the original oxaloacetic acid. The analogy of these reactions with the preceding ones is evident.

Mode of action of trisodium α-hydroxy-β-oxosuccinic acid (III) on aconitase. The first experiments were done to establish whether compounds (III) and (V) produced the same inhibition of aconitase activity as did equimolecular concentrations of glyoxylate plus oxaloacetate (Ruffo *et al.* 1959). Therefore crude and purified aconitase preparations were incubated with 10 μmoles of *cis*-

Fig. 2. Effect of compounds (III) and (V) on aconitase. For the experiments with crude aconitase, each incubation mixture contained, in a final volume of 4 ml., 10 μmoles of *cis*-aconitate, 3 ml. of diluted (1:50) liver extract in 0.1 M-phosphate buffer, pH 7.4, 0.9 % (w/v) with respect to KCl, and either ●, compound (III), or △, compound (V), as indicated; incubation was for 1 hr. at 38°. For the experiments with purified enzyme, each incubation mixture contained, in a final volume of 4 ml., 10 μmoles of *cis*-aconitate, 0.1 ml. of purified aconitase solution (corresponding to about 3 mg. of protein/ml.), 2.5 ml. of the same buffer as above, and ○, compound (III) as indicated; incubation was for 15 min. at 38°. For deproteinization, 3 ml. of freshly prepared 5 % (w/v) tungstic acid was used.

aconitate in the presence or absence of various concentrations of (III) and (V); citrate was determined at the end of the incubation.

The results (Fig. 2) showed that almost complete inhibition of aconitase activity was obtained with 1 μmole of (III)/ml., independent of the degree of purification of the enzyme; with purified aconitase more than 50 % inhibition was obtained with 0.125 μmole/ml. To test whether the inhibition was competitive, purified aconitase was incubated with suitable concentrations of (III) and increasing concentrations of *cis*-aconitate or citrate. Characteristic curves indicating competitive inhibition were obtained when either *cis*-aconitate or citrate was the substrate (Figs. 3 and 4). No inhibition of the crude aconitase by 1 μmole of disodium α-hydroxymethyl-β-oxosuccinate (V)/ml. was found (Fig. 2).

Fig. 3. Competitive inhibition of aconitase by oxalomalate. Incubation mixtures contained, in a final volume of 4 ml., 0.1 ml. of enzyme preparation (corresponding to about 4 mg. of protein/ml.), 2.5 ml. of 0.1 M-phosphate buffer, pH 7.4, 0.9% (w/v) with respect to KCl, and *cis*-aconitate as indicated; incubation was for 15 min. at 38°. ●, Control; ○, 2 μ moles of oxalomalate, added to incubation mixture.

and further tests with concentrations ten times that of trisodium α -hydroxy- β -oxalosuccinate (III) were also negative.

DISCUSSION

The experimental results showed that, under carefully controlled physiological conditions of pH and temperature, condensation between sodium oxaloacetate and sodium glyoxylate occurred, yielding a trisodium salt of a new tricarboxylic acid, which was identified with α -hydroxy- β -oxalosuccinic acid (oxalomalic acid). The reaction did not occur in acid medium, possibly because the condensation product was not stable in acid conditions. The reaction may be compared with that between formaldehyde and other aldehydes, discovered by Tollens in 1889, and more recently utilized by Fourneau *et al.* (1930) for the preparation of amino alcohols. We have also found that formaldehyde will condense with oxaloacetate: in this case a similar compound, but C_6 instead of C_8 , namely α -hydroxymethyl- β -oxosuccinic acid, was formed.

With regard to the biological effect of these new compounds, α -hydroxymethyl- β -oxosuccinate (V) did not affect aconitase activity, but oxalomalate (III) caused inhibition. These results confirmed work by Ruffo *et al.* (1959, 1960) which showed that, by incubation of aconitase with oxaloacetate plus glyoxylate, the enzyme was strongly inhibited.

Fig. 4. Competitive inhibition of aconitase by oxalomalate. Each incubation mixture, prepared directly in the quartz cuvettes of a Beckman spectrophotometer, contained citrate, as indicated, in 3 ml. of 0.05 M-phosphate buffer, pH 7.4, warmed to 20°. The reaction was started by mixing into the cuvette 0.1 ml. of enzyme preparation (corresponding to about 1.5 mg. of protein/ml.). ●, Control; △, 0.05 μ mole of oxalomalate added; ○, 0.01 μ mole of oxalomalate added.

Moreover, as was previously supposed, it has now been demonstrated that the condensation reaction is a non-enzymic one, and gives a new tricarboxylic acid. The results showed that 1 μ mole of oxalomalate (III)/ml. completely inhibited a purified aconitase preparation, and that the mechanism of the inhibition, with either *cis*-aconitate or citrate as substrate, was of the competitive type. This result is compatible with the structural similarity between oxalomalate and isocitrate. Since the chemical condensation occurred spontaneously under physiological conditions, and other results showed that (a) the inhibition of aconitase by glyoxylate plus oxaloacetate *in vivo* was reversible (Romano, Ruffo & Adinolfi, 1960) and that (b) glyoxylate appeared in considerable amounts in the organs and blood of rats given a thiamine-deficient diet (Liang, 1960, 1962), the condensation of glyoxylate with oxaloacetate in animal cells may be physiologically important. From this reaction a powerful inhibitor of aconitase is formed, which depresses the activity of the enzyme. Since the limitation of aconitase activity is expected to diminish the rate of the whole citric acid-cycle sequence, the results suggest that glyoxylate may control the rate of the terminal respirative processes in animal cells. This possibility has been examined experimentally (Ruffo, Adinolfi, Budillon & Capobianco, 1962).

SUMMARY

1. The rate of the condensation of glyoxylate and oxaloacetate was followed by determining the disappearance of the aldehyde group.

2. The reaction was complete after 3 hr. at 40° and pH 7.4, and did not occur at pH 3. The compound obtained was identified as the trihydrate of the trisodium salt of α -hydroxy- β -oxalosuccinic acid (oxalomalic acid).

3. A similar compound, but C₅ instead of C₆, namely α -hydroxymethyl- β -oxosuccinic acid, was obtained when formaldehyde was substituted for glyoxylate in the condensation.

4. Only oxalomalate was able to inhibit the activity of crude or purified preparations of aconitase. The inhibitory effect was of the competitive type, with either *cis*-aconitate or citrate as substrate.

5. A possible physiological role of glyoxylate to control the rate of the citric acid cycle, through the condensation with oxaloacetate, is suggested.

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Biochem. J. (1962) **85**, 593

Control of the Citric Acid Cycle by Glyoxylate

2. MECHANISM OF THE INHIBITION OF RESPIRATION IN LIVER AND KIDNEY PARTICLES

By A. RUFFO, ANNA ADINOLFI, G. BUDILLON AND G. CAPOBIANCO
Laboratory of Biochemistry, Institute of Tumours, Pascale Foundation, Naples, Italy

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The observation that the chemical reaction of glyoxylate with oxaloacetate produced the formation of oxalomalate, a new tricarboxylic acid, which inhibits the activity of crude and purified aconitase (Ruffo, Romano & Adinolfi, 1959; Ruffo, Testa, Adinolfi & Pelizza, 1962), led us to investigate the possible physiological significance of this reaction in animal tissues. Since both glyoxylate and oxaloacetate are cellular metabolites, it seems possible that glyoxylate might control the citric acid cycle, owing to its property of reacting with oxaloacetate to form a powerful inhibitor of aconitase. To investigate this problem, we determined the oxygen uptake, the P:O ratio and the formation of citrate in intact or damaged rat-liver mitochondria and in rabbit-kidney cyclophorase preparations, incubated with different substrates of the citric acid cycle in the presence and in the

absence either of glyoxylate (with and without oxaloacetate) or of oxalomalate.

The main results obtained showed that glyoxylate, alone or with oxaloacetate, and oxalomalate, always produced inhibition of the respiratory processes, but without an equivalent depression of the P:O ratio. The inhibition, in many cases, was accompanied by an accumulation of citrate.

EXPERIMENTAL

Preparation and incubation of intact and damaged mitochondria. The livers of adult albino rats were homogenized in 0.25M-sucrose with a Potter glass homogenizer and centrifuged for 10 min. at 600g to sediment nuclei and cell debris, according to Schneider (1949). The supernatant was centrifuged for 15 min. at 8500g, and the sedimented mitochondria were washed twice with 0.25M-sucrose and resuspended in the same solution. Samples