Al (200 µg/l) in the form of $AlCl_3$. This aluminium concentration is representative of that in the water of lakes and streams in areas receiving acid rain[4]. The acidity of the water was kept at pH 5 by addition of H_2SO_4. Controls were kept in tap water (pH 6.9). Water temperatures were 4–6 °C (winter) or 8–12 °C (summer) and the exposure lasted for 4–7 days. The methods used for determination of carbonic anhydrase and Na-K-ATPase were those described by Maren[7] and Johnson et al.[8], respectively. Chloride was determined by coulometric titration and Na^+ by atomic absorption spectroscopy.

We found a marked decrease in the activity of both carbonic anhydrase (table 1; $p < 0.01$) and Na-K-ATPase (table 2; $p < 0.05$) in the gills of exposed fish. The reduction in enzyme activity was associated with a reduction in the plasma concentrations of sodium and chloride (tables 1 and 2; $p < 0.01$, winter experiments in the salmon, $p < 0.05$). Reduction in enzyme activity and loss of Na^+ and Cl^- occurred before the exposed fish showed visible signs of being affected. A similar loss of ions in salmonids in aluminium-free water is observed after acidification to pH 4 by adding H_2SO_4, but at pH 5 the hydrogen ions themselves seemingly represent no physiological stress to the fish[4]. Our results point to the importance of the enzymes studied in fish death induced by aluminium at low pH.

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A new continuous optical assay for isocitrate lyase[1]

E. Giachetti, G. Pinzauti and P. Vanni

Istituto interfacoltà di Chimica biologica, Università di Firenze, viale Morgagni 50, I-50134 Firenze (Italy), 25 February 1983

Summary. A new continuous optical assay method for isocitrate lyase is reported. This is a coupled assay which requires lactate dehydrogenase as an ancillary enzyme. The method yields linear data up to 0.12 units/ml. The assay is also suitable for crude extracts.

Isocitrate lyase (EC 4.1.3.1) catalyzes the reversible cleavage of *threo*-D_s-isocitrate into glyoxylate and succinate. There are 2 widely-used methods for the assay of isocitrate lyase: one discontinuous[2], the other continuous, chemically coupled[3]. A 3rd method, which assays isocitrate lyase continuously in the direction of isocitrate formation (isocitrate dehydrogenase-coupled), has also been reported[4,5], but its application is limited because glyoxylate and succinate inhibit isocitrate lyase from a number of sources[5,6].

Lactate dehydrogenase, isoenzyme I, from pig heart has been reported to catalyze both the oxidation and reduction of glyoxylate[7]. Lactate dehydrogenase can, therefore, be used as an auxiliary enzyme for a continuous assay of isocitrate lyase. This paper is concerned with the reliability of the method. A comparison with other assays currently in use is also made.

Materials and methods. Chemicals and biochemicals. Lactate dehydrogenase (LDH) from pig heart (whole preparation) and NADH were obtained from Boehringer, Mannheim, FRG; lactate dehydrogenase isoenzyme I from pig heart and *threo*-D,L-isocitrate were obtained from Sigma, Chemical Company, USA, and all other chemicals were purchased from Merck, Darmstadt, FRG.

Isocitrate lyase preparation. Isocitrate lyase (ICL) was isolated from the endosperm of *Pinus pinea* germinating seeds by the method previously reported[6]. All the enzyme fractions used in this study were prepared by this purification procedure.

Enzyme assay. All procedures were carried out at 30 °C in 40 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethansulphonic acid) buffer (pH 7). The continuous assay, chemically coupled with phenylhydrazine, is described elsewhere[6]. The discontinuous assay was performed according to Roche et al.[8], with minor modifications of the buffer system (40 mM HEPES, pH 7). This is a colorimetric method which requires a standard curve. 1 unit of enzyme activity is here defined as the amount of enzyme which catalyzes the cleavage of 1 µmol of substrate per min at 30 °C. Statistical analysis of kinetic data was done in the manner described by Wilkinson[9] and Cleland[10].

Results and discussion. The principle of the LDH-coupled continuous method is based upon the following sequence of reactions:

$$\text{isocitrate} \xrightarrow{\text{ICL}} \text{glyoxylate} + \text{succinate} \qquad \text{(a)}$$

$$\text{glyoxylate} \xrightarrow[\text{NADH} + \text{H}^+ \;\; \text{NAD}^+]{\text{LDH}} \text{glycolate} \qquad \text{(b)}$$

Preliminary studies showed that not only isoenzyme I, but the whole preparation of lactate dehydrogenase from pig heart was suitable as an auxiliary enzyme. The amount of auxiliary enzyme required in the assay was determined by the use of the following equation[11]:

$$V_{max}(\text{LDH}) = -\frac{K_{ms} \ln(1 - Fs)}{t} \qquad \text{(c)}$$

where K_{ms} is the Michaelis constant for LDH (glyoxylate as substrate), $V_{max(LDH)}$ gives the amount of LDH required to obtain, in time t (the lag-phase), a fraction of glyoxylate at the steady-state (F_s) virtually equivalent to $[S]_{ss}$, the concentration of substrate at the steady-state.

K_m of LDH toward glyoxylate, under the same conditions of the coupled assay, was estimated to be 8.40 ± 0.90 mM, a value close to that reported for isoenzyme I (pig heart) under slightly different conditions[7]. We accepted $F_s = 0.99$ $[S]_{ss}$, as being experimentally equivalent to $[S]_{ss}$, and fixed the lag-phase (t) at 1 min. If required, a further reduction of the lag-phase is possible.

Reaction mixture composition. The reaction mixture for the LDH-coupled assay contains, in a final volume of 1 ml: 40 mM HEPES (pH 7), 6 mM $MgCl_2$, 4 mM *threo*-D,L-isocitrate, 0.28 mM NADH and 45 units lactate dehydrogenase from pig heart (glyoxylate as substrate). NADH oxidation is followed spectrophotometrically at 340 nm.

It should be noted that the pH of the LDH-coupled assay is also the pH optimum of lactate dehydrogenase for glyoxylate reduction[7] and of isocitrate lyase from *P. pinea* for isocitrate cleavage in HEPES buffer[6].

Interference in the test by the half-reaction of glyoxylate disproportionation can be excluded, even after NAD^+ release, for the pH optimum of LDH-catalyzed glyoxylate oxidation is very different[7] and the molecular activity of the enzyme for this reaction is about 0.27 of that for glyoxylate reduction[7].

The table shows the values of isocitrate lyase activity, obtained with 3 assay methods, tested on different biochemical preparations[6]. It would appear that the presence of some interfering compounds makes it impossible to use the discontinuous method for crude extracts and partially purified preparations. Moreover, this method is time-consuming and has the limitations of every discontinuous assay.

Comparison of LHD-coupled assay with the discontinuous and the phenylhydrazine-coupled assays

Isocitrate lyase fractions[a]	Discontinuous method (units/ml)	Continuous methods Phenylhydrazine-coupled (units/ml)	LDH-coupled (units/ml)
Crude extract	11.26 ± 0.50[b]	1.89 ± 0.03	2.17 ± 0.08
Acetone precipitate 33% ammonium sulfate precipitate	10.10 ± 0.49[b]	1.91 ± 0.09	2.14 ± 0.05
	1.90 ± 0.09	1.20 ± 0.03	1.59 ± 0.03
Purified enzyme	1.39 ± 0.06	1.24 ± 0.04	1.60 ± 0.05

[a] All the fractions refer to the purification procedure of isocitrate lyase from *Pinus pinea* previously reported[6].

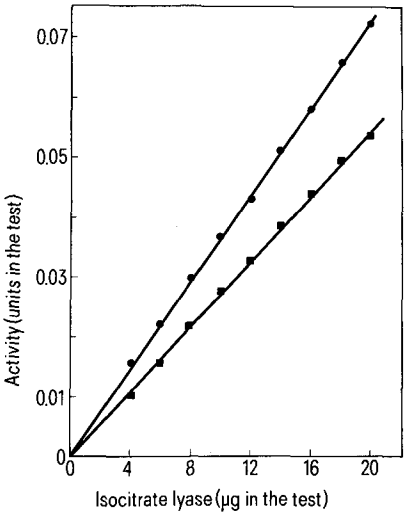
[b] Interfering compound(s) appear to be present in crude and partially purified preparations.

Each value is the mean of 4 tests ± SD.

On every isocitrate lyase fraction, the LDH-coupled assay gives higher activity values than those obtained with the phenylhydrazine-coupled assay, as is also shown in the figure, where the linearity of the 2 continuous methods is reported and compared. The lower values are probably due to the fact that the steady-state velocity in the latter assay is influenced by the coupled chemical reaction. On the other hand, we have not found higher concentrations of phenylhydrazine in the test to lead to an increase in the measurement of isocitrate lyase activity.

A comparison of K_m values for isocitrate lyase toward *threo*-D_s-isocitrate, obtained with the 2 continuous methods, shows the suitability of the LDH-coupled assay also for kinetic studies. In fact, we obtained K_m's equal to 0.15 ± 0.01 and 0.16 ± 0.01 mM, estimated by the LDH-coupled and the phenylhydrazine-coupled methods respectively.

In conclusion, the LDH-coupled assay reported here was found to be proportional over a wide range of enzyme concentrations (up to 0.12 units per ml in the test) and to provide a better and more reliable measurement of the enzyme activity.



Effect of enzyme concentration on isocitrate lyase activity, assayed with the LDH-coupled (●) and the phenylhydrazine-coupled (■) methods. The purified isocitrate lyase fraction of the table was used for this study. Linear regression analysis shows that both methods are proportional, but the activity values obtained with the LDH-coupled assay are higher than those obtained with the phenylhydrazine-coupled method. Results of linear regression analysis, assuming a straight line of the form $y = bx$, are as follows: (●) $r = 1.000$; $b = 0.00363$. (■) $r = 1.000$; $b = 0.00272$. Enzyme activity is expressed in terms of units in the reaction mixture.

Note added in proof.

During the preparation of our manuscript, Cleland (Biochemistry *21* (1982) 4420-4427) used LDH as ancillary enzyme for an ICL assay. No data were reported on the reliability and the optimal conditions for this assay.

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