D-Isoascorbic acid is active but distinctly weaker than ascorbic acid itself. Under the experimental conditions described, TPNH is not oxidized in the presence of ascorbic acid.

5. Fe^{+++} in complex form is reduced by DPNH in the presence of the enzyme.

6. The speed of the DPNH-oxidation by ascorbic acid is dependent on the O_2 content of the reaction medium.

7. On the basis of the experiments it appears that "monodehydroascorbic acid" acts as an intermediary electron acceptor. The system

$$\text{ascorbic acid} \rightleftharpoons \text{monodehydroascorbic acid}$$

forms the electron carrier between DPNH and O_2. Intermediary hydroxyl radicals probably arise in this way.

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THE ENZYMIC DETERMINATION OF *MYO*-INOSITOL

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myo-Inositol has been found to be widely distributed in living cells and to be a major constituent of the phospholipids. The lack of a direct, sensitive, and specific assay for *myo*-inositol has hindered studies as to its metabolism and function in biological systems. Earlier studies on *myo*-inositol metabolism by MAGASANIK have shown that capsulated strains of *Aerobacter aerogenes* are capable of using *myo*-inositol as a sole carbon source for growth[1]. Further work revealed that extracts of *myo*-inositol-grown *A. aerogenes* cells catalyzed the reduction of DPN* by *myo*-inositol[2]. Attempts by other workers to adapt this DPN-linked inositol dehydrogenase for the direct spectro-

* The following abbreviations are used: DPN = Diphosphopyridine nucleotide; TRIS = Tris (hydroxymethyl) aminomethane; DPNH = Reduced diphosphopyridine nucleotide.

References p. 611.

photometric assay of *myo*-inositol proved unsuccessful[3]. An enzymic method for the micro determination of *myo*-inositol has been reported by CHARALAMPOUS AND ABRAHAMS but it apparently does not function in the presence of oxygen[4]. This method requires the coupling of the inositol dehydrogenase obtained from *Acetobacter suboxydans* with pig-heart diaphorase and 2,6-dichorophenol indophenol under strict anaerobic conditions.

In an effort to develop a simple enzymic method for the determination of *myo*-inositol and its derivatives, we reinvestigated the DPN-linked inositol dehydrogenase obtained from inositol-grown *A. aerogenes* cells. This system which offers the advantages of simplicity and rapidity has now been adapted for the estimation of microgram amounts of *myo*-inositol by following the reduction of DPN directly in a spectrophotometer.

An *A. aerogenes* strain, 41124, obtained from the New York State Department of Health, was grown, as previously described[1], on *myo*-inositol as the sole carbon source. After growth for 18 h at 37°C with vigorous aeration, the cells were collected by centrifugation in a Sharples supercentrifuge at 0°C and washed with cold, distilled water. A cell-free extract was prepared by shaking 3 g of wet, packed cells with 10 ml of 0.1 *M* Tris buffer, pH 7.5, and 7 g of glass powder in the Nossal disintegrator for a total of 1 min at 0°C using 15 sec shaking periods[5]. The suspension was then centrifuged at 20,000 *g* in the Lourdes Model AT centrifuge for 30 min at 0°C and the supernatant solution was used directly in the assay. An alternate method of preparation consisted of grinding 3 g of wet, packed cells with 9 g of alumina A-301 for 15 min at 0°C. Following the addition of 15 ml of 0.1 *M* Tris, pH 7.5, the suspension was centrifuged as described above and the supernatant was used in the assay.

The stoichiometry and conditions of the reduction of DPN by *myo*-inositol in the presence of this crude extract are given in Fig. 1. Under the conditions of the assay, the maximum reduction of DPN by *myo*-inositol is proportional to the *myo*-inositol concentration, although complete stoichiometric reduction of DPN is not observed. At higher *myo*-inositol concentrations, above 10^{-4} *M*, this proportionality tends to fall off. This may, in part, be due to an accentuation of a DPNH-oxidase

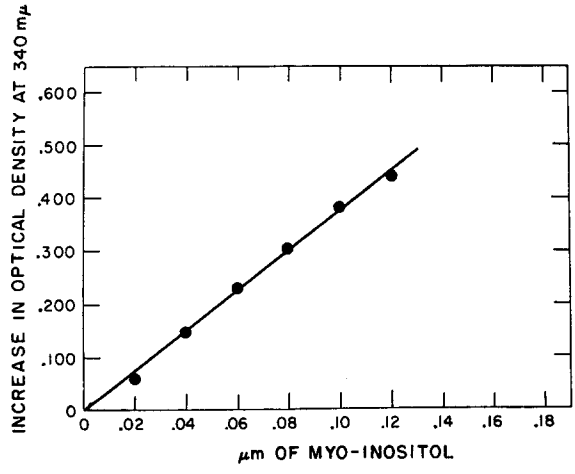

Fig. 1. Stoichiometry of the *myo*-inositol assay. The incubation mixture contained 50 μmoles of carbonate buffer, pH 9.5, 2 μmoles of DPN, 1.4 mg of protein (cell-free extract) and substrate as stated, in a total volume of 1.0 ml. The optical density was read in a Beckman DU spectrophotometer. The values given are the maximum values obtained 3 to 4 min after the addition of enzyme (*cf.* Fig. 2).

References p. 611.

system which seems to be present in the crude extract. This is indicated in Fig. 2 where the reduction of DPN in the presence of *myo*-inositol is followed with time. DPNH formation reaches a maximum value in 3–4 min after the addition of enzyme. Since the crude enzyme shows some absorption at 340 mμ, all readings are taken against a blank cuvette containing all the components of the system except the substrate.

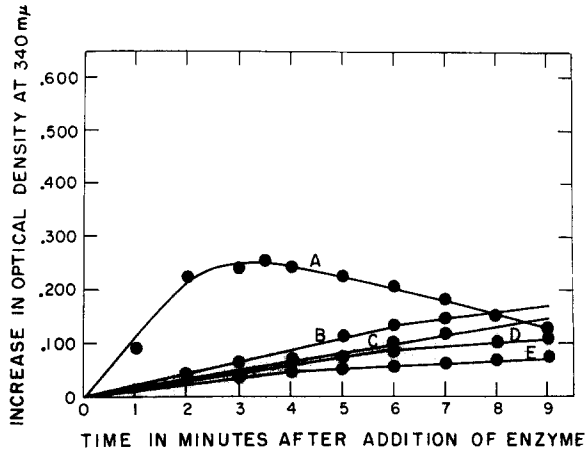

Fig. 2. Time curve for the oxidation of substrates by inositol dehydrogenase. Incubation mixtures used were the same as in Fig. 1. Curve A = 0.07 μmole *myo*-inositol, B = 0.07 μmole *neo*-inositol, C = 1.0 μmole glucose, D = 0.07 μmole of D-inositol, E = 0.10 μmole scyllitol.

TABLE I

REACTION OF SUBSTRATES IN THE *myo*-INOSITOL ASSAY

*Substrate**	*μM used in assay systems***	*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	0.040
D-L-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

* The cyclitols were kindly supplied by Dr. BERNARD AGRANOFF of the National Institute of Mental Health.

** Incubation mixtures are the same as in Fig. 1.

*** At 3½ min after addition of enzyme.

§ Obtained from the California Foundation for Biochemical Research.

References p. 611.

The substrate specificity of this cell-free extract, as shown in Table I, agrees, in general, with the results obtained by MAGASANIK with whole cells[1]. D-Inositol and *neo*-inositol react slowly in this system (Fig. 2) as might be expected since they contain axial hydroxyl groups, while sequoyitol (*myo*-inositol monomethyl ether) reacts like *myo*-inositol itself. Glucose, at low concentrations, does not react in this assay, though at higher concentrations, above 10^{-3} *M*, glucose, dihydroxyacetone, and glyceraldehyde react slowly. This is a shown for glucose in Fig. 2. It is interesting to note that scyllitol, which contains no axial hydroxyl group, reacts slowly in this system. This may be due to the possible presence of small amounts of *myo*-inositol in the scyllitol preparation. Contaminating amounts of *myo*-inositol may also explain the reaction of D-inositol and *neo*-inositol in this assay. Also, scyllitol does not inhibit the oxidation of *myo*-inositol in contrast to the results obtained with whole cells[1] and, in fact, none of the substrates tested in this assay, at the levels shown in Table I, inhibit the estimation of *myo*-inositol. Colchicine, however, will interfere with the dehydrogenation of *myo*-inositol in this system as might be expected from the studies of FRANZL AND CHARGAFF[6]. At a concentration of 10^{-4} *M* colchicine shows a 50% inhibition under the conditions of this assay. In the presence of crude heat-killed cell-free extracts of *E. coli*, a slight inhibition, 10–20%, has also been observed in this assay.

The cell-free extract used in these studies is stable for at least 4 months when stored at —20°C, and provides a convenient and rapid method for the determination of *myo*-inositol.

SUMMARY

An inositol dehydrogenase obtained from *myo*-inositol-grown *Aerobacter aerogenes* has been adapted for the determination of *myo*-inositol in microgram amounts. The other cyclitols and sugars tested do not interfere with this assay, though colchicine exerts a marked inhibitory effect.

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