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Effect of NAD$^+$ on Malate Oxidation in Intact Plant Mitochondria[1]

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ABSTRACT

Potato tuber mitochondria oxidizing malate respond to NAD$^+$ addition with increased oxidation rates, whereas mung bean hypocotyl mitochondria do not. This is traced to a low endogenous content of NAD$^+$ in potato mitochondria, which prove to take up added NAD$^+$. This mechanism concentrates NAD$^+$ in the matrix space. Analyses for oxaloacetate and pyruvate (with pyruvate dehydrogenase blocked) are consistent with regulation of malate oxidation by the internal NAD$^+$/NADH ratio.

It is well established that mitochondria isolated from a large variety of plant tissues oxidize malate rapidly in the absence of either glutamate or a source of acetyl-CoA (21, 24). This oxidation is attributed to malate dehydrogenase (L-malate: NAD$^+$oxidoreductase EC 1.1.1.37) and/or NAD$^+$-linked malic enzyme (L-malate:NAD$^+$oxidoreductase [decarboxylating] EC 1.1.1.39), discovered in plant mitochondria by Macrae and Moorhouse (15). The presence of these two enzymes in the matrix space (7) causes pyruvate and oxaloacetate to accumulate in the medium during malate oxidation. The rates and products of malate oxidation by plant mitochondria vary in response to changes in the pH of the incubation medium (16). In addition, the oxidation of malate which is coupled to three sites of ATP formation is stimulated under certain conditions by NAD$^+$ (6, 9, 20).

With the aim of further clarifying the mechanisms of malate oxidation in intact plant mitochondria this report details the effect of NAD$^+$ on malate oxidation in potato tuber and mung bean hypocotyl mitochondria. Some of these results have been presented elsewhere (17).

MATERIALS AND METHODS

Preparation of Mitochondria. Mitochondria from potato (*Solanum tuberosum* L.) tubers and etiolated mung bean (*Vigna radiata* L.) hypocotyls cut from bean seedlings grown for 5 days in the dark at 26 C and 60% RH were prepared and purified by methods previously described (10). All operations were carried out at 0–4 C. Following purification, the mitochondria appeared to be virtually free from extramitochondrial contamination and had a high degree of membrane intactness as judged by electron microscopy and by low activities of the inner membrane and matrix marker enzymes (antimycin A-sensitive NADH:Cyt *c* oxidoreductase and malate dehydrogenase) (10). In addition, the mitochondria were tightly coupled: average ADP/O ratios for succinate were 1.8 and respiratory control ratios for the same substrate were approximately 4.

O_2 Uptake Measurements. O_2 uptake was measured at 25 C using a Clark-type O_2 electrode (Hansatech DW O_2 electrode unit). The reaction medium (medium A) contained: 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer, 0.1% defatted BSA, and known amounts of mitochondrial protein. Unless otherwise stated, all incubations were carried out at pH 7.2.

Assay of Metabolic Products. Products of malate metabolism by intact mitochondria were routinely assayed at 25 C in a stirred cell containing medium A and known amounts of mitochondrial protein. The reaction was initiated by the addition of 15 mM malate. At various times 1-ml aliquots were taken and added to 0.3 ml of cold 20% $HClO_4$ containing 1 mM EDTA. After addition of 10 μl of an alcoholic solution of methyl orange (0.06% w/v) the samples were quickly neutralized with KOH (up to pH 5.5) and centrifuged for 10 min at 2,000*g* to remove $KClO_4$. The supernatant was used for pyruvate and oxaloacetate determination as described by Wedding *et al.* (23). Simultaneously the O_2 consumption of 1-ml aliquots was measured. In some experiments, the reaction was stopped directly in the electrode cell. In good agreement with Wedding *et al.* (23) we have observed that, after treatment with $HClO_4$, a low malate dehydrogenase activity remained in the extract, making oxaloacetate determination with this enzyme difficult. Oxaloacetate was therefore quantitatively decarboxylated with 10 mM $NiCl_2$ (23) for 5 min at 45 C in a second aliquot of the centrifuged extract and pyruvate thus formed determined.

Assay of NAD$^+$. Extraction and enzymic estimation of endogenous mitochondrial NAD$^+$ was carried out according to Klingenberg (13). It was important to maintain the total pyridine nucleotide pool in its oxidized state since any NADH present in the medium was destroyed due to its instability in $HClO_4$. Consequently, prior to extraction of NAD$^+$ mitochondria were incubated in the medium A containing: 150 μM ATP, 4 μM FCCP, 200 μg catalase, 100 μM H_2O_2. NAD$^+$ concentrations were measured using a Chance-Aminco dual wavelength spectrophotometer, the reference and assay wavelengths being 374 and 340 nm, respectively.

Pyridine Nucleotide Fluorescence. The redox level of the endogenous NAD$^+$ was monitored fluorometrically with an Eppendorf fluorometer equipped with a 366 nm primary filter for excitation and a secondary filter transmitting between 400 and 3,000 nm to pass the emitted light. The reaction medium was medium A (final volume 3 ml).

NAD$^+$ Uptake. The uptake of [^{14}C]NAD$^+$ by intact purified mitochondria was initiated by adding 10 μl of the mitochondrial suspension (about 1 mg protein) to 100 μl medium A containing

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^{14}C-labeled compounds ([^{14}C]NAD^+ or [^{14}C]sucrose) or ^3H_2O in a 400-μl capacity polypropylene microtube. The uptake was stopped by rapid centrifugation (Beckman, microfuge B) of the mitochondria through a layer of silicone oil (80 μl, Versilub F50, General Electric) into 50 μl 1.6 M $HClO_4$. The silicone oil has a density of 1.065g/ml, the density of 1.6 M $HClO_4$ is 1.08. For details on the silicone layer filtering centrifugation technique and on the evaluation of the uptake into the sucrose impermeable space, which is the space surrounded by the inner mitochondrial membrane, see Klingenberg and Pfaff (12). The intramitochondrial volume was estimated with [^{14}C]sucrose.

RESULTS

Effect of NAD^+ on Malate Oxidation. To inhibit the pyruvate dehydrogenase complex, 3 mM Na-arsenite was routinely added to the incubation medium. We have verified that Na-arsenite was practically without effect on malate dehydrogenase and NAD^+-linked malic enzyme activities.

The effect of pH and NAD^+ on O_2 consumption and on accumulated products (pyruvate and oxaloacetate) in potato tuber and mung bean hypocotyl mitochondria is shown in Figures 1 and 2. In the presence of 3 mM Na-arsenite, there is a good correlation between O_2 uptake and pyruvate + oxaloacetate formed. No citrate was detected in any of the incubations. Under state 3 conditions and at pH 6.5 the fraction of total O_2 uptake which can be accounted for by accumulated pyruvate is high and the fraction which appears as oxaloacetate is low. However, as the pH increases, the fraction of O_2 uptake appearing as oxaloacetate increases very rapidly whereas the fraction of O_2 uptake appearing as pyruvate decreases rapidly. Addition of a known amount of NaOH which causes a rapid increase of the pH from 6.5 to 7.5 immediately triggers oxaloacetate formation and stops pyruvate production (Fig. 3). Conversely, addition of a known amount of HCl which causes a rapid fall of the pH from 7.5 to 6.5 immediately triggers pyruvate formation (see 18).

Addition of NAD^+ to potato mitochondria oxidizing malate considerably enhanced O_2 consumption at all of the pH values tested (Fig. 1). At pH 6.5 the stimulation by NAD^+ of pyruvate production was much higher than that of oxaloacetate whereas at pH 7.5 the stimulation by NAD^+ of oxaloacetate production was much higher than that of pyruvate. In marked contrast and at all of the pH values tested the rate of pyruvate and oxaloacetate production by mung bean hypocotyl mitochondria oxidizing malate was not affected by the presence of exogenous NAD^+ (Fig. 2).

Effect of Rotenone on Malate Oxidation. In addition to measurements of O_2 consumption and products of malate metabolism, pyridine nucleotide fluorescence of mitochondrial suspensions has been used to evaluate rapidly the pyridine nucleotide oxidation reduction state. The addition of malate (15 mM) to potato mitochondria resulted in a prompt reduction of pyridine nucleotides (Fig. 4). With time the pyridine nucleotides were slowly reoxidized until a new intramitochondrial pyridine nucleotide oxidation reduction state was attained. When this was achieved, the NADH/NAD^+ ratio was much higher at pH 6.5 than that observed at pH 7.5. Addition of rotenone to mitchondria supplemented with malate caused immediate reduction of pyridine nucleotides at all the pH values tested (Fig. 4). Identical results were obtained with

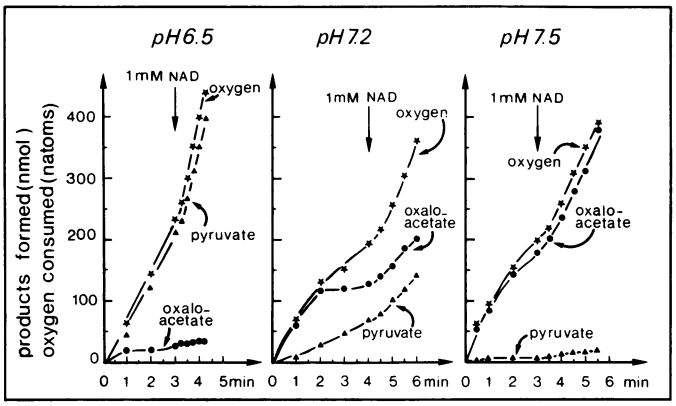

FIG. 1. Production of pyruvate (▲) and oxaloacetate (●) and O_2 consumption (★) during malate oxidation by mitochondria from potato tubers as a function of pH (measured at 25 C). The standard assay solution was used with 0.5 mg mitochondrial protein/ml, 15 mM malate, 1 mM ADP, and 3 mM Na-arsenite. As indicated 1 mM NAD^+ was added. The final volume of the reaction mixture was 10 ml.

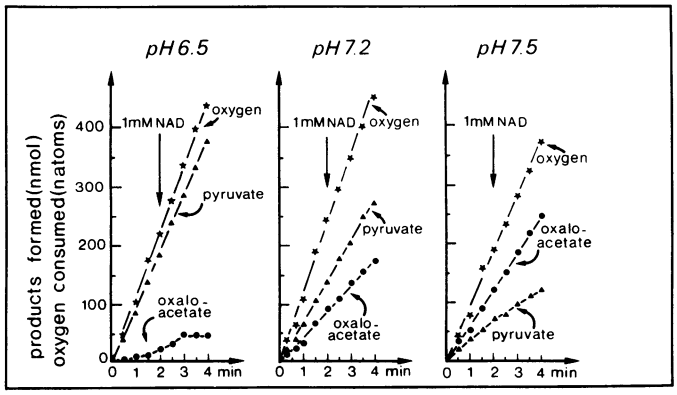

FIG. 2. Production of pyruvate (▲) and oxaloacetate (●) and O_2 consumption (★) during malate oxidation by mitochondria from mung bean hypocotyls as a function of pH (measured at 25 C). The standard assay solution was used with 0.4 mg mitochondrial protein/ml, 15 mM malate, 1 mM ADP, and 3 mM Na-arsenite. As indicated 1 mM NAD^+ was added. The final volume of the reaction mixture was 11 ml.

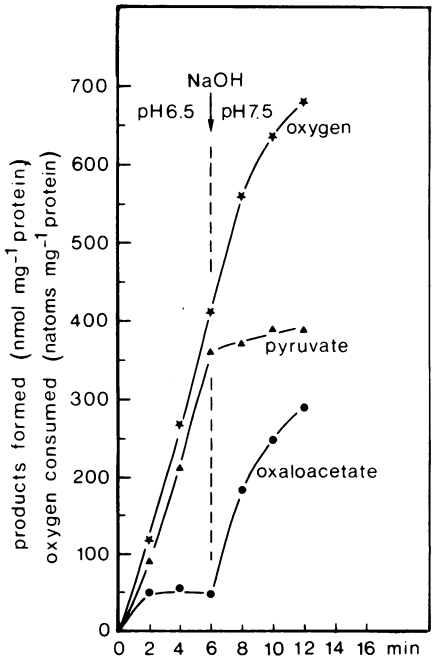

FIG. 3. Production of pyruvate (▲) and oxaloacetate (●) and O_2 consumption (★) during malate oxidation by mitochondria from potato tubers as a function of time. The standard assay solution was used with 0.5 mg mitochondrial protein/ml, 15 mM malate, 1 mM ADP, 2 mM NAD^+, and 3 mM Na-arsenite. Initial pH was 6.5; final pH was 7.5. The final volume of the reaction mixture was 7 ml.

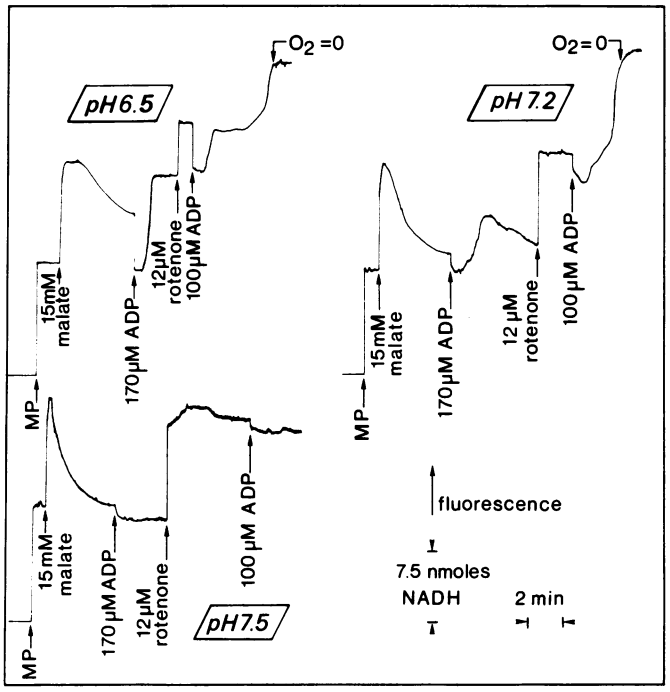

FIG. 4. Effect of rotenone on the oxidation state of endogenous NAD+ in purified potato tuber mitochondria oxidizing malate at various pH. The standard assay solution was used with 0.9 mg mitochondrial protein/ml and 3 mM Na-arsenite. The final volume of the reaction mixture was 3 ml.

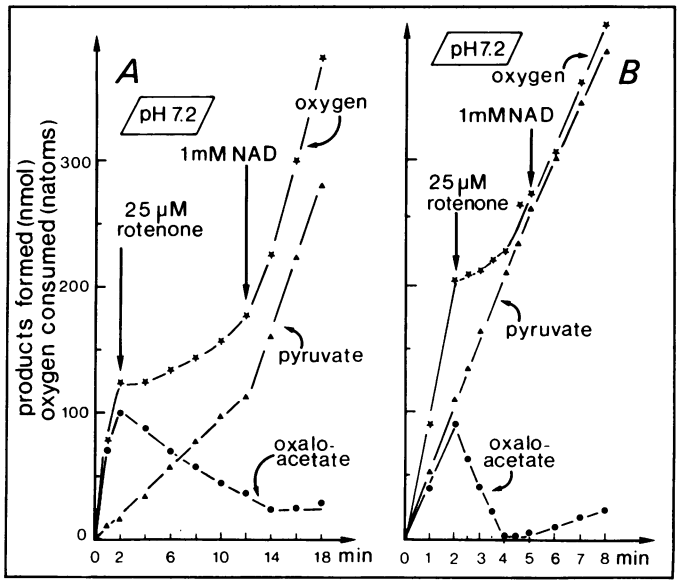

FIG. 5. Production of pyruvate (▲) and oxaloacetate (●) and O_2 consumption (★) during malate oxidation by mitochondria from potato tubers (A) and mung bean hypocotyls (B) as a function of time. The standard assay solution was used with 0.5 mg (A) and 0.4 mg (B) mitochondrial protein/ml, 15 mM malate, 1 mM ADP, and 3 mM Na-arsenite. As indicated 25 µM rotenone and 1 mM NAD+ were added. The final volume of the reaction mixture was 13 ml.

purified mung bean hypocotyl mitochondria.

In the presence of rotenone, pyruvate was excreted almost exclusively by plant mitochondria oxidizing malate at pH 7.2 (Fig. 5). This is in contrast with mitochondria oxidizing malate in the absence of rotenone (Figs. 1 and 2). Under these conditions it is clear that the NADH produced by the noninhibited malic enzyme is probably diverted from the respiratory chain to reduce the excess of oxaloacetate (see also 18). Again, addition of NAD+ to the medium enhanced the accumulation of pyruvate by potato tuber mitochondria oxidizing malate in the presence of rotenone (Fig. 5A) whereas NAD+ was practically without effect on the rate of pyruvate production during the course of the rotenone-insensitive malate oxidation by mung bean hypocotyl mitochondria (Fig. 5B).

Levels of Mitochondrial NAD+. All these observations together suggest that the rate of malate oxidation in potato tuber mitochondria contrary to mung bean hypocotyl mitochondria is limited by a lack of endogenous NAD+. It was therefore necessary to measure levels of NAD+ in potato tuber and in mung bean hypocotyl mitochondria. Table I shows that the total amount of NAD+ present in freshly prepared potato mitochondria was at least five times lower than that of isolated mung bean hypocotyl mitochondria. In fact, we have observed that whenever the total amount of mitochondrial NAD+ was low (*i.e.* below 2 mM), the rate of malate oxidation by plant mitochondria was considerably stimulated by exogenous NAD+.

Passage of NAD + through the Inner Mitochondrial Membrane. The mechanism by which exogenous NAD+ can stimulate an internally located enzyme such as malate dehydrogenase is not clear unless exogenous NAD+ can penetrate the inner mitochondrial membrane. Therefore with the aim of demonstrating further that the rate of malate oxidation is limited by a lack of endogenous NAD+, experiments were carried out to examine the passage of NAD+ through the inner mitochondrial membrane.

Figure 6A shows the measurement of the uptake of [^{14}C]NAD+ into purified intact potato mitochondria. [^{14}C]NAD+ was taken up rapidly into the sucrose impermeable ^3H_2O space (*i.e.* the matrix space). As the volume of the matrix space was measured (0.8—0.9 µl/mg of mitochondrial protein) the concentration of [^{14}C]NAD+ in the matrix space could be evaluated. At equilibrium the concentration of [^{14}C]NAD+ in the matrix space was found to be higher than in the medium. This was especially true for low external concentrations of NAD+. The uptake of NAD+ showed apparent first order kinetics (Fig. 6B) similar to other mitochondrial transport mechanisms (22) and this allowed calculation of the initial rate of entry of NAD+ into the mitochondria. By performing such experiments at various concentrations of NAD+, the concentration dependence of uptake was shown to obey Michaelis-Menten kinetics as indicated by the Lineweaver-Burk plot of Figure 7. The K_m value for NAD+ was determined as 0.30 ± 0.04 mM and V as 2.0 ± 0.5 nmol/min·mg mitochondrial protein. These results were obtained from five separate observations over a range of NAD+ concentrations from 5 µM to 2 mM.

The temperature dependence of NAD+ transport was investigated and is shown in Figure 8 in the form of an Arrhenius plot. The plot was linear above 5 C and the calculated activation energy was 75.6 kJ/mol. We have shown that the accumulation of NAD+ was insensitive to carboxyatractyloside and ADP.

Similar experiments with mung bean hypocotyl mitochondria yielded V values (0.5–0.7 nmol/min·mg protein) which were much lower than those obtained with potato tuber mitochondria. Consequently, it is very likely that the V value of the carrier might

Table I. *NAD Content of Potato Tuber and Mung Bean Hypocotyl Mitochondria*

Extraction and estimation of mitochondrial NAD+ were carried out as described. Results are for separate experiments.

Mitochondria	NAD Content	
	nmol/mg mitochondrial protein	
Potato tubers	1.4 1.1 0.8 0.6 0.8 1.7	Mean value: 1.1
Mung bean hypocotyls	5.6 5.5 6.0 5.8	Mean value: 5.7

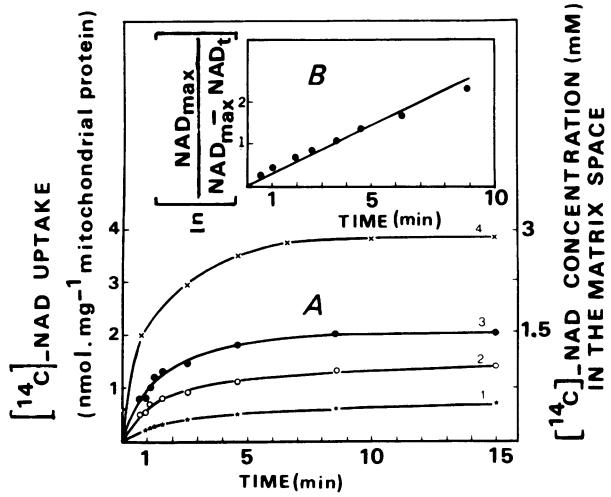

FIG. 6. Time course of NAD^+ uptake by potato mitochondria. [^{14}C]-NAD^+ uptake (A) was determined as described. The pH was 7.2; the temperature 22 C, and labeled NAD^+ was added at 0.05 (1), 0.2 (2), 0.5 (3), and 2 mM (4). Total uptake at equilibrium (NAD_{max}) was estimated, by extrapolation of data in A, to be 0.7, 1.4, 2.2, and 3.9 nmol/mg mitochondrial protein. NAD_t represents the NAD^+ uptake at time t. From B, the first-order rate constant K was calculated to be 0.3 min^{-1} (for example curve 1) and, hence, the initial rate of NAD^+ uptake was determined from the first-order rate equation $V = K(NAD_{max})$ to be 0.2 nmol/min·mg mitochondrial protein.

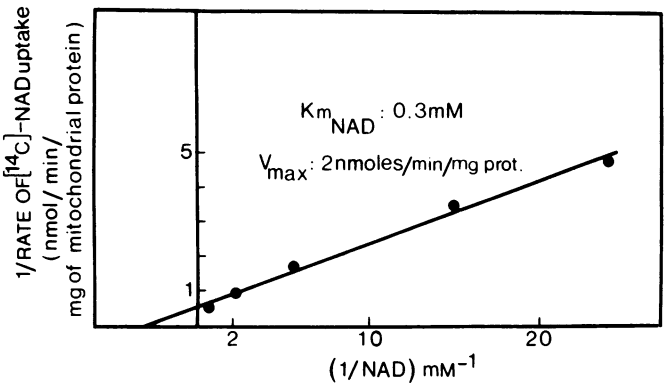

FIG. 7. Concentration dependence of [^{14}C]NAD^+ uptake by potato mitochondria. Labeled NAD^+ accumulation was measured at pH 7.2 and 22 C as described, at different concentrations of NAD^+. Initial rates of uptake were calculated as described in the legend of Figure 6 and are plotted as a Lineweaver-Burk plot.

be affected by the initial concentration of NAD^+ present in the mitochondria.

DISCUSSION

These results demonstrate for the first time that plant mitochondria possess a transport mechanism for the net accumulation of NAD^+. Recently, Abou-Khalil and Hanson (1, 2) have demonstrated a net adenosine diphosphate accumulation in plant mitochondria. This accumulation was energy linked and required the presence of phosphate. However it seems probable that we are dealing with a separate transport system because the accumulation of NAD^+ is not inhibited by ADP.

The transport of NAD^+ through the inner mitochondrial membrane readily explains the stimulation by exogenous NAD^+ of malate oxidation in intact plant mitochondria (17, 18). The stimulation observed is especially pronounced with mitochondria isolated from old potato tubers containing low amounts of endogenous NAD^+. With mitochondria containing high concentrations of endogenous NAD^+ this stimulation is hardly visible. To explain the stimulation by NAD^+ of malate oxidation in intact plant mitochondria two interpretations have been offered. Coleman and Palmer (6), Brunton and Palmer (4), and Palmer and Arron (20) have suggested that the NAD^+-linked malic enzyme, located presumably in the intermembrane space reduces externally added NAD^+ which is reoxidized by a specific NADH dehydrogenase located near the external face of the inner membrane (11). This NADH dehydrogenase is linked to the respiratory chain and is coupled to two sites of phosphorylation (19). In contrast, Day and Wiskich (7–9) indicated that malate had to penetrate the inner membrane in order to be oxidized. Consequently they proposed that a transmembrane transhydrogenase was responsible for the reduction of external NAD^+. Unfortunately there are observations already published or presented in this paper which cannot be explained by either of these hypotheses. Firstly, the response towards NAD^+ of plant mitochondria oxidizing malate is often variable and erratic. Secondly, if the NADH was reacting directly with the flavoprotein on the external face of the inner membrane of intact mitochondria one would expect a decrease in the ADP/O ratio during the course of malate oxidation in the presence of NAD^+. This is not seen unless the mitochondria are damaged (18). Thirdly, both the NAD^+-linked malic enzyme and malate dehydrogenase are located in the matrix space (7). These observations suggest that the rate of malate oxidation is limited by a lack of endogenous NAD^+. The same thing is true for the other NAD^+-linked Krebs cycle dehydrogenases (9). Thus no separate compartment for malic enzyme and malate dehydrogenase (8, 9) needs to be postulated to explain the effects of NAD^+ on malate oxidation.

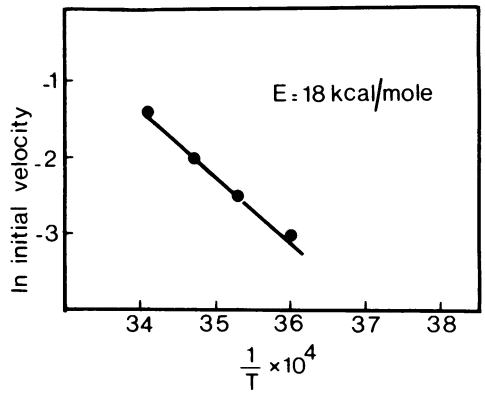

FIG. 8. Temperature dependence of [^{14}C]NAD^+ uptake by potato mitochondria. Labeled NAD^+ was added at 50 μM. The initial rate of NAD^+ uptake (V) (expressed as nmol/min·mg mitochondrial protein) was calculated from the first-order rate constant (see legend, Fig. 5).

The switch over in the products of malate oxidation with changing pH (Figs. 1–3) was formerly explained in terms of the pH activity profiles of the NAD^+-requiring malic enzyme (16). In fact, although the inner mitochondrial membrane constitutes an insulating barrier for protons, we have recently observed that the lower the pH of the external medium, the lower the pH of the matrix space and vice versa (18). In addition, we have also observed that, at alkaline pH, NAD^+-linked malic enzyme localized in the matrix space is strongly inhibited by the accumulation of bicarbonate (18).

The fact that exogenous NAD increases the rate of oxaloacetate formation during the course of malate oxidation by potato tuber mitochondria strongly supports the evidence that the regulation *in vivo* of malate dehydrogenase activity can be readily accounted for by equilibrium effect alone (3, 18). Thus, in the presence of

exogenous NAD^+ and under state 3 conditions the mitochondrial NAD^+ concentration will be raised (Fig. 4) and the equilibrium of the malate dehydrogenase reaction will move towards oxaloacetate formation, permitting excretion of significant levels of oxaloacetate (18). This evidence is strengthened by the fact that in the presence of rotenone, an inhibitor of one of the several non-haem iron centers associated with the internal NADH dehydrogenase complex (5), the mitochondrial level of NADH is raised (Fig. 4 and ref. 14) and the equilibrium of the malate dehydrogenase reaction is displaced towards malate formation and only small quantities of oxaloacetate accumulate (Fig. 5). In fact, whenever the NAD^+-linked malic enzyme activity is weakened or blocked due to a build up of bicarbonate in the matrix space (18) the NADH level will drop and the rate of oxaloacetate production will be much higher than that of pyruvate. Conversely, when the activity of the NAD^+-linked malic enzyme is high the mitochondrial NADH level will be raised and the rate of oxaloacetate production will be considerably reduced (Figs. 1, 2, 4, and 5).

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