Biochimica et Biophysica Acta, 293 (1973) 295–303

BBA 66813

INOSITOL DEHYDROGENASE FROM THE YEAST *CRYPTOCOCCUS MELIBIOSUM*

M. VIDAL-LEIRIA AND N. VAN UDEN
Laboratory of Microbiology, Gulbenkian Institute of Science, Oeiras (Portugal)

(Received August 30th, 1972)

SUMMARY

1. A *myo*-inositol:NAD^+ oxidoreductase from the yeast *Cryptococcus melibiosum* was purified about 35-fold by the use of polyethylene glycol 6000.

2. The enzyme preparation accepted *myo*-inositol, (+)-inositol and *scyllo*-inosose as substrates, NAD^+ and NADH as coenzymes but not quebrachitol, $NADP^+$ and NADPH.

3. Paper chromatography in 4 solvent systems indicated that *myo*-inositol is oxidized to *scyllo*-inosose. Thus the initial step of *myo*-inositol catabolism in the yeast appeared to be similar to that in *Aerobacter aerogenes*[6,7].

4. The kinetics of inhibition by reaction products was suggestive of a "Bi Bi Ordered" mechanism[14] with NAD^+ and NADH as the leading substrates. The kinetic constants defined by the model were estimated.

5. The equilibrium constant at 25 °C of *myo*-inositol–NAD^+ oxidoreduction as catalyzed by the enzyme preparation was estimated from Racker[11] plots which gave $5.3(\pm 2.5)\cdot 10^{-12}$.

INTRODUCTION

Of the 361 yeast species recognized in a recent monograph[1], 35 species include strains which are capable of utilizing *myo*-inositol as a source of carbon and energy.

The pathway of inositol catabolism in yeasts has not been definitely established. Sivak and Hoffman-Ostenhof[2] proposed that inositol is catabolized in the yeast *Schwanniomyces occidentalis* via the D-glucuronate-L-gulonate pathway, the initial oxidation of inositol being mediated by an inositol oxygenase. Thus inositol catabolism in yeasts would be similar to that reported for certain animal[3] and plant[4] tissues. Vidal-Leiria[5] tested for enzyme activities in cell-free extracts of inositol-adapted strains pertaining to 13 yeast species. Though all strains were active with respect to one or more enzymes of the glucuronate–gulonate pathway, inositol oxygenase

Nomenclature: *myo*-inositol, 1,2,3,5/4,6-inositol; *scyllo*-inosose, 2,4,6/3,5-pentahydroxycyclohexanone.

activity could not be detected in any of them. On the other hand, five of the strains showed NAD^+-dependent inositol dehydrogenase (*myo*-inositol:NAD^+ oxidoreductase, EC 1.1.1.18) activity. According to these findings, the initial catabolism of inositol in yeasts would be similar to that occurring in *Aerobacter aerogenes*[6,7].

Cryptococcus melibiosum I.G.C. 3939, one of the yeast strains tested by Vidal-Leiria[5] was selected for further study of its inositol dehydrogenase, the results of which are the subject of the present paper.

MATERIALS AND METHODS

Preparation of cells

The organism used is the type strain of *Cryptococcus melibiosum* (Shifrine and Phaff) Phaff and Fell[8]. It is maintained in the culture collection of the Gulbenkian Institute of Science under the number I.G.C. 3939, and is available for free distribution.

The yeast was grown at 25 °C for 48 h with shaking in 2-l Erlenmeyer flasks containing 1 l of the following medium: *myo*-inositol 0.5% (w/v), Bacto-peptone (Difco) 1% (w/v), Bacto-yeast extract (Difco) 0.5% (w/v), demineralized water. The flasks were inoculated with 25 ml of culture grown in the same medium, which in turn had been inoculated from stock cultures on slants of the same medium solidified with 2% agar.

About 90 g (wet weight) of washed cells were suspended in a double amount of 0.1 M potassium phosphate buffer (pH 7.0) and this suspension was poured into six times its volume of acetone at −17 °C. After stirring for 30 min, sedimentation was carried out for 5 min followed by filtration through paper (Whatman No. 50), washing with acetone at −10 °C followed by cold diethyl ether. Finally, the cell cake was dried in a vacuum over laminated paraffin and kept at ±2 °C in a desiccator over silica gel until use.

Preparation of the enzyme

Acetone-dried cells were ground at 4 °C with an equal amount of glass beads (*d* 0.25–0.30 mm) and added to 10 times the amount of 0.1 M potassium phosphate buffer (pH 7.0) containing 10^{-3} M EDTA and $5 \cdot 10^{-4}$ M glutathione. All subsequent purification steps were performed at 2 °C–4 °C with the same buffer.

The mixture was left standing overnight and subsequently centrifuged (1800 × *g*, 15 min). To the supernatant ("crude extract") solid polyethylene glycol 6000 (B.D.H.) was added to a final concentration of 30% (w/v) followed by magnetic stirring for 60 min. The precipitate was spun down (1800 × *g*, 5 min) and resuspended in buffer to a final volume of about 1/7 of the original crude extract. A 12.5% solution (w/v) of polyethylene glycol 6000 was slowly added to the preparation until a final concentration of 10% (w/v) was reached, followed by magnetic stirring during 30 min. The precipitate was spun down (7500 × *g*, 10 min) and discarded. Solid polyethylene glycol was added to the supernatant to a final concentration of 35% (w/v), followed by magnetic stirring for 1 h. The precipitate was spun down (17 000 × *g*, 3 h) and resuspended in buffer to a final volume of about 1/20 of the original crude extract. This preparation was used in the experiments. It did not appreciably lose activity when kept at −17 °C for 1 year. A summary of a typical purification run is given in Table I.

TABLE I

PARTIAL PURIFICATION OF INOSITOL DEHYDROGENASE

Step	*Vol. (ml)*	*Activity*		*Protein (mg/ml)*	*Specific activity (units/mg protein)*
		(units)*	*(units/ml)*		
Crude extract	73	52.6	0.72	15.3	0.047
30% PEG**	10	11.2	1.12	14.5	0.077
10% PEG	50	12.8	0.26	3.2	0.081
35% PEG	4	18.8	4.7	2.8	1.68

* One unit is defined as the ability to produce 1 μmole of NADH per min under standard conditions (pH 8.7, 25 °C).

** Polyethylene glycol 6000.

Unit and standard assay of the enzyme

One unit of *myo*-inositol dehydrogenase is defined as the ability to produce 1 μmole of NADH per min under standard conditions. The standard assay was performed in a cuvette having a 1 cm light path and containing a total volume of 3 ml with 82.5 mM *myo*-inositol, 0.35 mM NAD^+ and 16.7 mM tetrapotassium pyrophosphate buffer (pH 8.70). The reaction, which was normally carried out at 25 °C, was initiated by the addition of 0.010 to 0.025 ml of enzyme solution (about 2 mg/ml of protein). The change in absorbance at 340 nm was followed in a Unicam SP 500 spectrophotometer fitted with a Vitatron recorder, type UR 400.

Preparation and paper chromatography of product

Product was obtained as described by Berman and Magasanik[7] by running the reaction in the presence of an NAD^+-generating system consisting of diaphorase, methylene blue and oxygen. Samples were removed at 0, 14 and 20 h, treated with 50% trichloroacetic acid and centrifuged. 5-μl portions of the supernatants were spotted on Whatman No. 1 paper for chromatography together with the following controls: *myo*-inositol, *scyllo*-inosose, *myo*-inositol + *scyllo*-inosose, reaction mixture without *myo*-inositol, reaction mixture without NAD^+.

Descending chromatography was performed with the following solvent systems: Solvent 1, acetone–water, 85:15; Solvent 2, ethanol–acetic acid–water, 8:2:2; Solvent 3, phenol–water, 4:1; Solvent 4, pyridine–amyl alcohol–water, 7:7:6; Spots of *myo*-inositol and *scyllo*-inosose were visualized with a silver nitrate–acetone spray[9], and reducing compounds were visualized with 1% 2,3,5-triphenyltetrazolium in 0.5 M NaOH[10].

Determination of the equilibrium constant

The equilibrium constant of the reaction at a given temperature is defined by the activities of the reactants at equilibrium:

$$K_{eq} = \frac{[\text{NADH}]\,[\text{oxidized } \textit{myo}\text{-inositol}]\,[\text{H}^+]}{[\text{NAD}^+]\,[\textit{myo}\text{-inositol}]} \qquad (1)$$

K_{app}, a pH-dependent apparent equilibrium constant is obtained when the equilibrium constant is estimated omitting the proton activity from Eqn (1):

$$K_{app} = \frac{K_{eq}}{[H^+]} \quad (2)$$

From this relation follows immediately that

$$\log K_{app} = \log K_{eq} + pH \quad (3)$$

where pH refers to the proton activity at equilibrium (Racker[11]).

For practical purposes the activities of the reactants may be estimated as molar concentrations. The enzyme concentration should be low so that the amount of reactants complexed with the enzyme may be neglected in the calculations.

Estimates of K_{app} (25 °C) at various pH values were obtained as follows. Reactants and buffer were pipetted into cuvettes with a 1 cm light path to produce volumes of 3 ml with the following initial concentrations: *myo*-inositol, 19.98 mM; NAD^+, 95.3 μM, enzyme, 0.07 unit/ml; tetra-potassium pyrophosphate buffer, 16.7 mM. The initial pH was varied between 8.3 and 9.8 by using stock solutions of buffer adjusted with concentrated HCl to different pH values. The concentration of active NAD^+ in the stock solution was estimated by the procedure of Klingenberg[12].

The reaction was followed spectrophotometrically at 340 nm and was allowed to reach equilibrium. The equilibrium concentration of NADH was obtained from the optical density at equilibrium. The equilibrium concentration of oxidized *myo*-inositol was assumed to be identical to that of NADH; the equilibrium concentration of NAD^+ and *myo*-inositol were then obtained by subtracting the value of the NADH concentration from the initial concentration of the two substrates. The pH values at equilibrium were measured with a microelectrode (Pye Unicam 401-M5) connected to an E_07 model Pye Unicam 290 pH meter. For measuring pH each sample was placed in a cuvette thermostated at 25 °C and provided with a magnetic stirring device.

Miscellaneous

The sources of chemicals and reagents used were as follows: *myo*-inositol, *scyllo*-inosose, NAD^+, $NADP^+$, NADH, NADPH, pyruvic acid, yeast alcohol dehydrogenase, rabbit muscle lactate dehydrogenase, and *Clostridium kluyveri* diaphorase, from Sigma Chemical Co.; 2,3,5-triphenyltetrazolium chloride, (+)-inositol, from Calbiochem.; polyethylene glycol 6000, from B.D.H.; quebrachitol, from Mann Research Laboratories Inc. Other chemicals were obtained locally and were of the highest purity obtainable.

Protein concentration was estimated according to Warburg and Christian[13].

RESULTS AND CONCLUSIONS

Substrate specificity

The preparation catalyzed the reduction of NAD^+, but not of $NADP^+$, with either *myo*-inositol or (+)-inositol as the electron donor. Quebrachitol was not a suitable substrate. NADH, but not NADPH, was oxidized with *scyllo*-inosose as the electron acceptor.

Product identification

Samples containing oxidized *myo*-inositol prepared as indicated previously were run in descending paper chromatography with Solvent 1 (20 h), Solvent 2 (16 h),

Solvent 3 (100 h) and Solvent 4 (45 h) and gave spots that had the same R_F values as *scyllo*-inosose in each of the four systems. These results led us to conclude that the initial step of inositol catabolism in *Cryptococcus melibiosum* consists in the enzymatic oxidation of *myo*-inositol to *scyllo*-inosose with NAD^+ as the coenzyme.

Kinetics

The product inhibition pattern to be reported later in this section indicated a sequential mechanism of the type "Bi Bi Ordered" (Cleland[14]) with NAD^+ and NADH as the leading substrates. Using Cleland's[14] symbolism we may write for the initial velocity of the forward reaction:

$$v_1 = V_1 \frac{AB}{K_{ia}K_b + K_bA + K_aB + AB} \quad (4)$$

where the symbols have the following meaning: V, maximum velocity when both substrates are saturating; A, concentration of NAD^+; B, concentration of *myo*-inositol; K_{ia} dissociation constant of the enzyme–NAD^+ complex; K_a, Michaelis constant of NAD^+ when *myo*-inositol is saturating; K_b, Michaelis constant of *myo*-inositol when NAD^+ is saturating.

For the initial velocity of the reverse reaction we have:

$$v_2 = V_2 \frac{PQ}{K_{iq}K_p + K_pQ + K_qP + PQ} \quad (5)$$

where the symbols have analogous meanings, P and p referring to *scyllo*-inosose, Q and q to NADH.

Since hydrogen ions are a reaction product not accounted for in Eqns 4 and 5,

TABLE II

KINETIC CONSTANTS AT pH 8.7 AND 25 °C OF *myo*-INOSITOL:NAD^+ OXIDOREDUCTASE FROM *Cryptococcus melibiosum*

The values are the means (± S.D.) of estimates obtained from five sets of experiments.

K_a (NAD^+)	$6.9 (\pm 0.7) \cdot 10^{-5}$ M
K_{ia} (NAD^+)	$1.5 (\pm 0.6) \cdot 10^{-4}$ M
K_b (*myo*-inositol)	$5.1 (\pm 1.8) \cdot 10^{-3}$ M
K_p (*scyllo*-inosose)	$6.4 (\pm 1.9) \cdot 10^{-4}$ M
K_q (NADH)	$9.9 (\pm 9.5) \cdot 10^{-5}$ M
K_{iq} (NADH)	$1.7 (\pm 0.8) \cdot 10^{-5}$ M

the kinetic parameters of these equations are functions of pH. Accordingly, all kinetic experiments were performed at the same pH (8.7); the temperature was 25 °C. Fig. 1 depicts a set of primary and secondary plots with NAD^+ as the variable substrate and *myo*-inositol as the fixed substrate. Similar plots were prepared with respect to the three other possible combinations of substrates and products. The kinetic parameters obtained from these plots are listed in Table II.

Inhibition of the forward reaction by *scyllo*-inosose with NAD^+ as the variable substrate and *myo*-inositol at a saturating concentration was uncompetitive (Fig. 2). This behaviour is diagnostic for a "Bi Bi Ordered" mechanism with NAD^+ as the

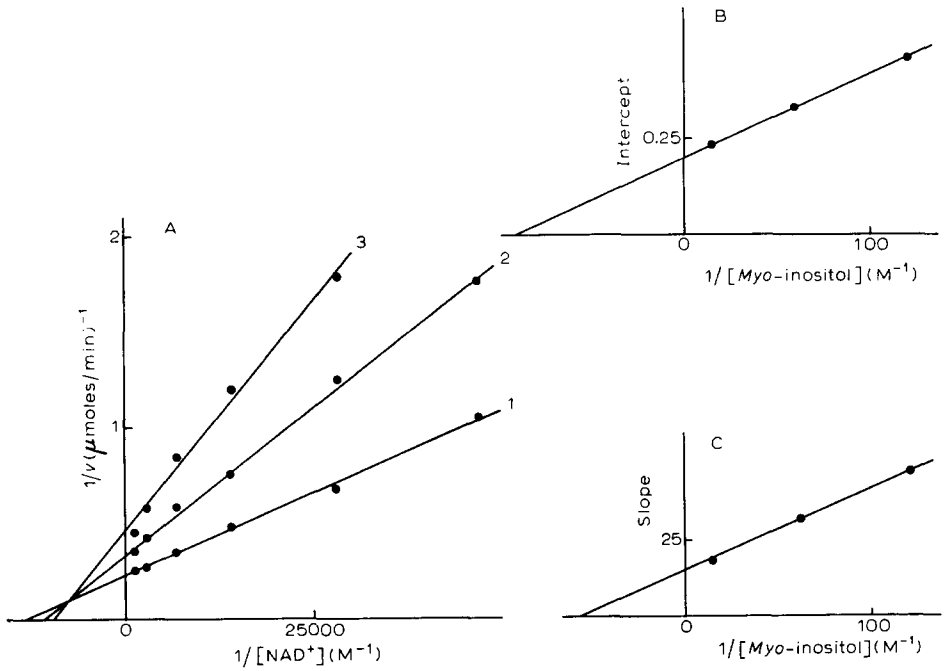

Fig. 1. A. Double reciprocal plot (least square fit) of the initial velocity with NAD^+ as the variable substrate at three concentrations of *myo*-inositol, 1: $6.6 \cdot 10^{-2}$ M, 2: $1.65 \cdot 10^{-2}$ M, 3: $0.825 \cdot 10^{-2}$ M. B. Secondary plot (least square fit) of the intercepts of plot A against the reciprocals of the corresponding *myo*-inositol concentrations. C. Secondary plot (least square fit) of the slopes of plot A against the reciprocals of the corresponding *myo*-inositol concentrations.

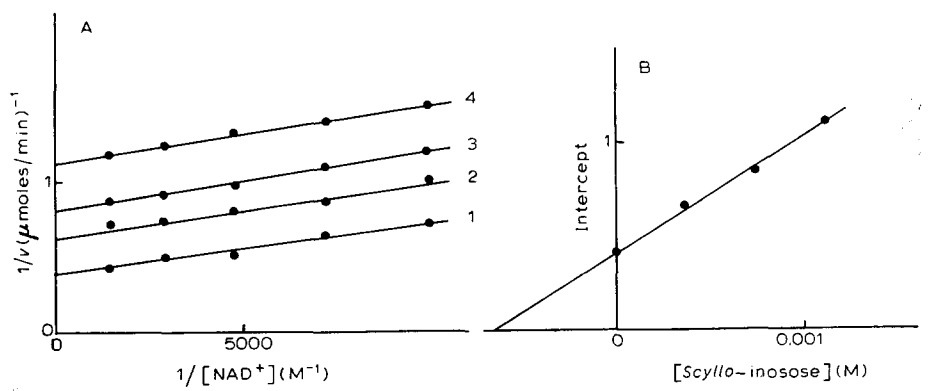

Fig. 2. A. Double reciprocal plot (least square fit) of the initial velocity with NAD^+ as the variable substrate, *myo*-inositol at a saturating concentration (8.33×10^{-2} M), and *scyllo*-inosose as the inhibitor at the following concentrations, 1: 0, 2: $0.374 \cdot 10^{-3}$ M, 3: $0.748 \cdot 10^{-3}$ M, 4: $1.222 \cdot 10^{-3}$ M. B. Secondary plot (least square fit) of the intercepts of plot A against the corresponding *scyllo*-inosose concentrations.

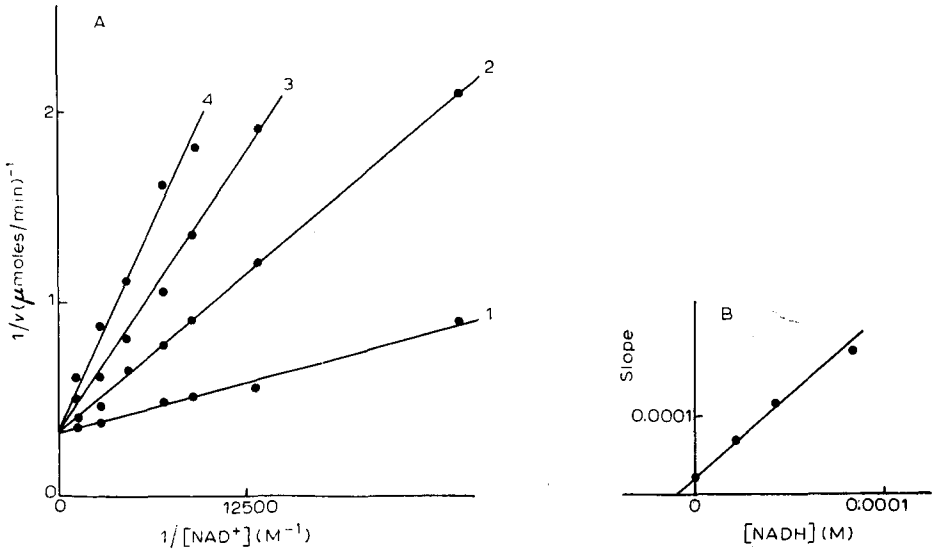

Fig. 3. A. Double reciprocal plot (least square fit) of the initial velocity with NAD^+ as the variable substrate, *myo*-inositol at a fixed concentration ($8.33 \cdot 10^{-2}$ M) and NADH as the inhibitor at the following concentrations, 1: 0, 2: $2.09 \cdot 10^{-5}$ M, 3: $4.18 \cdot 10^{-5}$ M, 4: $8.36 \cdot 10^{-5}$ M. B. Secondary plot (least square fit) of the slopes of plot A against the corresponding NADH concentrations.

leading substrate in this case (Cleland[14]). It may be expressed as follows:

$$\frac{1}{v_1} = \frac{1}{V_1}\left(1 + \frac{P}{K_{ip}}\right) + \frac{K_a}{V_1}\frac{1}{A} \tag{6}$$

where K_{ip} is an inhibition parameter referring to *scyllo*-inosose. A secondary plot of the intercepts (Fig. 2) gave an estimate of $6.5 \cdot 10^{-4}$ M for this parameter.

Inhibition of the forward reaction by NADH with NAD^+ as the variable substrate was competitive (Fig. 3). This is consistent with (though not diagnostic for[14]) a "Bi Bi Ordered" mechanism and may be expressed as follows[15]:

$$\frac{1}{v_1} = \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) + \frac{K_a}{V_1}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left(1 + \frac{Q}{K_{iq}}\right)\frac{1}{A} \tag{7}$$

A secondary plot of the slopes (Fig. 3) gave an estimate of $1.1 \cdot 10^{-5}$ M for K_{iq} which is of the same order of magnitude as an estimate of this parameter obtained by other means (Table II).

Inhibition of the forward reaction by *scyllo*-inosose with *myo*-inositol as the variable substrate and NAD^+ at a saturating concentration was non-competitive (Fig. 4). Again, this behaviour is consistent with (though not diagnostic for[14]) a "Bi Bi Ordered" mechanism and may be expressed as follows:

$$\frac{1}{v_1} = \frac{1}{V_1}\left(1 + \frac{P}{K_{ip}}\right) + \frac{K_b}{V_1}\left(1 + \frac{P}{\frac{K_pK_{iq}}{K_q}}\right)\frac{1}{B} \tag{8}$$

A secondary plot of the intercepts gave an estimate of $1.8 \cdot 10^{-4}$ M K_{ip} (Fig. 4) which is of the same order of magnitude as the K_{ip} value estimated from Fig. 2.

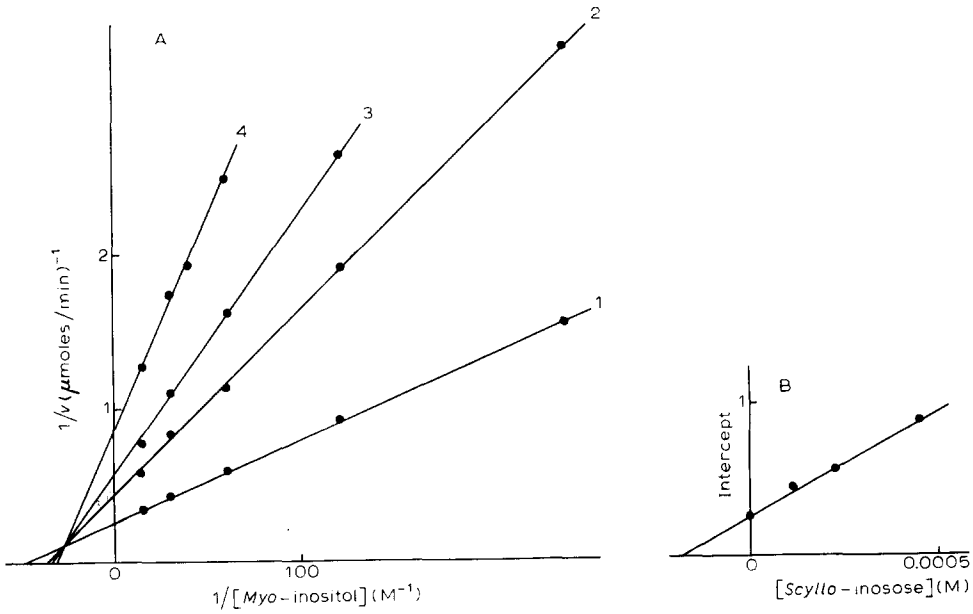

Fig. 4. A. Double reciprocal plot (least square fit) of the initial velocity with *myo*-inositol as the variable substrate, NAD^+ at a saturating concentration ($1.26 \cdot 10^{-3}$ M) and *scyllo*-inosose as inhibitor at the following concentrations, 1: 0, 2: $1.12 \cdot 10^{-4}$ M, 3: $2.24 \cdot 10^{-4}$ M, 4: $4.48 \cdot 10^{-4}$ M. B. Secondary plot (least square fit of the intercepts of plot A against the corresponding *scyllo*-inosose concentrations.

Equilibrium constant

The equilibrium constant of inositol-NAD^+ oxido-reduction catalyzed by the enzyme preparation at 25 °C was obtained from plots of quadruplicate sets of apparent equilibrium constants at various pH values (Fig. 5). The mean value ($\pm$ S.D.) of the estimates was $5.3(\pm 2.5) \cdot 10^{-12}$. Larner *et al.*[6] determined the equilibrium constant of

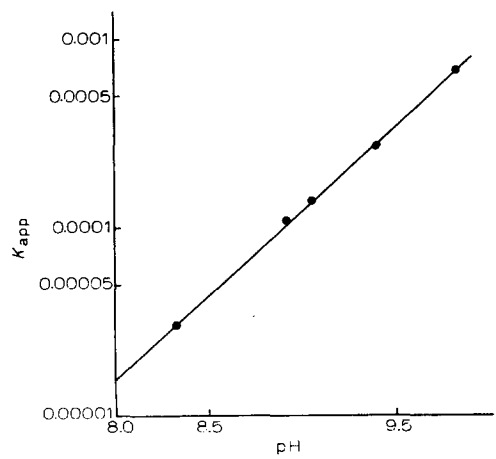

Fig. 5. Plot (least square fit) of apparent equilibrium constants of *myo*-inositol–NAD^+ oxido-reduction as catalyzed by inositol dehydrogenase from *C. melibiosum*, against the final pH values.

inositol–NAD^+ oxidoreduction catalyzed at "room temperature" by inositol dehydrogenase from *Aerobacter aerogenes*. Their estimate of $3.8 \cdot 10^{-12}$ is within the range of our estimates at 25 °C.

ACKNOWLEDGMENT

The technical assistance of Mrs Teresa Plácido is appreciated.

REFERENCES

1 Lodder, J. (1970) *The Yeasts*, 2nd edn, pp. 166–1352, North-Holland Publishing Company, Amsterdam and London
2 Sivak, A. and Hoffman-Ostenhof, O. (1961) *Biochim. Biophys. Acta* 53, 426–428
3 Charalampous, F. C. (1959) *J. Biol. Chem.* 234, 220–227
4 Loewus, F. A. and Kelly, S. (1962) *Proc. Natl. Acad. Sci. U.S.* 48, 421–425
5 Vidal-Leiria, M. (1969/1970) *Arq. Port. Bioquim.* 12, 403–404
6 Larner, J., Jackson, W. T., Graves, D. J. and Stamer, J. R. (1956) *Arch. Biochem. Biophys* 60, 352–363
7 Berman, T. and Magasanik, B. (1966) *J. Biol. Chem.* 241, 800–806
8 Phaff, H. J. and Fell, J. W. (1970) in *The Yeasts* (Lodder, J. ed.), 2nd edn, pp. 1130–1132 North-Holland Publishing Company, Amsterdam and London
9 Trevelyan, W. E., Procter, D. P. and Harrison, J. S. (1950) *Nature* 166, 444–445
10 Wallenfels, K. (1950) *Naturwissenschaften* 37, 491–492
11 Racker, E. (1950) *J. Biol. Chem.* 184, 313–319
12 Klingenberg, M. (1962) in *Methoden der enzymatischen Analyse* (Bergmeyer, H. U., ed.) pp. 528–530, Verlag Chemie, Weinheim
13 Warburg, O. and Christian, W. (1941) *Biochem. Z.* 310, 384–421
14 Cleland, W. W. (1963) *Biochim. Biophys. Acta* 67, 104–137
15 Cleland, W. W. (1963) *Biochim. Biophys. Acta* 67, 173–187