BBA 63448

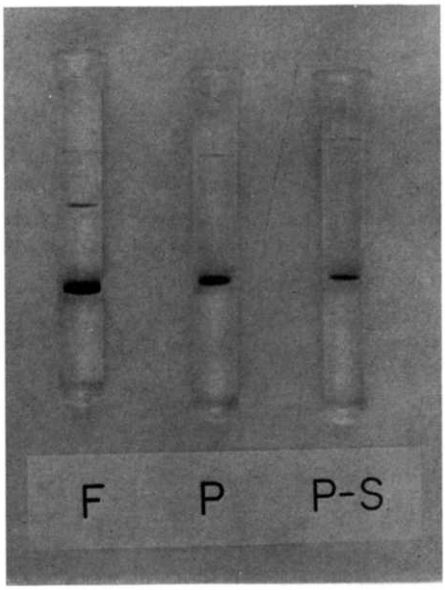
Effect of pH and buffer upon K_m and inhibition by phosphoenolpyruvate of isocitrate lyase from *Pseudomonas indigofera*

Isocitrate lyase (*threo*-D_s-isocitrate glyoxylate-lyase, EC 4.1.3.1) is a key enzyme in the glyoxylate cycle, which has an anaplerotic function in numerous microbes and germinating seedlings[1,2]. Catalysis by isocitrate lyase and its regulation is of particular significance in partitioning isocitrate between the glyoxylate cycle and the degradative tricarboxylic cycle in organisms having a single isocitrate pool.

A large assay-dependent difference in the Michaelis constant (K_m) for isocitrate lyase has been reported by two laboratories. Using a noncontinuous assay for one of the products, glyoxylate, the K_m for D_s-isocitrate was found to be 0.34–1.0 mM at pH 7.7 and 30° for essentially pure enzyme from *Pseudomonas indigofera*[3,4]. Employing a continuous assay, SYRETT AND JOHN[5] subsequently obtained a much lower value of 0.011 mM at pH 6.8 using partially purified enzyme from the same source. Although substrate depletion contributed to the discrepant values, it was evident that other factors were also of major importance.

We now describe a markedly more sensitive revision of the original noncontinuous assay[6] enabling estimation of K_m under conditions where substrate depletion is minimized. This assay can be used at the pH optimum of isocitrate lyase from most sources[7] in contrast to the continuous assay which is restricted to assays below pH 7. Also described are the effects of pH and buffer upon the K_m for isocitrate lyase and of pH upon inhibition by phosphoenolpyruvate.

Enzyme purification. Isocitrate lyase purified from *P. indigofera* ordinarily contains a trace ($< 1\%$) of slower gel electrophoretic component[8]. Occasionally this component is several percent of the final product. Accordingly the original purification[7,8] has been slightly improved and is now described. Isocitrate lyase from *P. indigofera* M1 (ref. 7) was purified through the stage of precipitation with alkaline $(NH_4)_2SO_4$ (Fraction LP) as described earlier[7]. Fraction LP contained 4130 units of isocitrate lyase of a specific activity[7,8] of 21.5. The precipitate (192 mg protein) was dialyzed against 0.01 M Tris–1 mM EDTA–1 mM β-mercaptoethanol buffer containing 5 mM $MgCl_2$, the protein concentration adjusted to 6–8 mg/ml with the same buffer, and the pH adjusted to 6.15–6.35 at 0° with the slow addition of 1 M acetic acid accompanied by vigorous stirring. The temperature of the solution contained in a 50-ml thin-walled flask immersed in a 50° water bath was then brought to 40° and maintained for 1 min or until the temperature reached 45°, whichever occurred first. During temperature adjustment the solution was continuously stirred. Subsequently, the suspension was chilled with stirring in an ice–water bath. The supernatant was separated by centrifugation at $8000 \times g$ for 15 min at 2°, and the pH was adjusted to 7.9 at 2° with 1 M Tris–HCl, pH 8.5 (25°); it contained 3580 units of enzyme of a specific activity of 26.8. Subsequent column chromatography on DEAE-cellulose and concentration of isocitrate lyase in the eluate were conducted as before[7,8]. Fig. 1 displays gel electrophoretograms for an unusually poor product (F) resulting from the former purification procedure[7,8] and for an enzyme preparation (P) isolated by the present modification. The activity of isocitrate lyase is completely stable to storage at −20° in 0.01 M Tris, pH 7.7 (25°), containing 1 mM EDTA, 3 mM dithiothreitol and



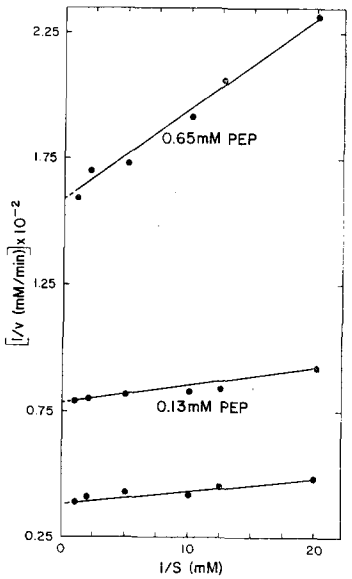


Fig. 1. Polyacrylamide disc gels were prepared from 7.5% acrylamide and electrophoresis conducted at pH 9.5 by the method of MITCHELL[9] with 2 mM β-mercaptoethanol added to the prerun buffer. Bands shown in F, P, P-S (see text) represent 50, 30 and 20 μg of protein, respectively. The gels were scanned with a Gilford spectrophotometer with a gel-scanning densitometer accessory. In F the absorbance of the upper band was 4% of the lower. No other minor bands were detectable.

Fig. 2. Double reciprocal plot for isocitrate cleavage in the presence and absence of phosphoenolpyruvate (PEP).

3 mM $MgCl_2$. Indeed no evidence of decomposition was noted after storage for 5 weeks (gel P-S, Fig. 1).

Assay and K_m's. To measure the K_m for isocitrate of the enzyme from *P. indigofera*, a procedure enabling the estimation of 8-fold lower concentrations of glyoxylate than was formerly the case was developed and is described below. Our former assay[8] was modified by increasing the final volume of the incubation mixture to 3.6 ml and by substituting 0.1 M morpholinopropane sulfonic acid (available from Calbiochem) for Tris where indicated; buffer pH was measured at 25°. Also 0.4 ml of 1 M oxalic acid was added to the incubation mixture in a 15–18-ml test tube to quench the reaction, and this was followed by the addition of 0.1 ml 5% phenylhydrazine·HCl. After mixing, the solution was brought to a boil within 30 sec in a hot sand bath and immediately cooled in an ice–water bath (0°) for 3–10 min. All operations prior to colorimetry were then conducted at 0°. 1.8 ml of 12 M HCl was added, immediately mixed, and within 5 min 0.1 ml 25% (w/v) $K_3Fe(CN)_6$ was added and mixed. The absorbance was then compared within 7–15 min with a reagent blank at 520 nm. The ε_M for sodium glyoxylate (normally obtained commercially as the monohydrate) in the final solution was $5.3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$.

To ensure that isocitrate depletion was not causing large errors in reaction velocity measurements, incubation mixtures were sampled at two different times at each isocitrate concentration. No decrease in rate was observed. Furthermore, the

average depletion at the lowest isocitrate concentration employed was only 10% and the maximum was 21%. The latter provides an upper limit for the error in the observed K_m from substrate depletion (provided that the correct v_{max} has been observed). The K_m for *threo*-(+)D_s-isocitrate in 0.1 M Tris was 0.11 mM at 30° and pH 7.7, appreciably lower than that obtained in earlier studies[3,4] but 10-fold higher than that reported for the same enzyme at pH 6.8 in imidazole buffer using the continuous assay[5]. Accordingly the K_m of this enzyme in 0.1 M Tris at pH 6.8 was measured by the assay described and found to be 0.04 mM at 30°. Investigation of the K_m in morpholinopropane sulfonic acid buffer resulted in much lower values of 0.04 and 0.005–0.007 mM at pH 7.7 and 6.8, respectively, yet the maximal velocities were closely similar to those obtained in Tris buffers. In both buffers the K_m decreases 3–7-fold in the pH interval of 7.7–6.8. The smaller pH dependence for Tris may reflect a subtle Tris-induced change in the structure of the enzyme. GOOD *et al.*[10] have observed inhibition of several enzymes by Tris. We recommend the use of buffers other than Tris or phosphate[11] in the assay of isocitrate lyase.

Inhibition by phosphoenolpyruvate. Phosphoenolpyruvate, which has been proposed as an allosteric inhibitor of isocitrate lyase[12], strongly inhibits the enzyme from Chlorella[5] and *E. coli*[12] at pH 6.8 yet has little effect upon the pseudomonad enzyme at pH 7.7 (ref. 3). Consequently we have examined the pH dependence of inhibition by phosphoenolpyruvate for the enzyme from *P. indigofera*. At pH 6.8, 7.1, 7.4 and 7.7 (maintained by 0.1 M morpholinopropane sulfonic acid) inhibition by 0.2 mM phosphoenolpyruvate of 80, 62, 29 and 20%, respectively, was obtained at saturating D_s-isocitrate (2 mM) confirming a trend first noted for the Chlorella enzyme[4]. Studies at pH 6.8 reveal that the inhibition is roughly uncompetitive at lower concentrations of phosphoenolpyruvate and mixed at higher concentration (Fig. 2). Clarification of the precise mechanism of action of phosphoenolpyruvate must await more diagnostic studies of the back reaction, *i.e.* the condensation of glyoxylate with succinate.

This work was supported by a Research Grant (GM-09039) and Research Career Development Award (2-K3-AI-5,268) to B. A. McF. from the National Institutes of Health and by a National Defense Education Act Predoctoral Fellowship to T. E. R.

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Received December 16th, 1969

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