THE STIMULATION OF EXOGENOUS NADH OXIDATION IN JERUSALEM ARTICHOKE MITOCHONDRIA BY SCREENING OF CHARGES ON THE MEMBRANES

S. P. JOHNSTON, I. M. MØLLER and J. M. PALMER
Department of Botany, Imperial College, Prince Consort Road, London SW7 2BB, England

Received 3 October 1979

1. Introduction

The ability of plant mitochondria to oxidize exogenous NADH is of great significance in the energy economy of the cell and it follows that the means of regulating such an oxidation must attract interest. The stimulation of exogenous NADH oxidation by various inorganic monovalent and divalent salts has been reported extensively [1], where divalent cations were noted to be effective at much smaller concentrations than monovalent cations, whereas the nature of the anion appeared to be unimportant. More recently, evidence presented in [2,3] was interpreted to indicate that the stimulation of exogenous NADH oxidation may be specifically dependent on Ca^{2+} (with Mn^{2+} and Sr^{2+} being much less effective). In the present study, using a range of salts which include inorganic and organic cations, the oxidation of exogenous NADH has been re-examined in conjunction with an investigation into the behaviour of cations in the diffuse layer associated with the mitochondrial membrane using the fluorescent monovalent cation 9-aminoacridine (9-AA). The results presented here are consistent with the view that electrical charges at the membrane surface influence the ease with which the substrate NADH may approach the membrane-bound dehydrogenase.

Abbreviations: 9-AA, 9-aminoacridine; $(DM)Br_2$, decamethylene-bis-trimethylammonium bromide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $(TEC)Cl_3$, tris(ethylenediamine)cobalt (III) chloride; TES, *N*-tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid

2. Materials and methods

2.1. *Materials*

NADH was obtained from C. F. Boehringer and Soehne GmbH, Mannheim. FCCP was a generous gift from Dr P. G. Heytler, Du Pont Chemicals. KCl, NaCl, $MgSO_4$, $MnCl_2$, $CaCl_2$ and $LaCl_3$ were obtained from BDH Chemicals Ltd, Poole, Dorset. $(DM)Br_2$ was from Sigma Chemical Co., $(TEC)Cl_3$ and 9-AA · HCl from Koch-Light Labs., Colnbrook, Bucks. and TES from Hopkin and Williams, Chadwell Heath, Essex.

2.2. *Isolation of mitochondria*

Mitochondria were isolated essentially as in [4]. They were then suspended in a medium containing 0.4 M sucrose, 2 mM TES (pH 7.2) (low salt medium) and removed by centrifugation at 48 000 × *g* for 2 min. The resulting pellet was finally suspended in a small volume of low salt medium to give a final preparation containing ~10 mg protein/ml.

2.3. *Oxygen consumption*

O_2 uptake by mitochondrial suspensions was measured in a Rank oxygen electrode assembly (Rank Bros., Bottisham, Cambridge) at 25°C in a low salt medium (0.3 M sucrose, 2 mM TES, pH 7.2). The concentration of oxygen was assumed to be 240 nmol O_2/ml. FCCP (2×10^{-7} M) and, where appropriate, the required amount of salt solutions were added to the oxygen electrode reaction vessel before O_2 consumption was initiated by the addition of 1 mM NADH.

2.4. *Protein determination*

The protein content of suspensions of mitochondria was measured by the Lowry method [5] using crystalline bovine serum albumin (fraction V) as the standard.

2.5. *Measurement of 9-AA fluorescence*

The fluorescence of 9-AA was measured at room temperature in a Perkin-Elmer MPF-3 fluorescence spectrophotometer as in [6]. The excitation wavelength was 398 nm (slitwidth 2 nm) and the emission was measured at 456 nm (slitwidth 5 nm). At a sensitivity setting of 1.0 this gave a reading of 72–74% full-scale deflection on the recorder with 20 μM 9-AA. The medium (1.5 ml) was the same low salt medium as used in the oxygen electrode. 9-AA (20 μM), mitochondria and aliquots of stock salt solutions were added consecutively and readings taken after each addition.

3. Results and discussion

Data presented in fig.1 demonstrate that in a low salt medium (0.3 M sucrose, 2 mM TES, pH 7.2) the rate of exogenous NADH oxidation may be enhanced by the addition of KCl, $(DM)Br_2$ and $(TEC)Cl_3$, illustrating that inorganic and organic cations of various valencies may be equally stimulatory. However, it can be seen that maximum stimulation of the rate of NADH oxidation caused by these cations occurred over different concentration ranges, i.e., 100 μM for $(TEC)^{3+}$, 1 mM for $(DM)^{2+}$ and 80 mM for K^+. These results are consistent with observations made [1,3] using monovalent and divalent inorganic cations and provide good evidence that the stimulation of NADH oxidation lacks chemical specificity towards the stimulating cation. It therefore seems likely that the ability of cations to stimulate is due to an electrostatic screening of fixed charges associated with the

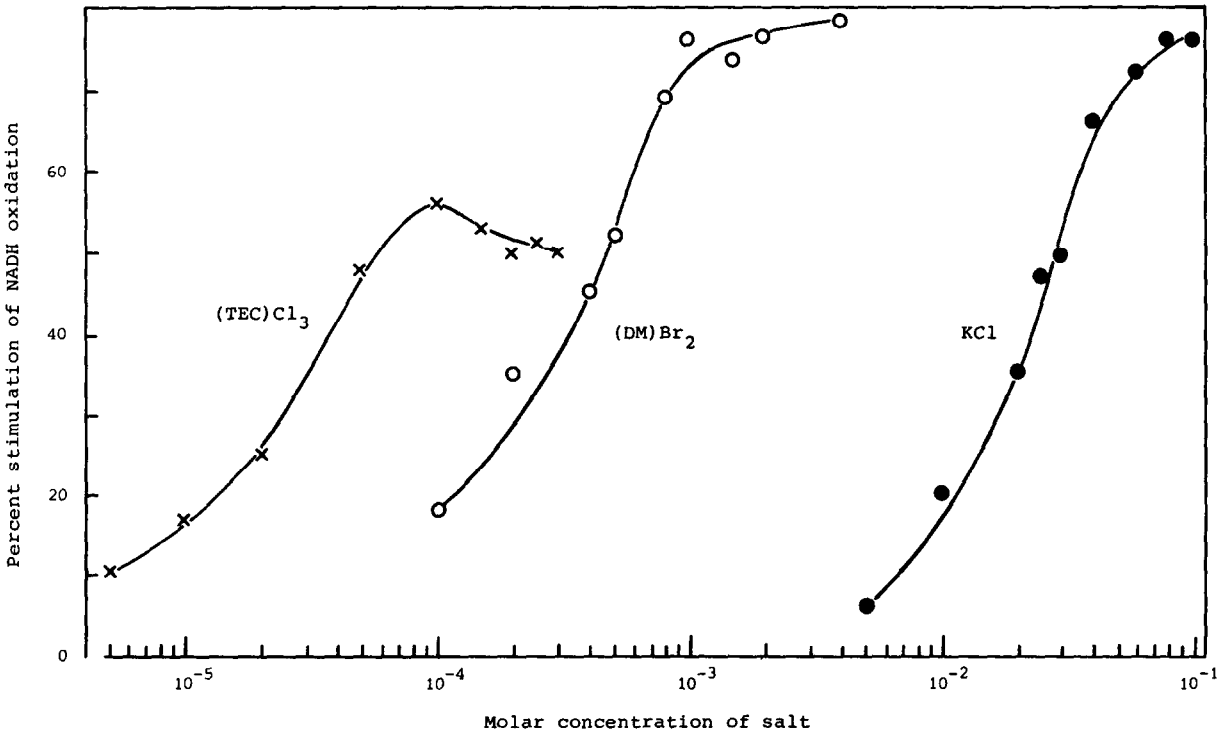

Fig.1. The stimulation, by salts, of the oxidation of exogenous NADH by Jerusalem artichoke mitochondria. The stimulation caused by $(TEC)Cl_3$ (×), $(DM)Br_2$ (○) and KCl (●) is expressed as the percent stimulation over the control containing no added salt. Each point represents a separate determination. The final protein concentration used in the assays was 0.4 mg/ml and the rate of oxygen consumption in the absence of salt was 134 nmol . min^{-1} . (mg protein)$^{-1}$.

membrane surface. This theory was investigated further using the fluorescent cation 9-AA. The addition of mitochondrial membranes to a low-salt reaction medium containing 20 μM 9-AA causes a partial quenching of fluorescence which has been assumed, as in the case with thylakoid membranes [6,7], to be due to the concentration quenching following the accumulation of 9-AA into the diffuse layer associated with the membranes. The quenched fluorescence can be released by increasing the concentration of other cations thus displacing 9-AA from the diffuse layer. Data presented in fig.2a,b compare the ability of Na^+ and Mg^{2+} to release the quenched fluorescence of 9-AA and stimulate the oxidation of exogenous NADH. The ability of the cations to release the quenching of fluorescence has been expressed as a percentage of the maximal release that can be achieved by very high concentrations of the ion in question. Both assays were carried out on the same preparation using equal concentrations of protein in each assay. It can be seen that the increase in fluorescence of 9-AA and the increase in NADH oxidation respond in a very similar manner to the increase in the concentration of both monovalent and divalent cations. This similarity in response suggests that in Jerusalem artichoke mitochondria the stimula-

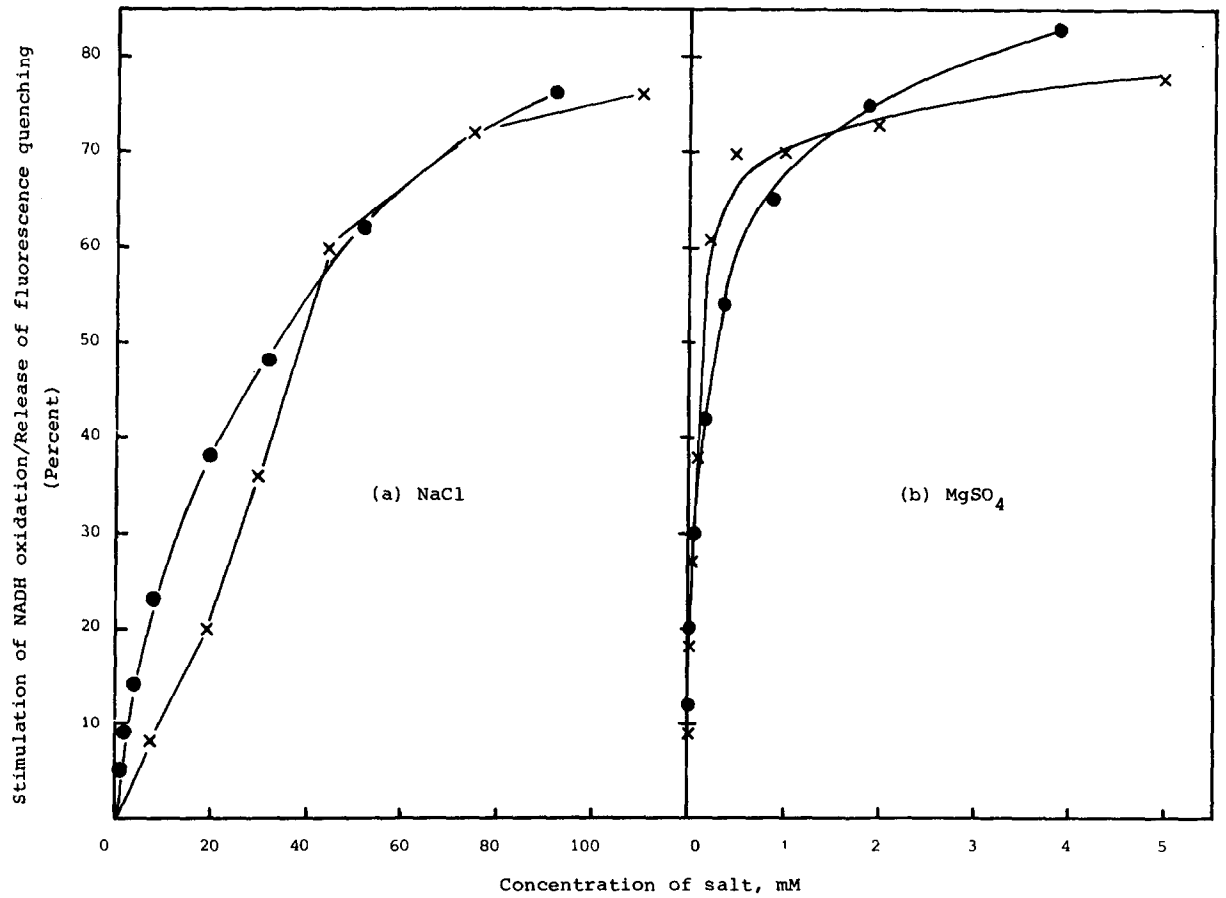

Fig.2. The effect of salts on the oxidation of exogenous NADH and the release of quenching of 9-AA fluorescence. The stimulation of oxidation of NADH (X) is expressed as the percent increase over the rate obtained without added salt. The release of quenching of 9-AA fluorescence (•) is expressed as the percentage of the maximal release obtainable with excess salt. The final protein level employed in all assays was 0.37 mg/ml and the rate of oxygen consumption in the absence of salt was 125 nmol . min^{-1} . $(mg\ protein)^{-1}$.

tion of oxidation of exogenous NADH may be due, in part at least, to the degree of screening of the fixed charges on the surface of the membrane by the cations in the diffuse layer. Since mitochondria cause the fluorescence of 9-AA to be quenched and because this can be released by a variety of cations it is suggested that, like thylakoid membranes [7,8], the membranes of the mitochondria are negatively charged. This has been confirmed by particle electrophoresis (I.M.M., H. Y. Nakatani, unpublished data). These fixed negative surface charges will attract cations into the diffuse layer adjacent to the membrane surface [9]. The cation composition of this diffuse layer will depend upon the electrolyte composition of the medium in which the mitochondrial membranes are suspended. The double layer theory as developed [10,11] predicts that the effectiveness of the cation in charge shielding is determined by its charge, with the following order of effectiveness:

$$C^{3+} > C^{2+} > C^{+}$$

and not by the chemical nature of the cation. It is clear that the data in fig.1,2 are consistent with the prediction of the classical double layer theory [12]. The data in table 1 underline the similarity between the concentrations of various cations required to stimulate both the rate of NADH oxidation and the release of 9-AA fluorescence. The values presented are the concentrations required to cause 50% of either maximum stimulation of the rate of NADH oxidation or maximum release of fluorescence. There is a close correlation between the values presented for monovalent and for trivalent cations. When considering the divalent cations, however, it can be seen that significantly lower concentrations of the inorganic divalents produce half-maximal stimulation of the rate of exogenous NADH oxidation than is required for half-maximal release of fluorescence quenching using 9-AA. Such a discrepancy could occur if the inorganic cations used were able to exert chemical selectivity and bind preferentially to those negative charges closely associated with bringing about the stimulation of NADH oxidation. It is significant to note that in the case of the organic divalent cation $(DM)^{2+}$, which may be expected to lack the required chemical specificity to bind to the required groups, there is less discrepancy between the concentrations necessary to stimulate the oxidation of NADH and release the quenching of fluorescence of 9-AA (table 1).

The data in fig.3 illustrate that certain cations may actually inhibit NADH oxidation, although release of 9-AA fluorescence caused by Mn^{2+} and La^{3+} is similar to that caused by cations which are stimulatory in NADH oxidation (I.M.M., unpublished data). In the case of Mn^{2+} very low concentrations stimulate the oxidation of NADH although inhibition soon becomes apparent as the concentration is increased. In the case of La^{3+} inhibition was observed at the lowest concentration tested. All the assays carried out in this study were conducted in the presence of FCCP and in the absence of P_i. If phosphate was present La^{3+} had little influence on the rate of NADH oxidation and a precipitate of lanthanum phosphate was obtained. It is assumed that both Mn^{2+} and La^{3+} are capable of

Table 1
Comparison between the effect of salts upon exogenous NADH oxidation and upon release of quenching of 9-AA fluorescence

Salt	Assay protein (mg/ml)	Concentration of salt at which fluorescence quenching is half-maximally released (mM)	Concentration of salt at which NADH oxidation is half-maximally stimulated (mM)
NaCl	0.37	35	32
KCl	0.42	–	21
$MgSO_4$	0.37	0.32	0.10
$CaCl_2$	0.47	0.37	0.12
$(DM)Br_2$	0.37	0.34	0.22
$(TEC)Cl_3$	0.37	0.031	0.023

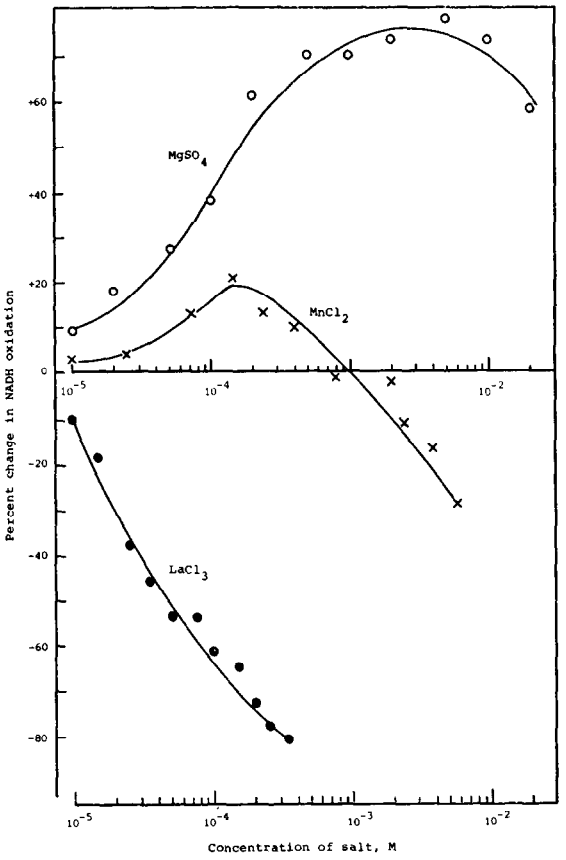

Fig.3. The effect of Mg^{2+}, Mn^{2+} and La^{3+} upon the oxidation of exogenous NADH. The change in rate of oxidation of NADH caused by $MgSO_4$ (○), $MnCl_2$ (×) and $LaCl_3$ (●) is expressed as a percent of the rate obtained in the absence of added salt. The protein concentration employed in the assays was ~0.4 mg/ml and the rate of oxygen consumption obtained in the absence of salt was 125 nmol . min^{-1} . (mg protein)$^{-1}$.

binding to sites on the mitochondrial membranes which influence adversely the oxidation of exogenous NADH.

The work in [13] has demonstrated that several enzymes located in various biological membranes (including outer and inner mitochondrial membranes) may be activated or inhibited by treatments which alter the density of the surface charge. At physiological pH, NADH is negatively charged and it is reasonable to assume that membranes with an overall negative charge will tend to repel NADH and other anionic substrates. The data here suggest that oxidation of exogenous NADH may be enhanced by treatments which alter the cation composition of the diffuse layer in such a way as to reduce the surface potential and allow this negatively charged substrate to approach the membrane surface.

Acknowledgements

We wish to thank Professor J. Barber and Dr W. S. Chow for introducing us to the concept of charge screening. I. M. Møller acknowledges receipt of a fellowship from the Danish Natural Science Research Council (no. 511-15019). The study was supported by grants from the Science Research Council, The Royal Society and the Central Research Fund, University of London.

References

[1] Hackett, D. P. (1961) Plant Physiol. 36, 445–452.
[2] Coleman, J. O. D. and Palmer, J. M. (1971) FEBS Lett. 17, 203–208.
[3] Earnshaw, M. J. (1975) FEBS Lett. 59, 109–112.
[4] Palmer, J. M. and Kirk, B. I. (1974) Biochem. J. 140, 79–86.
[5] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[6] Searle, G. F. W., Barber, J. and Mills, J. D. (1977) Biochim. Biophys. Acta 461, 413–425.
[7] Barber, J. (1977) in: Bioenergetics of Membranes (Packer, L. et al. eds) pp. 459–469, Elsevier/North-Holland, Amsterdam, New York.
[8] Davis, D. J. and Gross, E. L. (1975) Biochim. Biophys. Acta 387, 557–567.
[9] Barber, J., Mills, J. and Love, A. M. (1977) FEBS Lett. 74, 174–181.
[10] Gouy, G. (1910) Ann. Phys. (Paris) Ser. (4) 9, 457–468.
[11] Chapman, D. L. (1913) Phil. Mag. 25, 475–481.
[12] Barber, J. and Searle, G. F. W. (1978) FEBS Lett. 92, 5–8.
[13] Wojtczak, L. and Nalecz, M. J. (1979) Eur. J. Biochem. 94, 99–107.