Mass Spectrometric Service at the University of Göteborg.

3-Bromo-2-chloronitrobenzene prepared from 2-bromo-6-nitroaniline by the same method as described for 2-chloro-3-fluoronitrobenzene,[9] gave a yield of 81 % after steam distillation and one recrystallization from hexane, m.p. 57.0 – 58.5 °C, lit.[1] 57.0 – 58.0 °C.

3-Bromo-2-fluoronitrobenzene. 3-Bromo-2-chloronitrobenzene (14.2 g, 0.06 mol, dried over P_2O_5) was boiled for 7 days at 150 °C with 6.95 g (0.12 mol) of commercial anhydrous potassium fluoride (which was heated before use in order to remove all moisture) in 25 ml of dimethylformamide, dried over molecular sieves (4 Å). The reaction was followed by GLC. After 7 days the reaction had gone to 96 – 97 % completion. The yield was 10.0 g (76 %) after steam distillation. After two recrystallizations from hexane and two from methanol, the yield was 2.4 g, m.p. 29.5 – 30.5 °C. 3-Bromo-2-fluoronitrobenzene has apparently not been described in the literature. The mass spectrum revealed a 1:1 doublet at *m/e* 219 and 221, as expected for the desired compound.

^{19}F NMR spectrum for 3-bromo-2-fluoronitrobenzene in $CDCl_3$: 111 ppm upfield from CCl_3F (octet). J_{F-H_5} 1.48 Hz, J_{F-H_4} 5.75 or 6.45 Hz and J_{F-H_6} 6.45 or 5.75 Hz.

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The Role of Oxaloacetate as Feed-back Inhibitor of Isocitrate Lyase in Baker's Yeast

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It has been shown that oxaloacetate competitively inhibits purified isocitrate lyase from *Chorella pyrenoidosa*,[1] *Brevibacterium flavum*,[2] and *Candida guillermondii*.[3] For oxaloacetate to function as a feed-back inhibitor of isocitrate lyase (EC 4.1.3.1) *in vivo*, it must inhibit the lyase in the conditions existing inside the cell. Because the Michaelis constant for *threo*-D_s-isocitrate of isocitrate lyase from *Chlorella* is smaller (23 μM) than the *in vivo* concentration of isocitrate, John and Syrett [1] concluded that the enzyme was normally saturated with its substrate, and therefore that oxaloacetate, a competitive inhibitor, cannot cause significant inhibition *in vivo*. However, the Michaelis constant is much higher (300 μM) for the enzyme from *C. guillermondii*, and Hildebrandt and Weide [3] have concluded that oxaloacetate may be an effective regulator of isocitrate lyase in this organism. For both enzymes inhibition constants are small (37 and 50 μM, respectively). We have investigated the inhibition of isocitrate lyase from baker's yeast, *Saccharomyces cerevisiae* by several intermediates of the TCA cycle. Our results are quite similar to those of Hildebrandt and Weide for *C. guillermondii*,[3] except in the case of oxaloacetate. Reaction rates were estimated by a method which prevented contact between the oxaloacetate and phenylhydrazine reagent used to estimate the product, glyoxylic acid.

Material and methods. A_4 growth-stage baker's yeast from Alko Yeast Factory, Rajamäki, was used. The culture has been described in detail elsewhere.[4] Sodium glyoxylate was from Fluka AG (Buchs, SG., Switzerland), trisodium-DL-isocitrate from the Sigma Chemical Company (St.Louis, Mo., USA) and oxaloacetate from C. F. Boehringer & Soehne GmbH (Mannheim, West-Germany). All other reagents were of analytical grade from E. Merck AG (Darmstadt, West-Germany).

Isocitrate lyase was purified as follows. 100 g baker's yeast was suspended in 250 ml 0.2 M potassium phosphate buffer pH 7.5 Ballotini beads (40 ml, No. 31/12, diameter 0.25 mm) were added to 25 ml fractions and the cells were treated for 6 min in a Mini-mill disintegrator (Gifford-Wood Co., New Jersey, USA) using a rotor speed of 3300 rpm. The cup of the apparatus was kept in an ice-bath during the disintegration.

The protamine sulfate and ammonium sulfate precipitations, dialysis, and chromatography on

a DEAE column were performed according to Olson.[5] No heat treatment or ethanol precipitation was performed. The buffer solutions used were essentially the same as those of Olson,[5] but were 3 mM in $MgCl_2$, and 1 mM in cysteine and EDTA. A purification of about 40 fold was obtained.

The isocitrate lyase activity was determined at pH 6.0[5] and 30 °C, by the method of Dixon and Kornberg.[6] The reaction was initiated by adding DL-isocitrate. In the inhibition experiments the enzyme and inhibitor were incubated together for 10 min at 30 °C before the substrate was added. However, to prevent contact between oxaloacetate and phenylhydrazine when determining the effect of oxaloacetate on the enzyme activity, gloxylate formed in the reaction was estimated colorimetrically as follows.[7] At the end of the enzymic reaction 0.3 ml 0.5 M oxalic acid and 0.2 ml 1 % phenylhydrazine were added to 1 ml of reaction mixture, which contained 65 μM potassium phosphate buffer, pH 6.0, 2 μM cysteine, 1 – 5 μM oxaloacetate, 2.5 μM $MgCl_2$, about 5 μl enzyme and 0.5 – 5 μM DL-isocitrate. The mixture was held for 5 min at 105 °C and then in ice for at least 5 min. To the chilled mixture was added 1 ml concentrated hydrochloric acid and 0.5 ml 3 % potassium ferricyanate. The absorbance at 520 nm was measured on a Perkin-Elmer 124 double-beam spectrophotometer between 2 and 5 min after adding ferricyanate. At the same time an individual gloxylate standard was determined for each inhibitor concentration.

The protein content was estimated from the equation: protein content (mg/ml) = $0.77A_{280} - 0.38A_{260}$, where A_{280} and A_{260} are the absorbances at 280 and 260 nm, respectively.[8] The enzyme preparation used produced 3 μmol glyoxylate min^{-1} (mg protein)$^{-1}$ at 30 °C.

Results. The method of Dixon and Kornberg [6] gave a value of 0.71 mM for the Michaelis constant (Fig. 1). According to the manufacturer, *threo*-D_S-isocitrate, the real substrate for the enzyme, constitutes 49.5 % of the DL-isocitrate preparation used, and so the corrected value for the Michaelis constant for this substrate is 0.35 mM. Olson [5] reported that *threo*-L_S-isocitrate does not inhibit isocitratelyase. In Fig. 2a are Lineaweaver-Burk plots obtained at different concentrations of oxaloacetate. In Fig. 2b the slopes of the straight lines in Fig. 2a are plotted as a function of the inhibitor concentration. It can be seen that oxaloacetate causes linear competitive inhibition, with an inhibition constant of 1.1 mM.

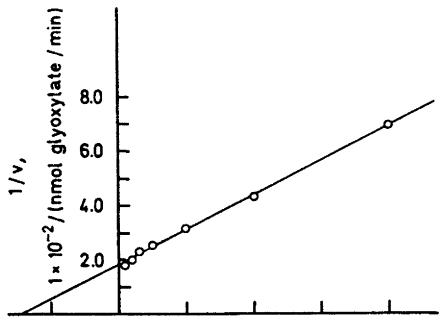

Fig. 1. Michaelis constant for DL-isocitrate at pH 6.0 and 30 °C. The data were obtained by the method of Dixon and Kornberg.[6]

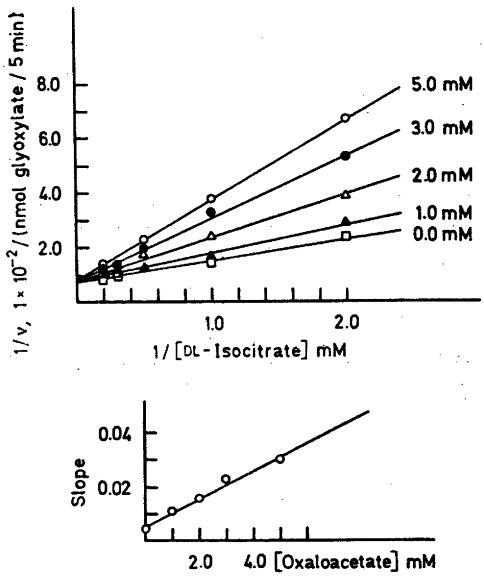

Fig. 2a. Oxaloacetate inhibition of inhibitor concentrations of 0, 1, 2, 3, and 5 mM.
Fig. 2b. Gradients of the straight lines obtained in Fig. 2a as a function of inhibitor concentration.

Discussion. The Michaelis constant obtained for *threo*-D_S-isocitrate (0.35 mM) is only a third the size of that reported (1.2 mM) by Olson for baker's yeast,[5] but is in good agreement with the value (0.3 mM) obtained by Hildebrandt and Weide.[9]

The oxaloacetate inhibition constant obtained is an order of magnitude larger than those reported by John and Syrett [1] and Hildebrandt and Weide,[3] and slightly larger than that (1 mM) of Ozaki and Shiio.[2] The experimental method employed differs from that of Hildebrandt and Weide [3] in that a different yeast species was used and that the phenylhydrazine reagent was not allowed to come in contact with the oxaloacetate until the enzymic reaction was complete. Ozaki and Shiio [2] used a scheme corresponding to the one described here.

Although they determined the inhibition at only one oxaloacetate concentration, Hildebrandt and Weide [3] supposed that the competitive inhibition by oxaloacetate of isocitrate lyase from *C. guillermondii* was linear. Our results show this to be correct for the enzyme from *S. cerevisiae*. When the inhibition of isocitrate lyase caused by oxaloacetate is small, the regulation mechanism suggested by Hildebrandt and Weide [3] for *C. guillermondii* is not possible for baker's yeast. However, according to the inhibition constant for oxaloacetate of 1.1 mM reported here, and a value for the concentration of oxaloacetate of less than 25 μM in baker's yeast grown on ethanol,[10] the maximum inhibition of isocitrate lyase by oxaloacetate (*i.e.*, at very low isocitrate concentrations) is only 2.5 %. Further, at 25 °C and pH 7.4 citrate and isocitrate make up 91 and 6 %, respectively, of the aconitase reaction equilibrium mixture.[11] The citrate concentration in baker's yeast grown on ethanol is about 2.5 mM,[12] so that the concentration of isocitrate may well be as high as 0.15 mM, which is comparable with, but rather lower than, its Michaelis constant. From these calculations we conclude that oxaloacetate cannot act as a feed-back inhibitor of isocitratelyase in baker's yeast.

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