myo-Inositol

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Principle

A crude inositol dehydrogenase preparation obtained from *Aerobacter aerogenes* which has been grown on a medium containing inositol, catalyses the oxidation of *myo*-inositol by diphosphopyridine nucleotide (DPN).

With this system microgram amounts of *myo*-inositol can be estimated rapidly and simply by following the reduction of DPN spectrophotometrically[1]. Other enzymic methods for the microdetermination of *myo*-inositol have been described by *Kean* and *Charalampous*[2] and *Charalampous* and *Abrahams*[3].

Inositol dehydrogenase catalyses the reaction:

(1) *myo*-Inositol + DPN^+ $\rightleftharpoons$ *scyllo-myo*-inosose + DPNH + H^+

Under the conditions of the method described here the reaction does not go to completion, but the maximum formation of DPNH is proportional to the *myo*-inositol concentration. Presumably the initial oxidation product is *scyllo-myo*-inosose, although in the presence of crude inositol dehydrogenase further reactions may occur[4]. Under suitable conditions *scyllo-myo*-inosose is quantitatively reduced by DPNH and inositol dehydrogenase[5].

Reagents

1. Sodium carbonate, Na_2CO_3
2. Sodium hydrogen carbonate, $NaHCO_3$
3. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.
4. *myo*-Inositol
   commercial preparation, see p. 1023.
5. Inositol dehydrogenase
   a crude preparation obtained from *Aerobacter aerogenes*[1]. See Appendix, p. 174.

Preparation of Solutions

I. Sodium carbonate buffer (0.5 M; pH 9.5):
Add 0.5 M sodium hydrogen carbonate solution (42 g. $NaHCO_3$/1000 ml.) to 0.5 M sodium carbonate solution (53 g. Na_2CO_3/1000 ml.) until the pH reaches 9.5 (glass electrode).

II. Diphosphopyridine nucleotide (2×10^{-2} M β-DPN):
Dissolve 73.5 mg. DPN in 5 ml. distilled water.

III. *myo*-Inositol (2×10^{-3} M):
Dissolve 18.0 mg. *myo*-inositol in distilled water and make up to 50 ml.

IV. Inositol dehydrogenase (about 30 mg. protein/ml.)

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[1] *A. Weissbach*, Biochim. biophysica Acta *17*, 608 [1958].
[2] *E. L. Kean* and *F. C. Charalampous*, Biochim. biophysica Acta *36*, 1 [1959].
[3] *F. C. Charalampous* and *P. Abrahams*, J. biol. Chemistry *225*, 575 [1956].
[4] *J. M. Goldstone* and *B. Magasanik*, Feder. Proc. *3*, 218 [1954].
[5] *A. Weissbach*, unpublished.

Stability of the solutions

Crude inositol dehydrogenase is stable for at least four months at −20° C. If during this period a precipitate forms it can be centrifuged down and discarded without affecting the enzyme activity. DPN and *myo*-inositol solutions are stable for many months at −20° C. The carbonate buffer is stable indefinitely at room temperature if kept in a tightly stoppered bottle.

Procedure

Experimental material

In a few cases crude extracts from heat killed bacteria inhibit the determination about 10−20%. It is recommended that when the samples for assay are very crude extracts, the bulk of the impurities should be removed by the Barium-Zinc method of *Agranoff* et al.[6]: Mix a solution of the sample, containing 0.01 to 1.0 μmole inositol, with

1 ml. 0.15 M $Ba(OH)_2$ solution,

heat 15 min. at 100° C, add

1 ml. 5% $ZnSO_4 \cdot 7\ H_2O$ solution

and centrifuge. Treat supernatant with

2.0 ml. of an aqueous slurry containing 1 ml. Amberlite IRA-400 (OH^--form) (as packed wet resin),

centrifuge again, concentrate the supernatant *in vacuo* to 1 ml. and use a portion of this solution for estimation of *myo*-inositol.

Standard curve

The *myo*-inositol content of the sample may be read directly from a standard curve determined for each enzyme preparation. However, it is usual to set up control cuvettes containing 0.02, 0.06 and 0.1 μmoles *myo*-inositol (= 0.01, 0.03 and 0.05 ml. solution III) when unknown samples are to be assayed. Optical densities of approximately 0.080, 0.240 and 0.400 should be obtained.

Spectrophotometric measurements

Wavelength: 340 mμ; light path: 1 cm.; final volume: 1.0 ml.

Read against a control cuvette containing enzyme solution since this absorbs slightly at 340 mμ. The sample should not contain more than 0.1 μmole inositol. The standard curve is only linear up to a concentration of 10^{-4} M inositol.

Pipette the solutions in the given order into the cuvettes:

Experimental cuvette	*Control cuvette*
0.20 ml. buffer (solution I)	0.20 ml. buffer (solution I)
0.10 ml. DPN solution (II)	0.10 ml. DPN solution (II)
0.05 ml. enzyme solution (IV)	0.05 ml. enzyme solution (IV)
Sample + dist. water to give 1.00 ml. total volume	0.65 ml. dist. water

The absorption due to formation of DPNH reaches a maximum value 3 to 4 min. after the start of the reaction. This value is used. The fall in optical density which occurs with longer reaction times is at least partly due to the presence of DPNH oxidase activity in the enzyme preparation (Fig. 1).

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[6] *B. W. Agranoff, R. M. Bradley* and *R. O. Brady*, J. biol. Chemistry *233*, 1077 [1958].

Calculations

The value for *myo*-inositol in μmoles/ml. assay mixture is read from the standard curve by use of the optical density obtained approximately 3 to 4 min. after the start of the reaction. To calculate the *myo*-inositol concentration of the sample it is necessary to divide by the volume of the sample. Any dilution of the sample occurring during the preliminary treatment has to be taken into account. 0.1 μmole Inositol = 18 μg.

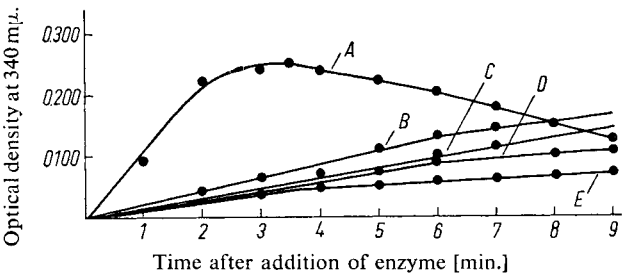

Fig. 1. Time curve for the oxidation of A: 0.07 μmoles *myo*-inositol — B: 0.07 μmoles *neo*-inositol — C: 1.0 μmole glucose — D: 0.07 μmoles D-inositol — E: 0.10 μmoles scyllitol. Enzyme: Inositol dehydrogenase.

Sources of Error and Specificity

The substrate specificity of crude inositol dehydrogenase (Table 1) in general reflects the specificity of whole cells[7]. Sequoyitol (= *myo*-inositol monomethyl ether) reacts like *myo*-inositol itself. Glucose in low concentrations does not react; above 10^{-3} M, glucose, dihydroxyacetone and glyceraldehyde react slowly (Fig. 1). It is surprising that scyllitol which contains no axial OH group reacts

Table 1. Substrate specificity of a crude inositol dehydrogenase preparation from *A.aerogenes*

Substrate	μmole	Increase in optical density at 340 mμ *)
myo-Inositol	0.07	0.255
Pinitol	0.07	0.031
cis-Inositol	0.07	0
epi-Inositol	0.07	0.010
allo-Inositol	0.07	0
Dambonitol	0.07	0
Quebrachitol	0.07	0
L-Inositol	0.07	0
neo-Inositol	0.07	0.075
D-Inositol	0.07	0.050
Scyllitol	0.10	0.050
DL-1-*O*-methyl-*myo*-inositol	0.07	0.010
Sequoyitol	0.08	0.300
Ribose	0.05	0
Glucose	0.05	0
Galactinol **)	0.10	0
Inositol monophosphate ***)	0.08	0
Inositol monophosphate after treatment with wheat phytase)	0.08	0.286

*) $3^1/_2$ minutes after the addition of the enzyme
**) α-D-galactosyl-*myo*-inositol
***) From the California Foundation for Biochemical Research.

7) *B. Magasanik*, J. biol. Chemistry *205*, 1007 [1953].

slowly. Possibly the preparation was contaminated with a small amount of *myo*-inositol, which could also explain the slow reaction of D-inositol and *neo*-inositol. None of the substrates listed in Table 1 inhibit the oxidation of *myo*-inositol. However 10^{-4} colchicine gave a 50% inhibition of the assay, which is to be expected from the studies of *Franzl* and *Chargaff*[8].

Other Methods

Charalampous et al.[3] determined *myo*-inositol by anaerobic oxidation with 2,6-dichlorophenolindophenol catalysed by inositol dehydrogenase and pig heart diaphorase. Inositol oxidase converts *myo*-inositol to glucuronic acid, which may be estimated by the orcinol reaction or by TPNH and a TPNH-linked dehydrogenase[2].

Appendix

Inositol dehydrogenase[1]

Aerobacter aerogenes, strain 41 124 *); culture medium[7]: 0.27% Na_2HPO_4; 1.58% KH_2PO_4; 0.02% $MgSO_4 \cdot 7\,H_2O$; 0.001% $CaCl_2$; 0.2% $(NH_4)_2SO_4$; 5×10^{-4} M myo-inositol; pH 6.0. Shake culture for 18 hours at 37° C with vigorous aeration. Wash the cells at the centrifuge with cold distilled water. Shake 3 g. cell paste with 10 ml. 0.1 M tris buffer (pH 7.5) and 7 g. glass powder in a Nossal shaker for 1 min. at 0° C (frequency: 15/sec.). Alternatively grind 3 g. cell paste with 9 g. alumina powder A-301 for 15 min. at 0° C, then add 15 ml. 0.1 M tris buffer (pH 7.5). Centrifuge the suspension for 30 min. at 0° C and 20000 g. Store the supernatant at −20° C.

*) From the New York State Department of Health.

[8] *R. Franzl* and *E. Chargaff*, Nature [London] *168*, 955 [1951].