BBA 66629

ELECTROPHORETIC MIGRATION AND REDOX BEHAVIOR OF MALATE DEHYDROGENASES FROM CELL SUSPENSION CULTURES OF TOBACCO

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(Received January 24th, 1972)

SUMMARY

1. The presence of one distinct mitochondrial malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37), at least three cytosol malate (NAD^+) dehydrogenase isoenzymes and only one cytosol malic enzyme component (L-malate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40) were demonstrated in preparations from plant suspension cells cultivated at 25 °C.

2. Bulk separation of the cytosol malate (NAD^+) dehydrogenase isoenzymes was achieved by serial fractionation with $(NH_4)_2SO_4$. Each of the three enzyme forms possessed different kinetic properties and one of the isoenzymes did not require phenazine methosulfate to reduce tetrazolium after gel electrophoresis.

3. Cultivating the plant suspension cells at temperatures at least 10 °C above or below 25 °C prevented expression of one or the other of three cytosol malate (NAD^+) dehydrogenase isoenzymes.

4. Treatment of the particulate preparation with deoxycholate increased the electrophoretic mobility of mitochondrial malate (NAD^+) dehydrogenase. Depending on the concentration or assay method used, deoxycholate either inhibited or reversed the reaction catalyzed by soluble as well as particulate forms of malate (NAD^+) dehydrogenase.

INTRODUCTION

Cytochemical localization and *in vivo* assays have been used to determine the extent of cell differentiation among cell populations of WR-132 cultures derived from tobacco[1]. Malate (NAD^+) dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) activity during passage of cultures at 25 °C was characterized by periodic fluctuations in maximal activity with respect to time and specific patterns of shifting distribution in terms of localization sites. These investigations have been extended in

Abbreviations: PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol.

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an attempt to correlate metabolic and enzymic behavior with the molecular properties of malate (NAD^+) dehydrogenase enzyme extracted from WR-132 cultures grown at different temperatures.

Since the distribution of malate (NAD^+) dehydrogenase has proved to be bimodal in all organisms examined to date[2-5], particulate and soluble fractions from WR-132 cells were separated after homogenization by means of differential centrifugation and studied individually. The mitochondrial enzyme was solubilized with deoxycholate and the cytosol enzyme precipitated with $(NH_4)_2SO_4$ to achieve further purification. The effects of these reagents on the electrophoretic mobility and the tetrazolium reductase capability of malate (NAD^+) dehydrogenase isoenzymes are reported.

MATERIALS AND METHODS

Plant cultures

Enzyme extracts were obtained from WR-132 suspension cell cultures (*Nicotiana tabacum* L., var Xanthi) grown and harvested under conditions described in a previous paper[1]. Cell suspensions were separated from growth medium by filtration through Miracloth, and homogenized in a buffered grinding medium at pH 6.8 containing 0.075 M phosphate buffer (Na_2HPO_4 and KH_2PO_4), 0.35 M mannitol, 0.5 mM EDTA, 1.0 mM cysteine, and 0.1% polyvinylpyrrolidone (M_r 360 000). Cells were disrupted in the cold with a Virtis No. 45 homogenizer*. After filtration through Miracloth, the homogenate was centrifuged at 100 × *g* for 10 min in a Sorvall RC2-B. From 150 g fresh weight of cells in 100 ml of grinding medium, 200 ml of supernatant were obtained. The supernatant was recentrifuged at 16 000 × *g* for 15 min to give a large pellet (plastid–mitochondrial) fraction and a soluble fraction. The pellet, suspended in Tris–maleate buffer (0.2 M at pH 7.0) with 0.5 M sucrose and 0.05 $MgCl_2$, was washed three times by repeated centrifugations at 16 000 × *g* and resuspension in buffer.

Solid $(NH_4)_2SO_4$ was added to the soluble fraction over a period of 15 min with constant stirring at 4 °C. After stirring for approximately 60 min the precipitate was centrifuged down at 16 000 × *g* for 15 min. Pellets were collected at 25, 65 and 90% saturation. The solution remaining after 90% saturation was saved for further analysis. The large 0–25% pellet (Fraction I) and 25–65% pellet (Fraction II) were suspended in 10 ml water and the smaller 65–90% pellet (Fraction III) was suspended in 5 ml water. These fractions were dialyzed in the cold against 2 l of 0.2 mM Tris–maleate buffer (pH 7.0). Three changes in buffer were made and dialysis was extended overnight. The pellets from II and III redissolved but Fraction I did not. The latter was analyzed for enzyme activity as a suspension. The remaining 200 ml of solution (90% saturated with $(NH_4)_2SO_4$) were dialyzed in the cold until no precipitate was obtained with $BaCl_2$ and then dehydrated to obtain a final volume of 75 ml by covering the dialysis bags with dry Carbowax (20-M). This fraction was designated the $(NH_4)_2SO_4$ soluble fraction (Fraction IV). The final protein content in these cytosol fractions as determined by the Folin–Ciocalteu method[6] was as follows: I: 5.1 mg/ml; II: 11 mg/ml; III: 2.2 mg/ml; and IV: 0.8 mg/ml. In one series of experiments the malate (NAD^+) dehydrogenase banding patterns of 45–65% fractions from 3-, 6- and

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10-day-old cultures were examined for isoenzyme banding. The electrophoretic behavior of soluble L-malate:$NADP^+$ oxidoreductase (decarboxylating) (EC 1.1.1.40) and particulate L-glutamate:NAD^+ oxidoreductase (deaminating) (EC 1.4.1.2) were also examined to provide a standard of comparison.

Electrophoretic separations

The Canalco analytic system consisting of 7% acrylamide in pH 8.9 Tris–chloride buffer, was used for disc electrophoresis[7,8]. Satisfactory electrophoretic separation was achieved after 45 min at 5 mA per tube. Enzyme localization was accomplished in a reaction mixture containing 0.5 M sodium malate, 10 vol.; 0.1 M NAD(P), 10 vol.; 1 mg/ml of nitro blue tetrazolium or 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide, 20 vol.; 0.15 M phosphate buffer at pH 7.0, 20 vol.; 0.1 M sodium azide, 5 vol.; 0.5 M $MgCl_2$, 5 vol.; and 0.3 mg/ml of phenazine methosulfate (PMS), 2 vol. When 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide was used, 1 vol. of $CoCl_2$ (0.5 M) was added after staining had commenced. For localizing glutamate (NAD^+) dehydrogenase, 0.2 M monosodium glutamate was substituted for malate. Preparations with high malate:NAD^+ oxidoreductase activity stained adequately within 20 min but glutamate:NAD^+ oxidoreductase localization required up to 2 h. Deoxycholate at 2 or 5% was added to the polyacrylamide solution prior to photopolymerization for some electrophoretic separations in proportions equal to that of the enzyme sample. Gel bands were scanned to determine relative positions and staining intensities with the Canalco Model F microdensitometer.

Spectrophotometric assays

Enzyme activity in the fractions was assayed by measurement of increase or decrease of NAD(P)H at 340 nm and by a coupled dye assay similar to that described by Glick[9]. The acceptor dye 2,6-dichlorophenolindophenol (DCIP) at 1.2 mM was used with other reaction components in concentrations as previously described in ratios as indicated below. In a typical assay system, the ratios of reaction components were as follows: phosphate buffer (pH 7.0), 5 vol.; PMS, 2 vol.; DCIP, 2 vol.; substrate, 2 vol.; NAD^+ or $NADP^+$, 2 vol.; NaN_3, 2 vol.; enzyme, 1 vol.; and water, 15 vol. Aliquots were measured by the drop, (approximately 50 μl), and substrate was added to microcuvettes (1.5-ml volume capacity) after equilibration. A Beckman DK-2 recording spectrophotometer equipped with a time gear was used at 600 nm to follow loss of color resulting from reduction of DCIP. The enzyme assay using DCIP was similar to that employed for cytochemical demonstration of dehydrogenases with tetrazoliums, except that with DCIP an insoluble end product is not formed and therefore the reaction is theoretically reversible. All chemicals were reagent grade or obtained from Sigma Chemical Company.

RESULTS

Effect of cultivation temperature on malate dehydrogenases

Cytosol malate (NAD^+) dehydrogenase migrated more rapidly during electrophoresis than did the $NADP^+$-specific malic enzyme obtained from WR-132 culture preparations. In addition, malate (NAD^+) dehydrogenase from this source was localized in gels as a wide band of activity in contrast to the narrow band associated with

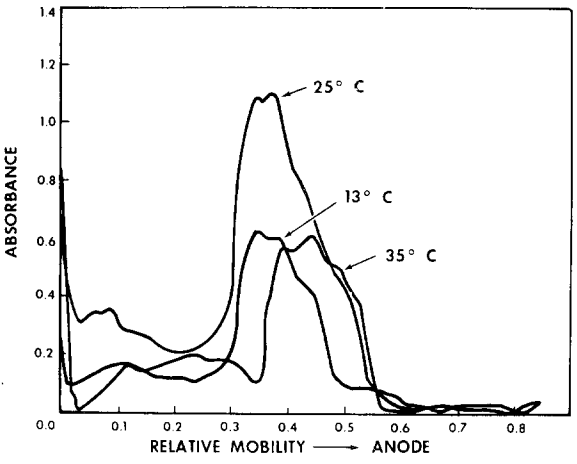

Fig. 1. Densitometer traces of malate (NAD+) dehydrogenase bands obtained from samples cultivated at 13, 25 and 35 °C. Note the absence of leading peaks in 13 °C derived material and the absence of trailing peaks in 35 °C derived material.

malate (NADP+) dehydrogenase activity. The NADP+-requiring enzyme from cultures grown at 13, 25 and 35 °C exhibited no difference in electrophoretic mobility whereas the NAD+-requiring enzyme from cultures grown at these temperatures exhibited unique isoenzyme patterns (Fig. 1). Enzyme samples from 13 °C cultures contained the slower moving components and 35 °C samples contained the more mobile isoenzyme components, while the complete range of enzyme heterogeneity occurred in 25 °C samples. No significant differences in migration behavior were found in mito-

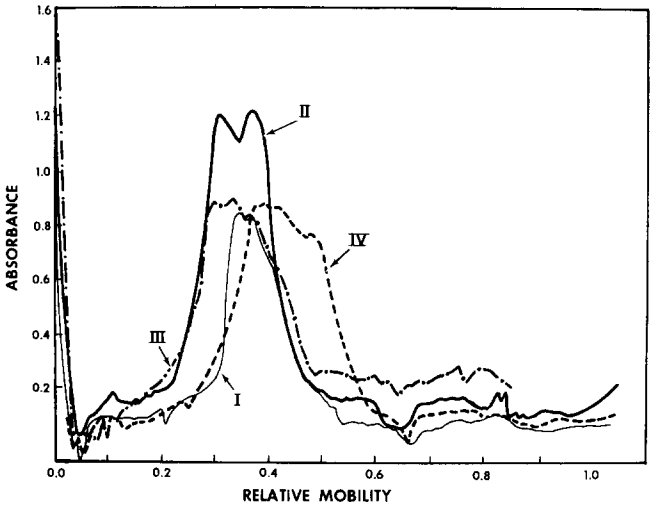

Fig. 2. Densitometer traces of malate (NAD+) dehydrogenase isoenzyme bands from fractions obtained by $(NH_4)_2SO_4$ precipitation of 25 °C cytosol material. Fractions as described in the text are I, 0–25%; II, 25–65%, III, 65–90%, and IV, soluble in 90% ammonium sulfate. There are three major regions of enzyme localization but a total of five peaks. The central peak is the only one common to all four fractions.

chondrial malate (NAD+) dehydrogenase obtained from cultures grown at the designated temperatures.

Separation of cytosol malate (NAD+) dehydrogenase by $(NH_4)_2SO_4$ fractionation

The cytosol fraction from 25 °C cultures was subdivided into four fractions by means of $(NH_4)_2SO_4$ fractionation. Although each of the four cytosol fractions contained malate (NAD+) dehydrogenase activity, the electrophoretic mobilities and kinetic properties of enzyme differed to some extent in each of the fractions. Disc electrophoresis and subsequent microdensitometric scanning of banded gels revealed the existence of five isoenzyme components or subunits (Fig.2). The most mobile isoenzymes were contained in Fraction IV, the slowest in Fractions II and III and a single isoenzyme with intermediate mobility in Fraction I. Although a total of five cytosol isoenzyme bands have been resolved by the method of disc electrophoresis only three were distinguished on the basis of their kinetic properties.

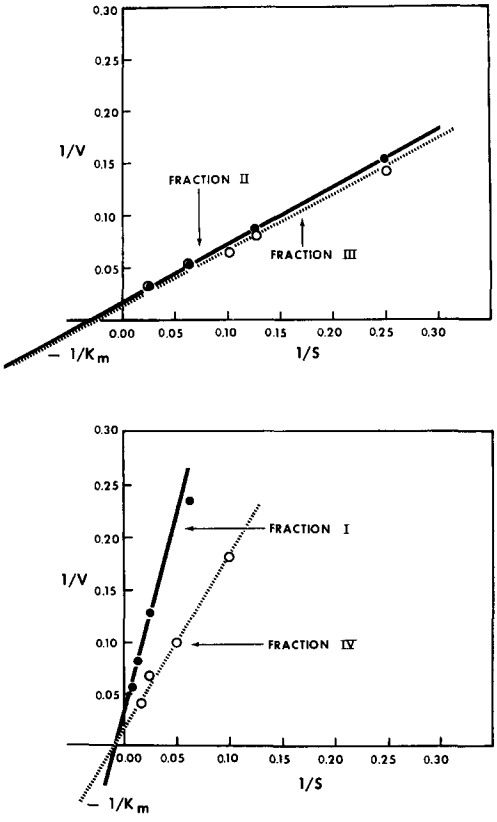

Fig. 3. Kinetic characteristics of malate (NAD+) dehydrogenases in $(NH_4)_2SO_4$ fractions prepared from 25 °C cytosol. Michaelis constants were determined by the coupled dye assay method using PMS and DCIP at pH 7.0 and varying only the malate concentration. These data confirm the existence of at least three distinct malate (NAD+) dehydrogenase isoenzymes in the cytosol. The K_m values for Fractions II and III are 9.2 mM malate and the K_m values for Fractions I and IV are 32 mM malate. While the enzyme constants for the latter two fractions are identical, the different slopes of the line K_m/V for Fractions I and IV suggest that Fraction I isoenzyme is a non-competitively inhibited form of Fraction IV isoenzyme.

The apparent K_m values for malate (NAD^+) dehydrogenase in each of the fractions were determined by replotting substrate concentration curves by using the double reciprocal method of Lineweaver and Burk[10], (Fig. 3). The apparent K_m and V for Fraction II and III were essentially identical. The apparent K_m values for Fractions I and IV were similar but larger than the K_m for Fractions II and III. However, the V for each of the former two enzymes was different (Fig. 3, lower graph). Furthermore, the degree of enzyme retention at the origin in the sample gel after disc electrophoresis, was noticeably significant with Fraction I, but negligible with Fraction IV.

"Diaphorase" capability of malate (NAD^+) dehydrogenase isoenzymes

Two forms of the NAD^+-requiring enzyme were able to reduce tetrazolium to formazan without the presence of an exogenous electron carrier such as phenazine methosulfate. Mitochondrial malate (NAD^+) dehydrogenase and one or two of the cytosol isoenzymes possessed this property (Fig. 4).

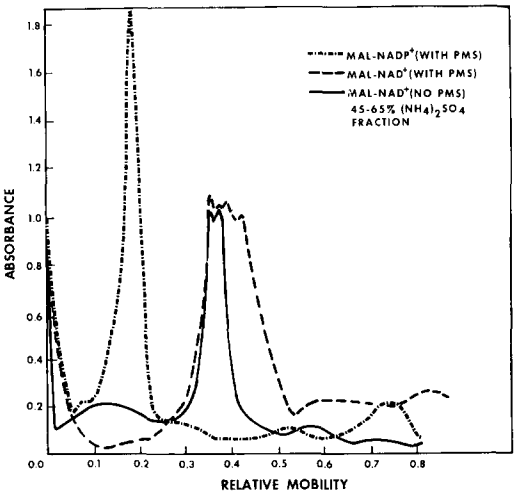

Fig. 4. Densitometer traces demonstrating the migration behavior and relative staining intensities of malate NAD^+- and $NADP^+$-linked dehydrogenases localized by formazan deposition in polyacrylamide gels after electrophoresis with or without PMS in the incubation medium. Samples were obtained from tobacco suspension cell cytosol extracts and fractionated with $(NH_4)_2SO_4$ at 45–65% saturation. Cytosol preparations from 3–6-day-old cultures always yield a single PMS-independent band but 10-day-old cultures yield two closely spaced bands.

Sharp banding for cytosol malate (NAD^+) dehydrogenase without PMS occurred only with samples from Fraction II. Fraction IV isoenzyme was completely dependent upon PMS for formazan localization, but Fraction I isoenzyme was only partially PMS dependent as judged by tetrazolium reductase ("diaphorase") activity.

Precipitation of cytosol material with 45–65% $(NH_4)_2SO_4$ and subsequent dialysis yielded the cleanest preparation of the least mobile malate (NAD^+) dehydrogenase isoenzyme. PMS was not required to visualize the enzyme activity in polyacrylamide gel after electrophoresis of these samples. The banding was narrow and singular when fractions were made from cultures harvested 3 or 6 days after inoculation but wider and doubled with cultures at least 10 days old (Fig. 4). It was observed that no

difference occurred in localization of cytosol malate ($NADP^+$) dehydrogenase from 3-, 6- and 10-day-old cultures but PMS was required to demonstrate activity.

The effect of deoxycholate on enzyme behavior

When deoxycholate was not present, the mitochondrial enzyme barely penetrated the separator gel, but if the surfactant was added to particulate suspensions before applying the sample, electrophoretic mobility of the mitochondrial enzyme was substantially increased (Fig. 5). Deoxycholate also improved resolution of the five individual cytosol bands but did not alter their position in the gel (Fig. 5).

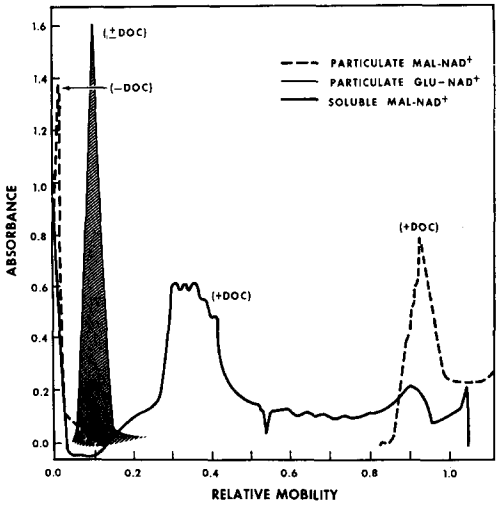

Fig. 5. Densitometer traces illustrating localization changes in mitochondrial and cytosol malate (NAD^+) dehydrogenases resulting from addition of deoxycholate (DOC) to the sample prior to electrophoresis. Mitochondrial isoenzyme is shifted from a proximal to a distal location in the gel. Cytosol enzyme is resolved into five peaks of approximately equal height (except for the two leading peaks) but no change in banding position can be observed. Glutamate (NAD^+) dehydrogenase is unaffected by deoxycholate. — — — —, mitochondrial malate (NAD^+) dehydrogenase; striped part, mitochondrial glutamate (NAD^+) dehydrogenase; and ———, cytosol malate (NAD^+) dehydrogenase.

When recombination experiments using deoxycholate were conducted with particulate and soluble enzyme mixtures, malate (NAD^+) dehydrogenase activity was localized in the central portion of the gel as 3–5 discrete closely spaced bands and two additional bands at either extremity of the gel. Using mitochondrial preparations, an incubation time of 30 min with 5% deoxycholate induced total conversion of the particulate enzyme to a soluble form which migrated into the distal portion of the gel (Fig. 5). Mobility of mitochondrial glutamate (NAD^+) dehydrogenase was unaltered by deoxycholate treatment (Fig. 5). Reduction of tetrazolium by glutamate (NAD^+) dehydrogenase was slow and required PMS but produced a narrow, intensely stained formazan band.

Non-linear reaction rates were characteristic of the NAD^+-linked enzyme but not of the $NADP^+$-requiring enzyme when assayed for increase of absorbance at 340 nm and at pH 7.0. For this reason subsequent assays of malate (NAD^+) dehydro-

genase by this method were made at the non-physiological pH of 10.0 for both the forward and reverse reactions. Under these conditions both malate → oxaloacetate and the reverse reaction were decreased by the addition of deoxycholate.

Deoxycholate interference was also examined at pH 7.0 by employing the coupled dye assay which depends upon the reduction of 2,6-dichlorophenolindophenol to a colorless form. Using this assay it was found that increased inhibition of malate (NAD^+) dehydrogenase activity resulted from increased concentrations of deoxycholate. Thus malate (NAD^+) dehydrogenase activity at pH 7.0 and deoxycholate concentrations of 0.0125, 0.050, 0.10 and 0.125% was 44, 30, 14 and 5%, respectively, of the original forward reaction. Increasing the deoxycholate concentration beyond 0.125% resulted in anomalous fluctuations in the activity including values in which the direction of the reaction reversed (*e.g.* at 0.175% deoxycholate activity was 4% of the original activity but in the opposite direction).

The maximum reversed activity with the coupled dye assay system was attained with deoxycholate at a final concentration of approximately 0.25%. At this level, the reversed rate was 10% of the original forward rate. The deoxycholate effect was obtained with crude, as well as with partially purified enzyme preparations. Deoxycholate is known to inhibit or stimulate some enzyme activities according to the concentration used, a response described as "biphasic"[11]. In the coupled dye assay, malate oxidation is measured by change in absorbance at 600 nm resulting from reduction of 2,6-dichlorophenolindophenol. Reoxidation of the dye turns it blue again. The possibility that deoxycholate reversal was an artifact of the assay system was eliminated by the results of experiments with Fraction II enzyme. Both malate (NAD^+) and ($NADP^+$) dehydrogenases occur in Fraction II making it simple to check the response

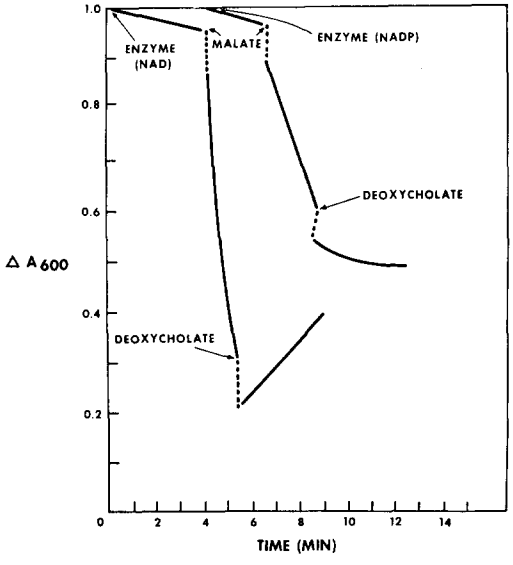

Fig. 6. Comparison of the effect of 0.25% deoxycholate on malate dehydrogenase activities in Fraction II assayed by the coupled dye method. Note abrupt reversal of the reaction when coenzyme NAD^+ is employed and total suppression of reaction when coenzyme $NADP^+$ is employed. The velocity of the reversed rate for malate (NAD^+) dehydrogenase is 10% that of the original forward reaction.

of the two enzymes to deoxycholate with comparable samples. Reversal of the $NADP^+$-requiring enzyme was not observed although activity was completely abolished by 0.25% deoxycholate (Fig. 6). Both mitochondrial and cytosol malate (NAD^+) dehydrogenase preparations responded similarly to deoxycholate.

DISCUSSION

Compartmentation of two different types of malate (NAD^+) dehydrogenase within particulate and soluble phases of plant and animal cells is well documented and may be physiologically significant[3,5,12-15]. It has been estimated that the cytosol enzyme accounts for 55–70% of the total malate (NAD^+) dehydrogenase activity in plants[18,19].

The two cytosol isoenzymes from WR-132 cells resembling each other the most in terms of electrophoretic and kinetic properties are separated out at opposite ends of the $(NH_4)_2SO_4$ gradient, *viz.* at 0–25% and 90%; therefore, it is unlikely that increasing the salt concentration caused formation of the isoenzymes. The occurrence of three cytosol malate (NAD^+) dehydrogenase isoenzymes may be related to differences in tertiary structure and the degree of coiling[20-22].

Villee[23] has speculated that multimolecular forms of malate (NAD^+) dehydrogenase exist as a result of conformational differences rather than hybridization although others dispute this concept[24]. Some workers[3,5] using ion exchange chromatography and starch gel electrophoresis failed to demonstrate subunits in mitochondrial and cytosol enzymes but as many as ten malate (NAD^+) dehydrogenases were reported for milled wheat fractions subjected to disc electrophoresis with polyacrylamide[25]. Mitochondrial and cytosol enzymes from animal sources differ in several fundamental respects[26-28]. Our work has demonstrated both differences and similarities between the two types of malate (NAD^+) dehydrogenases in plants.

Solubilization of the mitochondrial malate (NAD^+) dehydrogenase from WR-132 cells probably involves release of the enzyme protein from a lipid moiety as shown by the effects of deoxycholate with other membrane complexes[29,30]. This explains the greater electrophoretic mobility of mitochondrial enzyme after exposure to deoxycholate. Mitochondrial glutamate (NAD^+) dehydrogenase is apparently unchanged by deoxycholate. Apart from solubilization, deoxycholate has a secondary effect on both cytosol and mitochondrial malate (NAD^+) dehydrogenase. The kinetic evidence suggests that the surfactant causes structural perturbations near the active site without grossly altering overall configuration.

The original method for localizing dehydrogenases in electrophoresced gels required a "diaphorase" adjuvant in the reaction mixture[31]. The routine use of PMS to serve this purpose is a common procedure[32]. Cytochemical localization of dehydrogenases *in situ* is also achieved with the use of PMS[33,34]. Our results suggest that intracellular localization of malate (NAD^+) dehydrogenase with or without the inclusion of PMS could be used for discriminating between different forms of the enzyme *in situ*.

No correlation between the cytochemical heterogeneity for malate (NAD^+) dehydrogenase in WR-132 cells[1] and the isoenzyme patterns has yet been demonstrated. Since the levels of malate (NAD^+) dehydrogenase activity are altered by various factors during extraction and assay procedures[35,36], a direct relationship between the amount of *in vivo* activity and the amount of extractable enzyme would not

necessarily be observed. Presumably, endogenous substances analogous to deoxycholate could influence enzyme activity in the intact cell. Although concentrations of the cytosol isoenzymes reported here might coexist in equilibrium as conformers, their relative stability indicates that the mechanism for their isoenzyme interconversion would have to operate at or near the translation level of biosynthesis.

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