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# PROPERTIES OF THE OXIDATION OF EXOGENOUS NADH AND NADPH BY PLANT MITOCHONDRIA

## EVIDENCE AGAINST A PHOSPHATASE OR A NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE BEING RESPONSIBLE FOR NADPH OXIDATION

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**(1) The optimum pH for the oxidation of exogenous NADH by mitochondria from both Jerusalem artichoke (*Helianthus tuberosus*) tubers and *Arum maculatum* spadices was 7.0–7.1. NADPH oxidation had a lower optimum pH of 6.6 in *Arum* and 6.0 in Jerusalem artichoke mitochondria. In both types of mitochondria the rates of NADH and NADPH oxidation were identical below pH 6.0–5.5. (2) It is shown conclusively that neither a phosphatase converting NADPH to NADH nor a nicotinamide nucleotide transhydrogenase was involved in the oxidation of NADPH by these mitochondria. (3) Palmitoyl-CoA, an inhibitor of transhydrogenase activity in mammalian mitochondria, inhibits both NADH and NADPH oxidation by plant mitochondria with a $K_i$ of about 10 μM. (4) It is concluded that the known properties of NAD(P)H oxidation are best explained by assuming the presence of a second dehydrogenase specific for NADPH. At low pH, electron flow from the two dehydrogenases to oxygen shares a common rate-limiting step.**

## Introduction

Mammalian mitochondria do not oxidize exogenous NADH unless their membranes are damaged [1]. In contrast, mitochondria from higher plants and from microorganisms such as *Neurospora crassa* and yeast readily oxidize NADH, presumably by a dehydrogenase located on the outer surface of the inner membrane [2–4]. NADH oxidation by this enzyme is insensitive to rotenone and has an ADP/O ratio similar to that of succinate, indicating that the electrons are donated to the respiratory chain at the level of ubiquinone [3,4].

Mitochondria from *N. crassa* [3,5] and higher plants also oxidize exogenous NADPH. Koeppe and Miller [6] reported that NADPH was oxidized by corn shoot mitochondria and that the ADP/O ratios, respiratory controls and sensitivity to rotenone, antimycin and KCN were similar to those of NADH oxidation. However, based on the observation that amytal only inhibited the oxidation of NADPH, it was suggested that two dehydrogenases were present [6]. Arron and Edwards [7], using a wide variety of plant mitochondria, could not repeat the results with amytal but otherwise confirmed the findings by Koeppe and Miller [6]. In a later paper, NADPH

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Abbreviations: $(DM)Br_2$, decamethylenebis(trimethylammonium) bromide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mes, 4-morpholineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

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oxidation by potato mitochondria was reported to have the same pH optimum as NADH oxidation but to be more sensitive to chelators and to SH-group reagents [8]. The oxidation of both NADH [9] and NADPH [10] by plant mitochondria is $Ca^{2+}$-dependent at neutral pH, but the sensitivity to both chelators and mersalyl differs for the two substrates and this sensitivity varies with pH [10].

Thus, the general characteristics of NADH and NADPH oxidation by plant mitochondria are similar, but certain differences have been observed. The present study is an attempt to compare in more detail the mechanism by which NADH and NADPH are oxidized by plant mitochondria.

It is found that the pH optimum for NADPH oxidation, in contrast to previous results [8], is significantly lower than that for NADH oxidation in mitochondria from both *Arum maculatum* spadices and Jerusalem artichoke tubers. Below pH 6.0–5.5 the rates of oxidation with the two substrates are identical, suggesting a common rate-limiting step. Four different systems that may cause NADPH oxidation have been considered: (1) a phosphatase converting NADPH to NADH; (2) a transhydrogenase converting NADPH and $NAD^+$ into $NADP^+$ and NADH; (3) unspecific action by the NADH dehydrogenase; (4) a separate NADPH-specific dehydrogenase. The results make it possible to exclude the first two possibilities and, in conjunction with experiments published elsewhere [10], seem to favour the presence of two separate dehydrogenases. However, the possibility of the unspecific reaction, at lower pH, of the NADH dehydrogenase with NADPH cannot be excluded.

## Materials and Methods

*Preparation of mitochondria.* Mitochondria were prepared from Jerusalem artichoke (*Helianthus tuberosus*) tubers in a low-cation medium (approx. 1 mM $K^+$) by a procedure modified from that employed by Palmer and Kirk [11] as described previously [12].

Mitochondria from spadices of *A. maculatum* were either prepared in an EDTA-containing medium [13] or in a low-cation medium as described in Ref. 12.

*Measurement of NAD(P)H oxidation.* The oxidation of NAD(P)H was measured in an oxygen electrode (Rank Brothers, Cambridge, U.K.) at 25°C in a total volume of 1.0 ml. The medium employed and the additions made are specified for each experiment in the legends to the figures and tables. In all cases, except Fig. 6, the mitochondria were uncoupled by the presence of 2 or $4 \cdot 10^{-7}$ M FCCP.

*Pyridine nucleotide analysis.* A procedure similar to that described by Rydström [14] was followed. Mitochondria were incubated at 25°C in a total of 10–15 ml in a medium which will be described for the individual experiments. Samples of 2.0 ml were taken out at the appropriate time and mixed rapidly on a swirl-mixer with 0.5 ml of 20% $HCLO_4$ to stop the reaction. Highly acidic conditions destroy the reduced pyridine nucleotides [14]. After about 5 min the sample was neutralized with 0.4 ml of 20% KOH and 0.5 ml of 1 M Tris-acetate, pH 7.5 (cold), and left on ice for at least 5 min. The $KClO_4$ was removed by centrifugation (1000×*g*, 10 min) and the supernatant analyzed for $NAD^+$ and $NADP^+$.

An aliquot of the supernatant (0.2 ml) was mixed in a cuvette with 0.8 ml of 0.1 M Tris-acetate, 0.15 M ethanol, 4 mM DL-isocitrate, 2 mM $MgCl_2$, pH 8.0, and the $A_{340}$ recorded. Alcohol dehydrogenase (EC 1.1.1.1.) from yeast was added and the reaction allowed to go to completion. From the changes in $A_{340}$ the amount of $NAD^+$ in the original sample could be calculated. $NADP^+$-linked isocitrate dehydrogenase (EC 1.1.1.42) from pig heart was then added and the subsequent change in $A_{340}$ was used to calculate the amount of $NADP^+$.

*Activity of lactate dehydrogenase.* The activity of lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle was measured with 0.2 mM NADH and 5 mM pyruvate as the decrease in $A_{340}$. The composition and pH of the medium were identical to those of the medium to which lactate dehydrogenase was subsequently to be added (see Results).

*Protein determination.* The concentration of mitochondrial protein was determined by the method of Lowry et al. [15] after solubilizing the mitochondria with 0.5% (w/v) deoxycholate. Bovine serum albumin (Sigma No. A-8022) was used as the standard.

*Chemicals.* $NAD^+$, NADH, $NADP^+$, NADPH and alcohol dehydrogenase were obtained from Boehringer, palmitoyl-CoA, $(DM)Br_2$, isocitrate dehydrogenase ($NADP^+$-linked) and lactate dehydrogenase from Sigma and antimycin A from Calbiochem AG,

Lucerne, Switzerland. FCCP was a gift from Dr. P.C. Heytler, Du Pont Chemicals and *m*-chlorobenzhydroxamic acid (synthesized according to Ref. 16) a gift from Drs. A. Bergman and I. Ericson, Department of Biochemistry, University of Umeå, Umeå, Sweden.

## Results and Discussion

### *1. pH profile of NAD(P)H oxidation by plant mitochondria*

There is only one previous report in the literature on the response of NAD(P)H oxidation by plant mitochondria to pH. Arron and Edwards [8] found what appeared to be a rather broad pH optimum of 6.4–7.2 for the oxidation of both NADH and NADPH by potato mitochondria. However, since (a) only six pH values were assayed and (b) two different buffers were used without reporting the results of appropriate controls on possible differences in the effect of the two buffers, the results cannot be seen as conclusive [8]. In Figs. 1 and 2 the results are shown of a more comprehensive study of the effect of pH on NAD(P)H oxidation by two different types of plant mitochondria.

In *Arum* mitochondria the pH optimum for NADH oxidation is 7.1 whereas NADPH oxidation shows an optimum at pH 6.6 which is clearly lower than that for NADH oxidation (Fig. 1). The ratio of the rates, at optimal pH, for the oxidation of NADH and NADPH was normally about 2 : 1. At pH 7.2, where most studies on *Arum* mitochondria have been performed in this laboratory (e.g. Ref. 17), there is relatively little NADPH oxidation (Fig. 1).

The pH optimum for NADH oxidation by Jerusalem artichoke mitochondria is also about pH 7.0 whereas NADPH oxidation has a much lower optimum at pH 6.0. It should be noted that the relative rates of oxidation with the two substrates varied between different preparations of Jerusalem artichoke mitochondria. In Fig. 2, NADPH oxidation at pH 6.0 is only 40% of that at pH 7.0 whereas other preparations of mitochondria gave values up to 65% (not shown).

In agreement with previous results [6,7], antimycin completely inhibited NADPH (and NADH) oxidation by Jerusalem artichoke mitochondria at both pH 7.2 and 5.5 (not shown). A similar inhibition of NADH and NADPH oxidation by *Arum* mito-

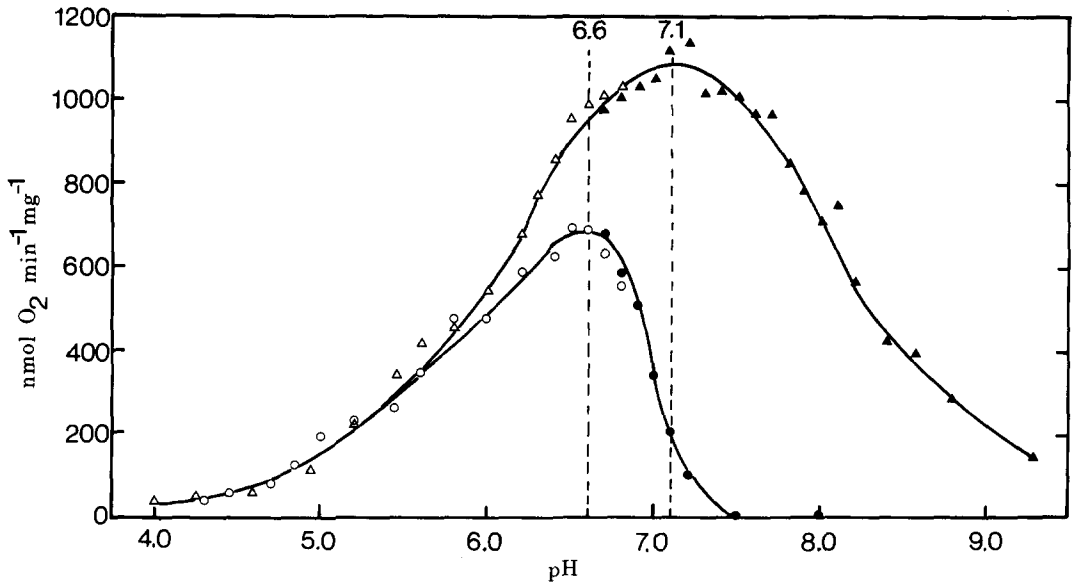


Fig. 1. NAD(P)H oxidation by *Arum* mitochondria as a function of pH. *Arum* mitochondria were prepared in a low-cation medium. The rate of $O_2$ consumption was measured in 0.3 M sucrose, 0.1 M MES (△, ○) or 0.1 M Tes (▲, ●), $2 \cdot 10^{-7}$ M FCCP, 2 mM $(DM)Br_2$ using 0.1 mg mitochondrial protein/ml. The reaction was started by the addition of 1 mM NADH (▲, △) or NADPH (○, ●).

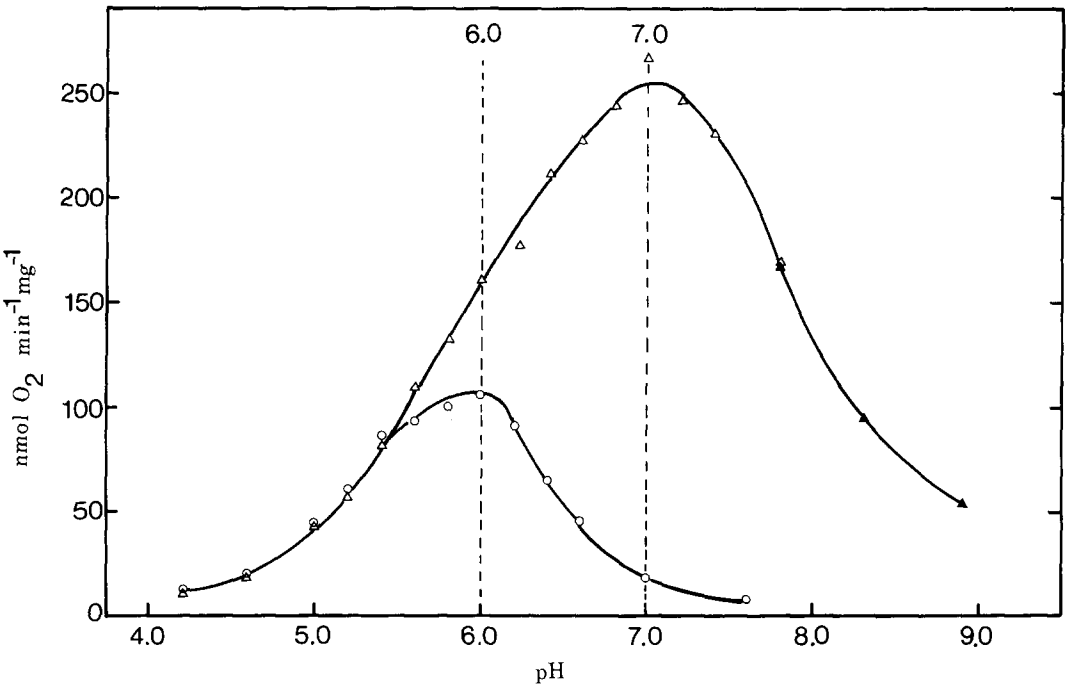


Fig. 2. NAD(P)H oxidation by Jerusalem artichoke mitochondria as a function of pH. The rate of $O_2$ consumption was measured in 0.3 M sucrose, 0.1 M Mes (△, ○), or Mops (▲), $4 \cdot 10^{-7}$ M FCCP, 2 mM (DM)$Br_2$ with 0.59 mg mitochondrial protein/ml. The reaction was started by the addition of 1 mM NADH (△, ▲) or NADPH (○).

chondria at pH 6.7 in the presence of antimycin plus *m*-chlorobenzhydroxamic acid (inhibitor of the alternative oxidase pathway; see Ref. 18) was reported by Møller and Palmer [19].

An interesting feature of the response of NAD(P)H oxidation to pH in both types of mitochondria is that below pH 6.0–5.5 the oxidation rates of NADH and NADPH were identical within experimental error (Figs. 1 and 2). Clearly, the oxidation of the two substrates shares a common rate-limiting step at acid pH. At higher pH the reactions imposing rate limitation for the oxidation of the two substrates are different. Several explanations are possible to account for these characteristics of NAD(P)H oxidation: (1) one dehydrogenase changing specificity with pH: (2) the presence of an acid phosphatase converting NADPH to NADH; (3) the presence of a pyridine nucleotide transhydrogenase – all of which assume the presence of only one dehydrogenase – and (4) two separate dehydrogenases which at acid pH share a common rate-limiting step in the electron-transport chain. Each of these possibilities has been considered.

*2. The possible presence of an acid phosphatase*

Koeppe and Miller [6] considered the possibility of a phosphatase being the cause of NADPH oxidation by corn mitochondria at pH 7.0 but found only $NADP^+$ and no $NAD^+$ produced during the assay. In Fig. 3 the results are shown from a similar experiment carried out at pH 6.7, the pH optimum of NADPH oxidation by *Arum* mitochondria (Fig. 1). Mitochondria were incubated with NADPH and samples taken out at the times indicated and analyzed for $NAD^+$ and $NADP^+$ as described in Materials and Methods. In agreement with the results of Koeppe and Miller [6], no $NAD^+$ production was detected although a small amount of $NAD^+$ was always present, probably originating from the NADPH solution. In a parallel experiment, $O_2$ consumption was measured (continuos line in Fig. 3) and the production of $NADP^+$ was clearly stoicheiometric with the consump- of $O_2$. The ratio was 2.0 $NADP^+/O_2$ consumed, showing that the final product of the oxidation was $H_2O$. Similar experiments were carried out with *Arum* mitochondria at pH 5.9 and with Jerusalem

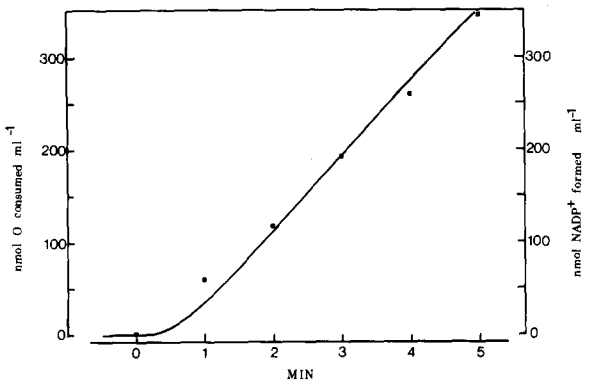


Fig. 3. Product analysis during NADPH oxidation by *Arum* mitochondria. *Arum* mitochondria were prepared in a low-cation medium and incubated at 0.06 mg protein/ml in 0.3 M sucrose, 10 mM Mops (pH 6.7), $2 \cdot 10^{-7}$ M FCCP and 2 mM $(DM)Br_2$. NADPH (1 mM) was added at 0 min and samples taken out at the times indicated and analyzed as described in Materials and Methods. The amount of $NADP^+$ formed is plotted against time (■). The concentration of $NAD^+$ was below 20 μM in all samples and no increase was observed (not shown). An oxygen electrode experiment was run in parallel under identical conditions and the $O_2$ consumption (nmol O) is given as the continuous line.

artichoke mitochondria at pH 5.7 and the results confirmed those reported in Fig. 3. It can therefore be concluded that a phosphatase is not involved in the oxidation of NADPH by plant mitochondria at either acid or neutral pH.

*3. Assays for nicotinamide nucleotide transhydrogenase activity*

The term nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) is used to denote enzymes that catalyze the reversible transfer of hydrogen between the two naturally occurring nicotinamide nucleotides NAD(H) and NADP(H), i.e., the reaction:

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH \quad (1)$$

without the mediation of any further substrates [20].

Ragland and Hackett [21] found no transhydrogenase activity at pH 7.5 in intact plant mitochondria, but digitonin caused a release of a low activity (less than 10 nmol/min per mg). A similar low activity was reported by Wilson and Bonner [22] to be present at pH 7.2 in submitochondrial particles prepared from mung bean mitochondria. This activity was reported to be partly ATP dependent in the forward direction of Eqn. 1 which is similar to that found for energy-linked membrane-bound transhydrogenases from bacteria and mammalian mitochondria [20,23]. Transhydrogenase activity has been reported to be present in a variety of green leaves [20,24], but at least in one case the activity was due to a chloroplast enzyme [20].

Thus, there is some evidence in the literature for the presence of low activities of transhydrogenase in plant mitochondria at neutral pH, which is the optimal pH for the activity of transhydrogenases [20]. However, no attempt has been made to correlate transhydrogenase activity with the capacity of the mitochondria to oxidize NADPH.

Palmitoyl-CoA is a potent inhibitor of the transhydrogenase in mammalian mitochondria with a $K_I$ of only 0.15 μM [23]. Since it is competitive with respect to NADP(H) [23] and unable to penetrate the inner mitochondrial membrane, it was thought to be a convenient inhibitor for NADPH oxidation by plant mitochondria. In Fig. 4 the effect is shown of

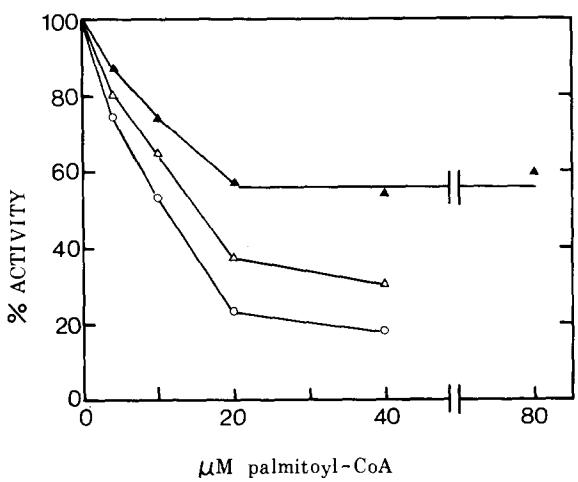


Fig. 4. Inhibition of NAD(P)H oxidation by palmitoyl-CoA in Jerusalem artichoke mitochondria. The oxidation of NAD(P)H was measured in 0.3 M sucrose, 5 mM Tes (pH 7.2) or 50 mM Mes (pH 5.7), $4 \cdot 10^{-7}$ M FCCP, 2 mM $(DM)Br_2$, 0.40 mg mitochondrial protein/ml plus palmitoyl-CoA as indicated. The reaction was started with 1 mM NAD(P)H. Uninhibited rates (100%) were 219 and 148 nmol/min per mg for NADH at pH 7.2 (▲) and 5.7 (△), respectively, and 136 nmol/min per mg for NADPH at pH 5.7 (○).

palmitoyl-CoA on the oxidation of NAD(P)H by Jerusalem artichoke mitochondria. NADH oxidation at pH 7.2 is inhibited by about 50% whereas the effect on the oxidation of both NADH and NADPH at pH 5.7 is stronger, maximal inhibition being 70–80% at 40 μM palmitoyl-CoA. In all cases the $K_I$ was about 10 μM. Palmitoyl-CoA (40 μM) also inhibited NADH and NADPH oxidation strongly (by more than 70%) in *Arum* mitochondria at pH 5.5, 6.7 and 7.2 (not shown). Thus, palmitoyl-CoA is not a specific inhibitor that can be used to distinguish between NADH and NADPH oxidation by plant mitochondria and the results do not provide any evidence for or against the presence of a transhydrogenase responsible for NADPH oxidation. However, the inhibition of NAD(P)H oxidation in plant mitochondria shown in Fig. 4 adds to the list of mitochondrial functions inhibited by palmitoyl-CoA as discussed recently by Beatrice and Pfeiffer [25].

It has been argued [6,7] that the presence of a transhydrogenase should be detectable by a stimulation of NADPH oxidation upon addition of $NAD^+$ and little or no evidence was found for this [6,7]. However, it is possible that there is sufficient $NAD^+$ in the mitochondrial matrix or bound to the enzyme to allow for maximal transhydrogenase activity. If the $NAD^+$ is converted into NADH by transhydrogenation from NADPH (see Eqn. 1), any system which can remove the NADH formed before it is reoxidized will inhibit the consumption of $O_2$ during NADPH oxidation. Lactate dehydrogenase plus pyruvate has been used to remove NADH formed by transhydrogenase activity [23] and was used in the experiments presented in Table I. Sufficient lactate dehydrogenase was added to allow a rate of NADH oxidation (measured in a separate assay at 0.2 mM NADH) comparable to that of NADPH oxidation measured. Furthermore, it was checked that this amount of lactate dehydrogenase did not cause a significant oxidation of NADPH directly due to a relative lack of specificity, particularly at low pH (see later). This NADH-removing system did not affect NADPH oxidation by either Jerusalem artichoke or *Arum* mitochondria. It should be noted that the addition of $NAD^+$ actually inhibited NADPH oxidation by *Arum* mitochondria (Table I).

The results presented in Table I would be conclusive if the NADH formed by transhydrogenation were released from the transhydrogenase into solution before being reoxidized by the NADH dehydrogenase. However, under uninhibited conditions this may not be the case, so as a final control experiment mitochondria were incubated with NADPH and $NAD^+$ (or NADH and $NADP^+$) in the presence of terminal oxidase inhibitors to prevent the reoxidation of any NADH (NADPH) formed. Samples were taken out after various time intervals and assayed for $NAD^+$ and $NADP^+$. If a transhydrogenase were active under the conditions employed one would expect to see the formation of $NADP^+$ and NADH if starting with NADPH and $NAD^+$ (reverse reaction in Eqn. 1).

The reduced forms are destroyed by the $HClO_4$ used to stop the reaction [23] and one will see an appearance of $NADP^+$ in the assay mixture and a concomitant and stoicheiometric disappearance of $NAD^+$.

The results from such an assay are shown in Fig. 5. The concentration of $NAD^+$ is constant whereas there is an increase in the concentration of $NADP^+$ (the $NADP^+$ present at 0 min derives from the NADPH

TABLE I

THE EFFECT OF AN NADH-REMOVING SYSTEM ON NADPH OXIDATION BY PLANT MITOCHONDRIA

The rate of oxidation was measured in an oxygen electrode. EDTA-prepared *Arum* mitochondria were suspended at 0.16 mg/ml in 0.3 M sucrose, 0.1 M Mops (pH 6.7), 2 mM (DM)-$Br_2$, $2 \cdot 10^{-7}$ M FCCP and 100 μM $CaCl_2$ (to remove the inhibition by EDTA [10]). NADPH (1 mM) was added to start the reaction. Other additions were 1 mM $NAD^+$, 5 mM pyruvate (pyr) and lactate dehydrogenase (LDH) at an activity of 350 nmol NADH/min. Jerusalem artichoke mitochondria (0.40 mg/ml) were suspended in 0.3 M sucrose, 50 mM Mes (pH 6.0), $4 \cdot 10^{-7}$ M FCCP and 2 mM (DM)$Br_2$. NADPH was added to start the reaction. Other additions were 5 mM pyruvate and lactate dehydrogenase at an activity of 180 nmol NADH/min. Oxidation rates expressed as nmol $O_2$/min per mg.

| | Mitochondria from | |
|---|---|---|
| | *Arum maculatum* | Jerusalem artichoke |
| NADH | – | 184 |
| NADPH | 647 (no $NAD^+$)<br>481 (+ $NAD^+$) | 153 |
| NADPH + LDH + pyr | 525 (+ $NAD^+$) | 143 |

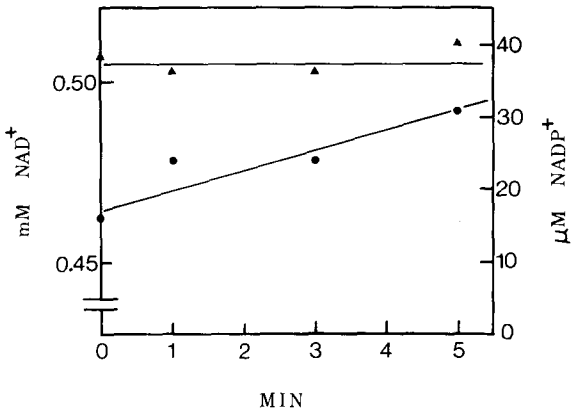


Fig. 5. Assay for transhydrogenation between NADPH and $NAD^+$ by *Arum* mitochondria. The assay was carried out at 25°C in 0.3 M sucrose, 0.1 M Mops (pH 6.7), $2 \cdot 10^{-7}$ M FCCP, 2 mM $(DM)Br_2$, 0.18 μM antimycin A, 1 mM *m*-chlorobenzhydroxamic acid, 0.5 mM NADPH and 0.5 mM $NAD^+$. The reaction was started by the addition of mitochondria (0.10 mg/ml) prepared in a low-cation medium. A sample was taken out just before and just after (10 s) the addition of miktochondria to determine the initial concentrations of $NAD(P)^+$. This was done as described in Materials and Methods. Other samples were taken out at the times indicated and the concentrations of $NAD^+$ (▲) and $NADP^+$ (●) are shown. The mitochondria oxidized NADPH at a rate of about 500 nmol $O_2$/min per mg of which about 40 nmol/min per mg was insensitive to antimycin A plus *m*-chlorobenzhydroxamic acid.

solution). The rate of appearance of $NADP^+$ is equivalent to about 30 nmol/min per mg and even if this were the result of a transhydrogenase activity it would be far too small to account for the NADPH oxidation of 500 nmol/min per mg measured on the same mitochondria in a separate experiment. The rate of $NADP^+$ appearance in Fig. 5 is, in fact, of the same order of magnitude as the rate of NADPH oxidation by *Arum* mitochondria in the presence of both antimycin and *m*-chlorobenzhydroxamic acid to inhibit the cytochrome oxidase and the alternative oxidase pathways, respectively [18]. In other words, the data in Fig. 5 indicate the presence of a certain amount of inhibitor-insensitive NADPH oxidation by *Arum* mitochondria but there is no evidence of transhydrogenase activity.

The experiment shown in Fig. 5 was carried out in the presence of FCCP. However, similar experiments were carried out on both Jerusalem artichoke (pH 5.5) and *Arum* (pH 6.7) mitochondria in the presence or absence of FCCP, with NADH + $NADP^+$ as well as NADPH + $NAD^+$. Under some of these conditions the addition of AMP or ATP was also attempted, since both have been reported to affect the activity of transhydrogenases [23,26]. In none of the experiments were concomitant and stoicheiometric changes in the concentrations of $NAD^+$ and $NADP^+$ observed.

All of the available evidence thus argues against a transhydrogenase being responsible for exogenous NADPH oxidation by plant mitochondria.

*4. A single unspecific NAD(P)H dehydrogenase*

It seemed possible that a dehydrogenase specific for NADH at pH 7 could become less specific as a result of a protonation. This protonation could be on the active site of the dehydrogenase or on the NADPH itself. NADPH contains a 2′-phosphate group which has a p*K* of 6.2–6.3 [27]. Navazio et al. [28] found that lactate dehydrogenase increased its activity with respect to NADPH compared to NADH markedly as the pH was lowered from 7.2 to 6.4, presumably due to a protonation of this phosphate group to make NADPH more like NADH. NADPH oxidation by Jerusalem artichoke mitochondria shows a similar response with an apparent p*K* of 6.4 (Fig. 2). However, the increase in NADPH oxidation by *Arum* mitochondria as the pH was lowered had an apparent p*K* of about 6.9 which cannot be ascribed to the protonation of NADPH.

Differences between NADH and NADPH oxidation in sensitivity to chelators and to SH-group reagents [8,10] are difficult to explain in terms of a single active site for both NADH and NADPH. Finally, the observed variability between preparations in the relative size of the rate of oxidation with NADH and NADPH at their optimum pH (see section 1) also argues against the concept of one unspecific dehydrogenase, since the ratios between these activities should then be constant.

*5. Two different dehydrogenases*

The model which seems to fit the available data best is one in which there are two dehydrogenases, one specific for NADH and the other for NADPH. The $K_m$ for NADPH in several different types of plant mitochondria has been reported to be 70 μM, or about twice that found for NADH [6,7]. In *Arum*

mitochondria the $K_m$ for NADPH (pH 6.7) was found to be 370 $\mu$M (not shown), which is 20-times higher than for NADH [19]. The reason for this very significant difference between *Arum* mitochondria and other mitochondria is possibly connected with the very special metabolism of *Arum* apadices [29].

Both dehydrogenases are presumably located on the outer surface of the inner membrane [2,4] and the electrons join the respiratory chain around ubiquinone to be coupled to Sites 2 and 3 of phosphorylation [4,6,7]. In Figs. 1 and 2 it was shown that a common rate-limiting step was present under uncoupled conditions below pH 6.0–5.5. The results in Fig. 6 show that also in the absence of FCCP the rates of oxidation with NADH and NADPH were identical. This indicates that the common rate-limiting step is at one of the last two coupling sites. Jerusalem artichoke mitochondria appear to require less FCCP to cause maximal uncoupling of NADH oxidation at pH 5.5 than at pH 7.2 (Fig. 6). The p$K$ of FCCP is 5.9 [30] and this means that there is more of the protonated form at the lower pH. Since FCCP is assumed to act by the movement across the inner membrane of the protonated form, this may be the reason for its greater efficiency at pH 5.5.

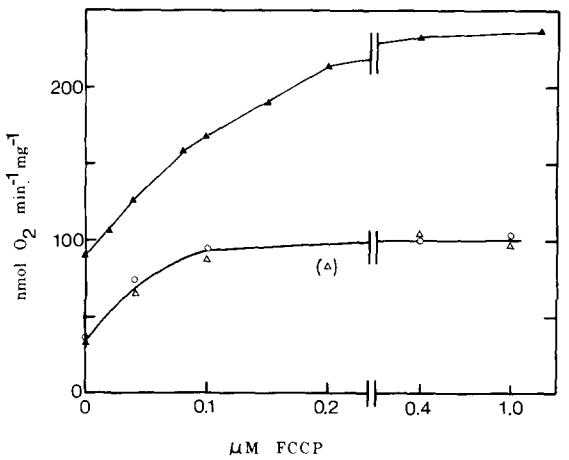


Fig. 6. Effect of FCCP on the oxidation of NAD(P)H by Jerusalem artichoke mitochondria. NADH oxidation at pH 7.2 (▲) was measured in 0.3 M sucrose, 5 mM $P_i$, 5 mM Tes, 2.5 mM $MgCl_2$ using 0.59 mg mitochondrial protein/ml. At pH 5.5 NADH (△) and NADPH (○) oxidation was measured in 0.3 M sucrose, 50 mM Mes, 2 mM $(DM)Br_2$ using 0.70 mg protein/ml. In all cases the reaction was started by the addition of 1 mM NAD(P)H and FCCP was added at 70% $O_2$.

However, above pH 6.0 the two dehydrogenases no longer share the same rate-limiting step as the flow of electrons from NADPH becomes progressively smaller compared to that from NADH (Figs. 1 and 2). It would therefore appear as if a step between NADPH and ubiquinone not shared by the NADH dehydrogenase becomes rate-limiting for NADPH oxidation at more alkaline pH. A reasonable candidate for this step would be the postulated NADPH dehydrogenase.

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## References

1 Lehninger, A.L. (1955) in The Harvey Lectures 1953–54, pp. 176–215, Academic Press, New York
2 Von Jagow, G. and Klingenberg, M. (1970) Eur. J. Biochem. 12, 583–592
3 Weiss, H., Van Jagow, G., Klingenberg, M. and Bücher, T. (1970) Eur. J. Biochem. 14, 75–82
4 Palmer, J.M. and Passam, H.C. (1971) Biochem. J. 122, 16–17p
5 Schwitzguébel, J.-P. and Palmer, J.M. (1981) FEMS Microbiol. Lett. 11, 273–277
6 Koeppe, D.E. and Miller, R.J. (1972) Plant Physiol. 49, 353–357
7 Arron, G.P. and Edwards, G.E. (1979) Can. J. Biochem. 57, 1392–1399
8 Arron, G.P. and Edwards, G.E. (1980) Plant Physiol. 65, 591–594
9 Møller, I.M., Johnston, S.P. and Palmer, J.M. (1981) Biochem. J. 194, 487–495
10 Møller, I.M. and Palmer, J.M. (1981) Physiol. Plant., in the press
11 Palmer, J.M. and Kirk, B.I. (1974) Biochem. J. 140, 79–86
12 Møller, I.M., Chow, W.-S., Palmer, J.M. and Barber, J. (1981) Biochem. J. 193, 37–46
13 Cammack, R. and Palmer, J.M. (1977) Biochem. J. 166, 347–355
14 Rydström, J. (1979) Methods Enzymol. 55, 261–275

15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Renfrow, W.B. and Hauser, C.R. (1937) J. Am. Chem. Soc. 59, 2308–2314
17 Passam, H.C. and Palmer, J.M. (1972) J. Exp. Bot. 23, 366–374
18 Schonbaum, G.R., Bonner, W.D., Storey, B.T. and Bahr, J.T. (1971) Plant Physiol. 47, 124–128
19 Møller, I.M. and Palmer, J.M. (1981) Biochem. J. 195, 583–588
20 Rydström, J., Hoek, J.B. and Ernster, L. (1976) in The Enzymes (Boyer, P.D., ed.), 3rd edn., vol. 13, pp. 51–88, Academic Press, New York
21 Ragland, T.E. and Hackett, D.P. (1964) Arch. Biochem. Biophys. 108, 479–489
22 Wilson, S.B. and Bonner, W.D. (1970) Plant Physiol. 46, 31–35
23 Rydström, J. (1977) Biochim. Biophys. Acta 463, 155–184
24 Keister, D.L., San Pietro, A. and Stolzenbach, F.E. (1960) J. Biol. Chem. 235, 2989–2996
25 Beatrice, M.C. and Pfeiffer, D.R. (1981) Biochem. J. 194, 71–77
26 Höjeberg, B. and Rydström, J. (1977) Eur. J. Biochem. 77, 235–241
27 Theorell, H. (1935) Biochem. Z. 275, 11–18
28 Navazio, F., Ernster, B.B. and Ernster, L. (1957) Biochim. Biophys. Acta 26, 416–421
29 Meeuse, B.J.D. (1975) Annu. Rev. Plant Physiol. 26, 117–126
30 Hopfer, U., Lehninger, A.L. and Thompson, T.E. (1968) Proc. Natl. Acad. Sci. U.S.A. 59, 484–490