The Inhibition, by Intermediary Metabolites, of Isocitrate Lyase from *Chlorella pyrenoidosa*

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1. Intermediary metabolites, arising from the glyoxylate by-pass, were tested for their effect on the activity of isocitrate lyase, the first enzyme of the by-pass. 2. Oxaloacetate and pyruvate inhibited the enzyme when present at concentrations similar to that of the substrate. 3. Inhibition was competitive and did not depend on a non-optimum pH. The affinities determined at the pH optimum were: K_i (oxaloacetate), $3.7 \times 10^{-5} \text{ M}$; K_i (pyruvate), $6.6 \times 10^{-5} \text{ M}$. 4. The significance of the inhibitions is discussed with particular reference to the known inhibition by phosphoenolpyruvate.

The activity of isocitrate lyase (EC 4.1.3.1) diverts intermediates of the tricarboxylic acid cycle into the glyoxylate cycle. The regulation of this pivotal enzyme is therefore of interest. Ashworth & Kornberg (1963) reported that phosphoenolpyruvate, which may be regarded as a product of the glyoxylate cycle, inhibits the enzyme from *Escherichia coli* at less than 1 mM, and Harrop & Kornberg (1966) described similar inhibition of the enzyme from *Chlorella vulgaris*. These observations were confirmed and extended by Syrett & John (1968), who reported an inhibition of the enzyme from *Chlorella pyrenoidosa* at the acidic pH of the Kornberg (1965a) assay, but also showed that the inhibition by phosphoenolpyruvate was strongly dependent on an acidic pH and was decreased to one-tenth at the pH optimum of the enzyme. The investigations now reported sought to establish whether other products of the glyoxylate cycle might also act as inhibitors of this first reaction peculiar to the cycle.

MATERIALS AND METHODS

Chemicals. DL-Isocitrate (trisodium salt) and glyoxylate (sodium salt) were purchased from British Drug Houses Ltd., Poole, Dorset; dimer-free pyruvate (sodium salt), 2-oxoglutaric acid, oxaloacetic acid, phosphoenolpyruvate (trisodium salt), 3-phosphoglyceric acid and 2-phosphoglyceric acid were from Sigma (London) Chemical Co. Ltd., London, S.W. 6; α -hydroxyglutarate (zinc salt) was from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; dithiothreitol (Cleland, 1964) was from Calbiochem Ltd., London, W. 1. All other chemicals were of reagent grade and were purchased from British Drug Houses Ltd.

Enzyme purification. The method of John & Syrett (1967) was employed.

Enzyme assay. The procedure was based on that of John & Syrett (1967) and measured the quantity of glyoxylate formed during 10 min. incubation at 30°. The incubation mixture contained (in a final volume of 1 ml.): 50 μ moles of imidazole, adjusted with HCl to pH 7.6 or to pH 6.8, or 50 μ moles of tris, adjusted with HCl to pH 8.5; 5 μ moles of MgCl_2 ; 2 μ moles of dithiothreitol; 1 μ mole of EDTA; 10 μ moles of DL-isocitrate. The reaction was started by the addition of isocitrate and stopped by the addition of 0.2 ml. of 20% (w/v) trichloroacetic acid. Glyoxylate was measured by the method of Kramer, Klein & Baselice (1959) by the addition of 1 ml. of freshly prepared 1% (w/v) phenylhydrazine hydrochloride, and then, after 60 min. at 30°, 1 ml. of conc. HCl and 1 ml. of 1% (w/v) $\text{K}_3\text{Fe}(\text{CN})_6$. Extinction at 520 m μ was determined within 2–4 min.

When lower concentrations of isocitrate were employed the volume at incubation was increased to 3 ml. at the same concentrations of the reagents, the reaction was stopped with 0.6 ml. of 20% (w/v) trichloroacetic acid and the final assay volume was minimized by the addition of 0.3 ml. of 10% (w/v) phenylhydrazine hydrochloride and, after 60 min. at 30°, 1 ml. of conc. HCl was again added, followed by 0.3 ml. of 10% (w/v) $\text{K}_3\text{Fe}(\text{CN})_6$.

Glyoxylate formed was determined by comparison with simultaneously prepared standards of glyoxylate. Where metabolites were included in the assay, separate blanks and standards were simultaneously determined for each concentration of metabolite. Assays containing pyruvate were measured 60 min. after colour development.

RESULTS

In view of the effect of pH on the inhibition of isocitrate lyase by phosphoenolpyruvate (Syrett & John, 1968), this investigation was extended to an acidic and an alkaline pH, in addition to the optimum of pH 7.6 (see Table 1).

Table 1. *Effect of intermediary metabolites on the activity of isocitrate lyase from Chlorella pyrenoidosa*

Metabolites were adjusted to the appropriate pH with HCl or NaOH. α -Hydroxyglutarate was obtained as the free acid by dissolving the zinc salt in an equivalent volume of H_2SO_4 before passing the solution through an acid-activated Zeo-Karb column. The standard assay procedure used for series A was modified in series B so that the incubation mixture contained 100 mM-triethanolamine hydrochloride-NaOH, pH 7.6, 5 mM-MgCl₂, 10 mM-GSH and 20 mM-DL-isocitrate.

		Inhibition (%)								
Metabolite	Concn. ...	pH 6.8			pH 7.6			pH 8.5		
		1 mM	5 mM	10 mM	1 mM	5 mM	10 mM	1 mM	5 mM	10 mM
Series A										
Phosphoenolpyruvate		63	90	95	8	29	50	2	15	25
Succinate		46	76	94	13	39	54	4	14	30
Pyruvate		1	8	21	16	56	75	63	88	95
Oxaloacetate		18	73	91	13	50	88	20	64	70
2-Oxoglutarate		1	—	10	5	—	36	12	—	73
3-Phosphoglycerate		1	—	26	13	—	29	0	—	30
Malate		5	—	17	5	—	12	8	—	52
Fumarate		5	—	7	9	—	12	4	—	17
Citrate		3	—	11	6	—	15	15	—	38
Aspartate		1	—	5	0	—	3	2	—	1
Glutamate		2	—	4	1	—	4	1	—	3
Series B										
2-Phosphoglycerate		—	—	—	—11	—	1	—	—	—
α -Hydroxyglutarate		—	—	—	—15	—	5	—	—	—

Most of the metabolites tested had little or no effect on enzyme activity, and, although 10 mM-malate and 10 mM-2-oxoglutarate inhibited by 52% and 73% respectively at pH 8.5, they inhibited by only 12% and 36% respectively at the pH optimum and are therefore considered improbable natural inhibitors.

Inhibition of more probable significance *in vivo* was observed for phosphoenolpyruvate, succinate, pyruvate and oxaloacetate, each of which, at 10 mM, inhibited enzyme activity by more than 50% at the pH optimum.

Low concentrations of phosphoenolpyruvate inhibited only at an acid pH. By contrast, oxaloacetate inhibited isocitrate lyase strongly at all three pH values. Pyruvate is more inhibitory at an alkaline pH, whereas succinate is more inhibitory at an acid pH; succinate is never the most effective inhibitor.

Of these metabolites only phosphoenolpyruvate has been investigated with respect to the nature of the inhibition (Syrett & John, 1968). Accordingly, the nature of the inhibition due to oxaloacetate, pyruvate and succinate, with respect to substrate binding, was investigated. The results are recorded according to the double-reciprocal plot of Lineweaver & Burk (1934) in Fig. 1. The three metabolites tested are all competitive inhibitors of this enzyme and their affinities are as follows: K_i (oxaloacetate), 3.7×10^{-5} M; K_i (pyruvate),

6.6×10^{-5} M; K_i (succinate), 5.8×10^{-4} M. At a substrate concentration of 0.5 mM-DL-isocitrate, which gives 88% of the V_{max} rate, 0.5 mM-oxaloacetate inhibited enzyme activity by 68% and 0.5 mM-pyruvate gave 60% inhibition. Slighter inhibition is apparent in Table 1, but this is a consequence of the high substrate concentration, which is about 160 times the K_m and so overcomes the effect of low concentrations of competitive inhibitors. α -Hydroxyglutarate is not an inhibitor of the *Chlorella pyrenoidosa* enzyme, although Rabin, Reeves, Wegener, Megraw & Ajl (1965) report, without specifying concentration, that it inhibits the isocitrate lyase from *E. coli* 26.

DISCUSSION

The competitive inhibition of isocitrate lyase by low concentrations of oxaloacetate and pyruvate has not previously been described. McFadden & Howes (1963) and Rao & McFadden (1965) failed to show appreciable inhibition of the enzyme from *Pseudomonas indigofera* after testing many intermediary metabolites, but they omitted pyruvate and oxaloacetate. Succinate has been reported to inhibit the enzyme from *Pseudomonas aeruginosa* (Smith & Gunsalus, 1957) and that from *Micrococcus* (Kornberg, Collins & Bigley, 1960). Inhibition in both cases was non-competitive, but the affinity for succinate was low (K_i 7.0×10^{-3} M and 3.1×10^{-3} M

Fig. 1. Effect of substrate concentration on the rate of the cleavage reaction of isocitrate lyase from *Chlorella pyrenoidosa*: ○, without added metabolite; ▽, with 0.5 mm-oxaloacetate; △, with 0.5 mm-succinate; □, with 0.5 mm-pyruvate; ●, with 0.17 mm-pyruvate + 0.17 mm-oxaloacetate. Assays of 3 ml. incubation volume contained 50 mm-imidazole-HCl buffer, pH 7.6, 5 mm-MgCl₂, 2 mm-dithiothreitol and 1 mm-EDTA. The enzyme was purified according to the standard procedure.

respectively), and it remains possible that these enzymes are more sensitive to phosphoenolpyruvate, oxaloacetate or pyruvate, which have since been reported to inhibit other enzymes at lower concentrations than does succinate.

One group of workers has, however, persuasively described a feedback control of isocitrate lyase and so of the glyoxylate cycle. Kornberg and co-workers report strong inhibition by phosphoenolpyruvate of the enzyme from *E. coli* (Ashworth & Kornberg, 1963). This enzyme is also inhibited, but to a smaller extent, by pyruvate (Kornberg, 1966).

Different circumstances apply in *Chlorella pyrenoidosa*. Phosphoenolpyruvate does inhibit at physiological concentration under acid conditions, but at the pH optimum of 7.6 inhibition is decreased to about one-tenth (Syrett & John, 1968). Inhibition by oxaloacetate, however, remains strong at the pH optimum and inhibition by both oxaloacetate and pyruvate exceeds inhibition by phosphoenolpyruvate at pH 7.6. When oxaloacetate and pyruvate are each present at 0.17 mm and with 0.25 mm-substrate, the enzyme is inhibited by 50%, and thus inhibition of isocitrate lyase *in vivo* must be considered the probable result if concentrations

of oxaloacetate and pyruvate approach the concentration of isocitrate. The data of Millbank (1954) show that the concentrations of inhibitor employed here are indeed physiological, since the intracellular concentrations of pyruvate in this strain of *Chlorella* range between 0.1 and 1.0 mm. There is an apparent excess of isocitrate lyase activity in this alga in comparison with the activity necessary for growth (John & Syrett, 1968), and it seems reasonable to suppose that the excess of enzyme represents latent activity that, *in vivo*, is inhibited as a result of the fine control of the glyoxylate cycle.

It is noteworthy that oxaloacetate and pyruvate are competitive inhibitors of isocitrate lyase, since they do not resemble the C₆ substrate *threo*-D₈-isocitrate and, further, 2-oxoglutarate, although it is closer in size to the substrate, is not an effective inhibitor. It is possible that the specificity of response to inhibitors results from a separate inhibitor-binding site, as described by Changeux (1963) to account for the competitive inhibition of *E. coli* L-threonine deaminase by leucine. An alternative and simpler explanation is that pyruvate and oxaloacetate resemble, not isocitrate, but the keto-carboxyl grouping of glyoxylate and so interfere with the formation of enzyme-product complex. On this interpretation the small oxaloacetate and pyruvate molecules would be sterically acceptable to the active site, but the larger 2-oxoglutarate would conflict with the region of the active site that binds C-4, C-5 and C-6 of isocitrate, because it lacks a carboxyl group at C-4, lacks a branched configuration of C-3, C-4 and C-5 and bears a carboxyl group at C-5 instead of C-6. The suggestion that inhibition is due to a similar binding of glyoxylate, pyruvate and oxaloacetate is supported by recent investigations of an analogous C-Clyase: 4-hydroxy-2-oxoglutarate aldolase. This enzyme can bind either pyruvate or glyoxylate at one site (Kobes & Dekker, 1966) and can also bind oxaloacetate at this site (Kobes & Dekker, 1967).

Kornberg (1965b) has emphasized the importance of anaplerotic reactions to the continuance of biosynthesis. Isocitrate lyase is the key enzyme in diverting the catabolic tricarboxylic acid cycle to the anaplerotic glyoxylate cycle, and so may be regarded as occupying a pivotal position in the control of intermediary metabolism. Such key enzymes, which regulate branch points in metabolism, are frequently susceptible to inhibition by a product of the sequence of reactions they initiate. The accepted model for such inhibition is that the metabolites, which need bear no resemblance to the substrate, operate as so-called allosteric effectors, but the regulation of isocitrate lyase in *Chlorella pyrenoidosa* does not entirely follow this pattern. Although the inhibition produced by phosphoenolpyruvate has been shown to be non-competitive

(Syrett & John, 1968) and so is consistent with a response to an allosteric effector, the inhibition reported here for oxaloacetate and pyruvate is competitive and more probably results simply from binding at the active site.

The relative importance of the competitive inhibition due to oxaloacetate and pyruvate and the non-competitive inhibition due to phosphoenolpyruvate is difficult to assess. Pyruvate and oxaloacetate can act to maintain the balance of intermediates common to the glyoxylate cycle and the tricarboxylic acid cycle. The withdrawal of 2-oxoglutarate, succinate or oxaloacetate for biosynthesis is expected to lower the concentration of oxaloacetate and of pyruvate and so to result in a compensating increase in isocitrate lyase activity. Oxaloacetate, which is the most effective inhibitor *in vitro* at the pH optimum, may be the more important natural inhibitor, since its concentration should be affected most directly by the greater part of biosynthesis, and this inhibitor is effective over the whole range of pH 6.8–8.5.

These competitive inhibitors can control the internal balance of intermediates common to the glyoxylate cycle and the tricarboxylic acid cycle, but cannot hold them to any absolute concentration since an excess of the products of the glyoxylate cycle leads, via the reactions of the tricarboxylic acid cycle, to an increase in isocitrate concentration, which would overcome competitive inhibition. Under such conditions inhibition due to phosphoenolpyruvate would be particularly effective, since it is unaffected by high concentrations of substrate. It is attractive that a rise in concentration of the tricarboxylic acid-cycle acids should favour the acid pH at which phosphoenolpyruvate could inhibit isocitrate lyase, and so curb their further accumulation. A pH-dependence has also been reported by Atkinson and co-workers in the adenylate control of citrate synthase (Jangaard, 1966).

In *E. coli* it is less likely that pyruvate or oxaloacetate are important natural inhibitors. Kornberg (1966) has shown that, although the *E. coli* enzyme is inhibited by pyruvate, phosphoenolpyruvate inhibits at lower concentrations. Umbarger (1960) describes inhibition of the *E. coli* enzyme by oxaloacetate, but the significance of this observation is not clear, since it refers to the back-reaction and since no details of the concentration of metabolites are given. Oxaloacetate does inhibit the forward reaction of isocitrate lyase from *E. coli*

(J. Pollock & P. C. L. John, unpublished work), but 5 mM-oxaloacetate is necessary to produce 62% inhibition of activity and so the enzyme is again more sensitive to phosphoenolpyruvate. Indeed, the enzyme can be inhibited *in vivo* even when oxaloacetate cannot be synthesized, as follows the addition of glucose to a strain of *E. coli* lacking phosphoenolpyruvate carboxylase (Ashworth & Kornberg, 1963).

In *Chlorella pyrenoidosa* therefore the feedback control of glyoxylate by-pass activity is more complex than work with other organisms has so far indicated.

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