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VANADATE-STIMULATED NADH OXIDATION IN PLASMA MEMBRANE

T. RAMASARMA, W.C. MacKELLAR and F.L. CRANE

Department of Biological Sciences, Purdue University, W. Lafayette, IN 47907 (U.S.A.) and Department of Biochemistry, Indian Institute of Science, Bangalore 560012 (India)

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The rate of NADH oxidation with oxygen as the acceptor is very low in mouse liver plasma membrane and erythrocyte membrane. When vanadate is added, this rate is stimulated 10- to 20-fold. The absorption spectrum of vanadate does not change with the disappearance of NADH. The reaction is inhibited by superoxide dismutase, and there is no activity under an argon atmosphere. This indicates that oxygen is the electron acceptor and the reaction is mediated by superoxide. The vanadate stimulation is not limited to plasma membrane. Golgi apparatus and endoplasmic reticulum show similar increase in NADH oxidase activity when vanadate is added. The endomembranes have significant vanadate-stimulated activity with both NADH and NADPH. The vanadate-stimulated NADH oxidase in plasma membrane is inhibited by compounds, which inhibit NADH dehydrogenase activity: catechols, anthracycline drugs and manganese. This activity is stimulated by high phosphate and sulfate anion concentrations.

Introduction

Vanadium compounds have recently come into prominence because of their profound effects on enzymes of plasma membranes. Orthovanadate has been found to be as potent an inhibitor as cardiac glycosides on $(Na^+ + K^+)$-ATPase from kidney, red blood cells, skeletal muscle and heart [1–6], giving 50% inhibition at 40 nM [2]. This inhibitory effect was found to be completely reversed by catecholamines [2,7]. Vanadium compounds form stable complexes with transferrin and calf intestinal alkaline phosphatase, and the activity of the latter was inhibited 50% at a concentration of 5 μM [8]. Other enzymes reported to be inhibited by vanadium are monoamine oxidase, succinate dehydrogenase and ribonuclease [9]. Metavanadate was shown to have a stimulatory effect on adenyl cyclase of rat fat cell membranes [10] and rat and guinea-pig atria and ventricles [11]. This activation was intrinsic to adenyl cyclase and not due to inhibition of ATPase, and it was not mediated by the adrenergic or histamine receptors.

Positive vanadyl and negative vanadite ions were oxidized by mitochondria or purified cytochrome oxidase, involving a copper site, but not by a pathway involving cytochrome a_3 [12]. A stimulatory effect of metavanadate on oxygen consumption by slices of rat or guinea-pig liver was described as early as 1938 [13].

In addition to $(Na^+ + K^+)$-ATPase and adenyl cyclase, plasma membranes have a powerful NADH dehydrogenase distinct in properties from NADH oxidation systems in other cellular organelles (see Ref. 14 for a review). Mouse liver plasma membrane possesses NADH dehydrogenase activity, which is 10- to 15-fold higher with ferricyanide as the acceptor than with oxygen or cytochrome *c* [15]. It is also subject to hormonal regulation by triiodothyronine, glucagon and insulin [16,17]. In our studies on the properties of this enzyme with different acceptors, we found that oxidation of NADH, measured either

by disappearance of NADH or as oxygen uptake, was stimulated several fold when vanadate was present. The properties of vanadate-stimulated NADH oxidation are described in this report.

Materials and Methods

Chemicals. The following chemicals used were from the sources indicated: NADH, disodium salt (grade III); NADPH, tetrasodium salt (grade II); cytochrome *c* (grade III); dichlorophenolindophenol, sodium salt; quinacrine hydrochloride; triiodothyronine; bathophenanthroline sulfonate, sodium salt; xanthine, sodium salt (grade III); L-adrenalin; DL-isoproterenol hydrochloride; caffeic acid; xanthine oxidase; catalase; superoxide dismutase from bovine blood were obtained from Sigma Chemical Co. Vanadium pentoxide was obtained from K&K Laboratories. Sodium orthovanadate and other reagent grade chemicals were obtained from Fisher Scientific Co.

Membrane preparations. Plasma membrane was prepared from mouse liver according to the method of Yunghans and Morré [18]. Smooth and rough endoplasmic reticulum and Golgi apparatus were prepared from mouse liver by the methods described by Morré [19]. Erythrocyte membranes were obtained from pig blood by the method of Steck [20].

Enzyme assays. The standard assay system consisted of NADH (0.1 mM), vanadate (0.5 mM), potassium phosphate buffer (0.05 M, pH 7.0) and plasma membrane (0.01–0.04 mg protein) in a total volume of 1 ml. The decrease in absorbance at 340 nm was followed in a Beckman Spectrophotometer with a Gilford attachment.

Results

Non-enzymic oxidation of NADH by vanadate

Solutions of orthovanadate or metavanadate show unspecific end-absorption in the ultraviolet region at pH 7.0. At 340 nm, an absorbance of 0.13 was obtained for a 0.13 mM solution prepared by dissolving vanadium pentoxide in alkali. Addition of this solution to NADH (0.1 mM) in phosphate buffer (0.05 M, pH 7.0) produced a marked increase in the non-enzymic oxidation of NADH measured by the change in absorbance at 340 nm. These non-enzymic rates of

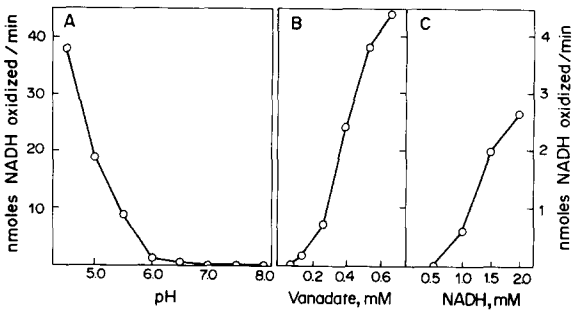

Fig. 1. Non-enzymic oxidation of NADH by vanadate. The assay system consisted of NADH (0.1 mM), vanadate (0.13 mM) and potassium phosphate buffer (0.05 M, pH 7.0) in a total volume of 1 ml. In each of the experiments A, B or C, the pH or concentrations of vanadate or NADH are changed as specified, holding the other two constant, as in the standard reaction mixture. The reaction was started by adding vanadate solution and was followed by measuring the decrease in absorbance at 340 nm. The rates of oxidation of NADH are given as nmol/min.

NADH oxidation increased with increased concentrations of NADH and vanadate and also on lowering the pH (Fig. 1). Thus, vanadate can enhance NADH oxidation non-enzymatically. The absorbance obtained with vanadate remained the same at the end of the reaction after all NADH was oxidized.

Stimulation of NADH oxidase by vanadate

Mouse liver plasma membrane showed poor rates of oxidation of NADH with oxygen as electron acceptor, measured either by decrease in absorbance at 340 nm or by oxygen uptake. These rates were stimulated 20-fold when 0.33 mM vanadate was added (Table I), approaching the rates found when ferricyanide was used as the acceptor. Rates even higher than those with ferricyanide can be obtained with increasing vanadate concentrations at pH 7.0. These rates were an order of magnitude higher at pH 5.0.

In the above experiment the absorbance change at 340 nm was measured to indicate the disappearance of NADH. As in the case of non-enzymic oxidation of NADH by vanadate, the plasma membrane catalyzed reaction did not affect the absorbance due to vanadate. Addition of plasma membrane progressively decreased the absorption spectrum and at the end of the reaction the initial spectrum of vanadate was retained (Fig. 2A). Fig. 2B shows the initial non-enzymic oxidation of NADH and the increased rate

TABLE I

RELATIVE RATES OF NADH OXIDATION BY MOUSE LIVER PLASMA MEMBRANE IN THE PRESENCE OF VARIOUS ACCEPTORS

The assay system consisted of NADH (0.1 mM), plasma membrane (0.042 mg protein), the acceptors at stated concentrations and potassium phosphate buffer (0.05 M, pH 7.4) in a final volume of 1 ml. The activity was measured by the decrease in absorbance at 340 nm and it is expressed as nmol NADH oxidized per min per mg protein.

Acceptor	Concn.	Enzyme activity
$K_3Fe(CN)_6$	0.2 mM	250
Cytochrome *c*	0.1 mg/ml	20
Dichlorophenolindophenol	1.0 mM	23
Oxygen	0.29 mM	10
Vanadate (oxygen)	0.33 mM	196

of NADH oxidation on the addition of plasma membrane. When plasma membrane, which had been boiled for 5 min, was added there was no increase in the rate above that of the blank. Similar results were obtained when lactate dehydrogenase was substituted for the plasma membrane; there was no increase in the rate above that of the blank. To determine if the presence of the membrane was required for the vanadate stimulation a 1% Triton X-100 extract was made from erythrocyte membranes. It can be seen from Table II that a soluble form of the enzyme can be made from erythrocyte membranes, which shows vanadate stimulated NADH oxidase activity.

In Fig. 2C oxygen uptake is shown under similar conditions as used for measuring NADH disappearance. This uptake of oxygen, accompanying NADH disappearance by the vanadate-stimulated NADH oxi-

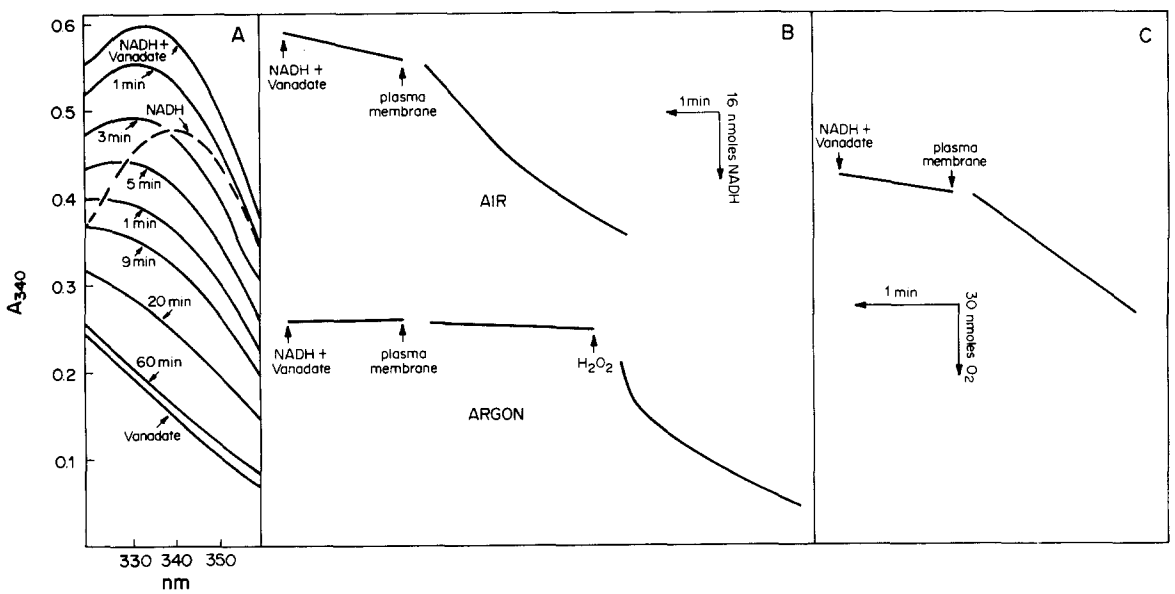

Fig. 2. Vanadate-stimulated oxidation of NADH by plasma membrane in air and under an argon atmosphere. The assay system consisted of potassium phosphate buffer (0.05 M, pH 7.0), which was flushed with argon for 3 h for the argon experiment, with the following additions in a total volume of 1 ml. (A) NADH (0.1 mM), vanadate (0.13 mM) and plasma membrane (0.02 mg protein). (B) NADH (0.1 mM), vanadate (0.05 mM) and plasma membrane (0.04 mg protein). (C) NADH (0.2 mM), vanadate (0.67 mM) and plasma membrane (0.11 mg protein). The reactions were started in all cases by adding the plasma membrane. In A, the spectra in the region of 320–360 nm were recorded periodically after adding the enzyme, in an Aminco DW-2a recording spectrophotometer. The lines shown as NADH (broken line) and vanadate correspond to the spectra of these before mixing. After mixing the absorption peak shifts to 334 nm (shown on the top). In B, the absorbance at 340 nm was measured in a Beckman spectrophotometer with a Gilford recording attachment. For the argon experiment, the cuvettes were filled with the buffer, NADH and vanadate, taking care to see that the opening was sealed by parafilm under a stream of argon. After recording the initial readings, plasma membrane (2 μl, 0.01 mg) was injected into the cuvette to start the reaction. 10 μl of H_2O_2 (10 mM) was injected where indicated. In C, the uptake of oxygen was measured in a Gilson Oxygraph fitted with a Clark electrode.

dase activity, suggests the need for molecular oxygen for the reaction. This is confirmed by showing that the NADH disappearance obtained in air could not proceed in an argon atmosphere, until a small quantity of H_2O_2 was added to provide oxygen (Fig. 2B). Under identical conditions, an argon atmosphere had no effect on the rate of oxidation of NADH by plasma membrane with ferricyanide or cytochrome *c* as the acceptor.

Active form of vanadate

Sodium orthovanadate in water gave a clear, alkaline solution. This solution showed slight activation in the standard system. On dissolving orthovanadate in potassium phosphate buffer (pH 7.0), a pale yellow color developed, and this solution showed a slight stimulation of the enzymatic activity. When the alkaline solution of ortho-vanadate was acidified by HCl, it turned yellow, and this preparation gave a 10-fold stimulation of the enzymatic activity. The best activation was obtained with a solution made by dissolving vanadium pentoxide in NaOH. This solution was yellow in color and had a neutral pH. It seems likely that the formation of the yellow color and the active species of vanadate are related. Since details of this reaction are not known, the concentrations of vanadate are given as orthovanadate.

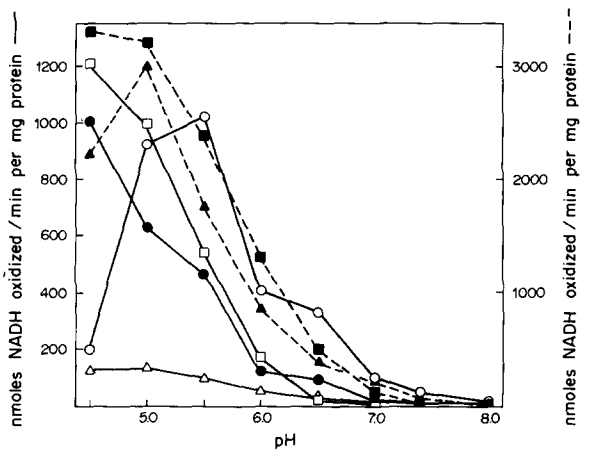

Fig. 3. Vanadate-stimulated NADH oxidation by plasma membrane and endomembranes at different pH values. The assay system consisted of NADH (0.1 mM) or NADPH (0.085 mM), vanadate for liver plasma membrane (0.033 mM) or vanadate for erythrocyte membrane and endomembranes (0.13 mM) and potassium phosphate buffer (0.05 M) at different pH values in a total volume of 1 ml. The protein concentration varied in the range of 0.01 to 0.04 mg for each membrane preparation, depending on the rates at different pH values. The calculated specific activity values are given. ■------■, Smooth endoplasmic reticulum; ●———●, rough endoplasmic reticulum; □———□, Golgi apparatus; ○———○, erythrocyte membrane; ▲———▲, liver plasma membrane (NADH); △———△, liver plasma membrane (NADPH).

Variation with pH

The data in Fig. 3 shows that the vanadate-stimulated activity of plasma membrane was highest at pH 5.0. At this pH the non-enzymatic oxidation

TABLE II

VANADATE-STIMULATED NADH OXIDATION BY ENDOMEMBRANES

The assay system consisted of NADH (0.1 mM) or NADPH (0.085 mM), vanadate (0.13 mM), the membrane preparations (0.01–0.5 mg protein) and potassium phosphate buffer (0.05 M, pH 7.0 or 5.0 as stated) in a total volume of 1 ml. For comparison, the activity with ferricyanide as the acceptor (instead of vanadate) was also measured. Enzyme activities are expressed as nmol oxidized per min per mg protein.

Membrane preparation	pH	Enzyme activity		NADH/ NADPH ratio
		NADH	NADPH	
A				
Plasma membrane	7.0	250	24	10.4
Smooth endoplasmic reticulum	7.0	466	152	3.1
Rough endoplasmic reticulum	7.0	303	42	7.2
Golgi apparatus	7.0	60	10	6.0
Erythrocyte membrane	7.0	151	4	37.8
Erythrocyte membrane Triton extract	7.0	169	–	–
B				
Plasma membrane	5.0	2640	545	4.8
Smooth endoplasmic membrane	5.0	5505	1284	4.3
Rough endoplasmic membrane	5.0	3232	307	10.5
Golgi apparatus	5.0	4760	122	66.1
Erythrocyte membrane	5.0	2006	163	12.3
C. Ferricyanide as acceptor				
Plasma membrane	7.0	248	18	13.8
Plasma membrane	5.0	151	2	75.5

also increased significantly. The activity at pH 5.0 is 12-fold higher than that at pH 7.0, while with ferricyanide as the acceptor, it decreased (see Table II). The activity with NADPH under comparable conditions was 1/10 as high as NADH, but the stimulation by low pH was clear even in this case.

Vanadate-stimulated NADH oxidation by endomembrane systems

The vanadate-stimulated activity is not limited to plasma membrane. Mouse liver smooth endoplasmic reticulum, rough endoplasmic reticulum and Golgi apparatus and pig erythrocyte membrane showed a similar increase in NADH oxidase activity, when vanadate was added. The activity measured by the decrease in absorbance at 340 nm was very low, when no acceptor was added. In the presence of vanadate the oxidation of NADH or NADPH increased several fold. The data in Table II show that endoplasmic reticulum has significant vanadate-stimulated activity with both NADH and NADPH. Erythrocyte membrane has high activity with NADH and low activity with NADPH. The ratio of activities for NADH/NADPH is high for plasma and erythrocyte membranes, in agreement with the properties described before [14]. The vanadate-stimulated activity was further enhanced at pH 5.0 in all endomembranes tested, and markedly with the Golgi apparatus.

The profiles of activity of vanadate-stimulated NADH oxidase in these membranes at different pH showed some differences between them. While plasma membrane showed a pH optimum of 5.0, erythrocyte membrane had a pH optimum of 5.5; other membranes showed no peak, but the activity increased with acidity (Fig. 4).

Effect of concentration of vanadate and NADH

The stimulation of NADH oxidase of plasma membrane is dependent on vanadate concentration (Fig. 4). The apparent K_m for half-maximal activation is 1.3 mM as calculated from the double-reciprocal plot. It is interesting to compare the effective concentrations of vanadate for this system to those observed for other enzymes. Activation was obtained with concentrations as low as 10 μM, and saturation was not reached at 1 mM in the case of adenylate cyclase; 50% maximal inhibition was obtained for $(Na^+ + K^+)$-ATPase with a concentration as low as 50 μM.

The vanadate-stimulated activity also increased with increasing NADH concentrations. A K_m value of 0.16 mM for NADH was calculated from the double-reciprocal plot (Fig. 4). This activated enzyme has a higher K_m for NADH compared to the values for the activities with ferricyanide and cytochrome *c* of 0.05 mM and 0.0012 mM, respectively.

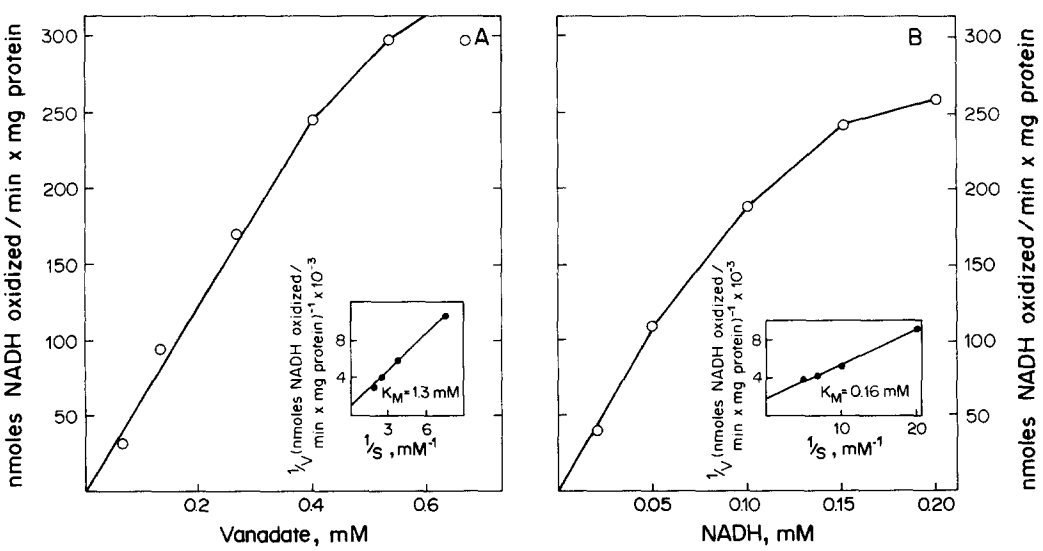

Fig. 4. The effect of various vanadate and NADH concentrations on vanadate-stimulated NADH oxidase in plasma membrane. The rate of oxidation of NADH was measured with varying concentrations of vanadate (A), or of NADH (B). The Lineweaver-Burk plots are shown in the inserts, from which K_m values were calculated.

Requirement of anions for vanadate-stimulated NADH oxidase

When the NADH oxidase of plasma membrane was tested in 0.005 M phosphate buffer (pH 7.0), both activity and stimulation by vanadate were low. Addition of 0.1 M potassium phosphate or 0.1 M sodium sulfate (pH 7.0) increased activity from 93 to over 700 nmol/min per mg protein. It appears that the stimulation is caused by something other than just the ionic strength since potassium phosphate and sodium sulfate stimulate activity, while KCl does not.

Maximum activity was obtained with concentrations of 0.05–0.15 M potassium phosphate and 0.075 M Na_2SO_4 (Fig. 5). At higher salt concentrations the activity decreased.

Effect of miscellaneous compounds

Selective inhibitions of the plasma membrane NADH dehydrogenase have been found, which are distinct from those of mitochondria and microsomes. Thus plasma membrane NADH dehydrogenase was shown to be more sensitive to quinacrine and azide than membranes from other sources, and it also responded to insulin and glucagon [16,17]. The vanadate-stimulated NADH oxidase was also found to be sensitive to quinacrine and azide (Table III),

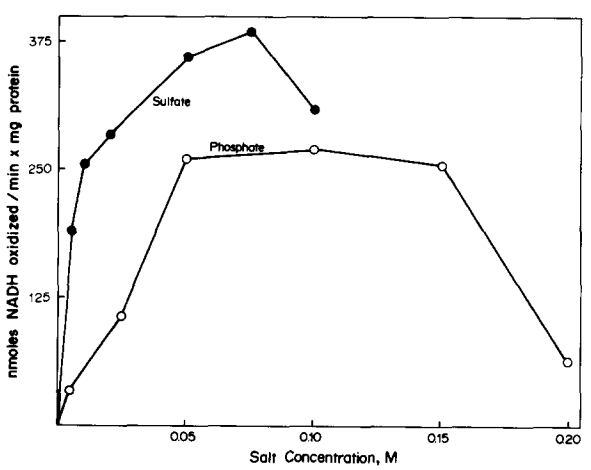

Fig. 5. The effect of various salt concentrations on vanadate-stimulated NADH oxidation in plasma membrane. The standard assay conditions were used, except 5 mM potassium phosphate buffer was used. Increasing concentrations of Na_2SO_4 or potassium phosphate (pH 7.0) were added as specified and the activity measured.

TABLE III

THE EFFECT OF VARIOUS COMPOUNDS ON VANADATE-STIMULATED NADH OXIDATION IN PLASMA MEMBRANE

The assay system consisted of NADH (0.1 mM), vanadate (0.5 mM), plasma membrane (0.4 mg protein) and potassium phosphate buffer (0.05 M, pH 7.0) in a total volume of 1 ml. The specified concentrations of various compounds were added before starting the reaction with plasma membrane. The activity is expressed as nmol NADH oxidized/min per mg protein. Change: –, inhibition; +, activation.

Additions		Activity	Change (%)
Compound	Concn.		
None	–	298	–
Quinacrine	40 μM	120	–60
	60 μM	80	–73
	80 μM	20	–93
NaN_3	25 mM	170	–43
	50 mM	110	–63
	100 mM	45	–85
Triiodothyronine	1 μM	238	–20
	2 μM	215	–28
EDTA	0.1 mM	350	+18
Bathophenanthroline sulfonate	10 μM	108	–64
Ascorbic acid	0.05 mM	110	–63
None	–	245	–
Antimycin A	2 μg/ml	243	–1
Catalase	5 μg/ml	140	–43
Adrenalin	0.2 mM	60	–75
Noradrenalin	0.1 mM	0	–100
Caffeic acid	10 μM	39	–84
Isoproterenol	10 μM	47	–81
None	–	265	–
Na_2SO_3	0.2 mM	425	+60
$CdCl_2$	0.1 mM	268	+1
$Co(NO_3)_2$	0.1 mM	220	–17
$Pb(NO_3)_2$	0.1 mM	345	+30
$MnCl_2$	0.1 mM	0	–100
NaCOOH	0.1 M	333	+25
Mannitol	0.1 M	133	–50
Mannitol	0.025 M	320	+20
Mannitol	0.01 M	298	+12

giving maximum inhibitions at 80 μM and 100 mM concentrations, respectively. Triiodothyronine had little effect on vanadate-stimulated activity in contrast to other systems, where it is known to stimulate oxygen uptake or to inhibit dichlorophenol-

indophenol reduction [14]. Antimycin A and EDTA had little effect. Bathophenanthroline sulfonate, a chelating agent, inhibited, whereas $CdCl_2$, a dithiol-binding agent, showed no effect at the concentrations tested. Catalase showed considerable inhibition. Adrenalin, noradrenalin and isoproterenol, all catecholamines, showed strong inhibition. Caffeic acid, a catechol, also inhibited, while $Co(NO_3)_2$ and $Pb(NO_3)_2$ showed small changes, but $MnCl_2$ gave a strong inhibition. Formate, a quencher of hydroxyl radical, showed a small activation at 0.1 M, whereas mannitol gave 50% inhibition. Mannitol showed little activation at lower concentrations (Table III).

Effect of manganese

It was found that manganese is a potent inhibitor even at μM concentrations, with 50% inhibition at about 0.3 μM (Fig. 6). The inhibition was measured at different concentrations of vanadate and a fixed $MnCl_2$ concentration (1 μM). The results indicated apparent stimulation by vanadate over the control rate.

Inhibition by $MnCl_2$ could also be reversed by EDTA (0.1 mM). The small amount of stimulation observed by the addition of EDTA (Table III) may, indeed, be the result of such a reversal of the action of an inhibitor present in the reaction mixture.

The inhibition by $MnCl_2$ was very specific for vanadate-stimulated NADH oxidase of plasma membrane. The oxidation of NADH by ferricyanide, cytochrome *c* or dichlorophenolindophenol by plasma membrane was not affected by $MnCl_2$ even at a concentration of 0.1 mM.

Effect of noradrenalin

Noradrenalin inhibited vanadate-stimulated NADH oxidation by plasma membranes (Fig. 7) in a concentration-dependent manner, with 50% inhibition at about 2 μM. At 10 μM noradrenalin, vanadate-dependent oxidation of NADH was nearly completely inhibited, whereas that obtained with ferricyanide and cytochrome *c* as acceptors was inhibited only partially (Table IV). Noradrenalin inhibition was obtained at different concentrations of vanadate (0.4–1.6 mM), and the results indicated apparent competition.

Noradrenalin inhibition was also obtained for the vanadate-stimulated NADH oxidation of the endomembranes tested (Table IV). It is instructive to recall that vanadate catalyzes the non-enzymic oxidation of noradrenalin [21] and, therefore, either an oxidation product of noradrenalin is the inhibitor, or in the process of this oxidation, the active species of vanadate may be lost, thus annulling the vanadate effect. This may be a general phenomenon concern-

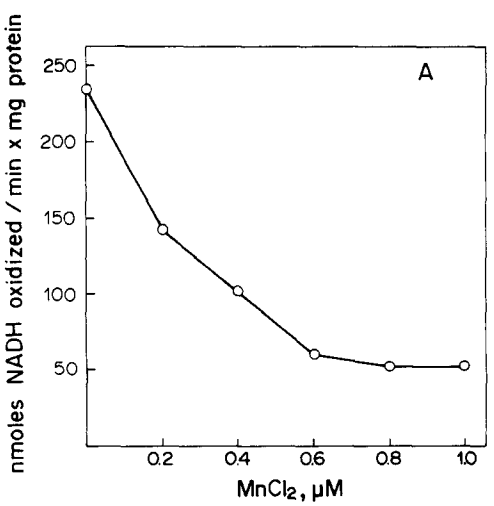

Fig. 6. The effect of various $MnCl_2$ concentrations on vanadate-stimulated NADH oxidation in plasma membrane. Specified concentrations of $MnCl_2$ were added in the standard assay system.

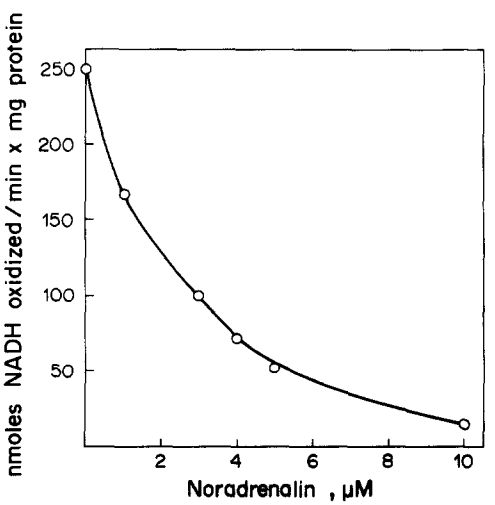

Fig. 7. The effect of various noradrenalin concentrations on vanadate-stimulated NADH oxidation in plasma membrane. The standard assay conditions were used with varying concentrations of noradrenalin.

TABLE IV

INHIBITION OF VANADATE-STIMULATED NADH OXIDATION IN VARIOUS ISOLATED MEMBRANES BY NORADRENALIN (NA)

The assay system consisted of NADH (0.1 mM), vanadate (0.5 mM), membrane preparations (0.01–0.04 mg protein) and potassium phosphate buffer (0.05 M, pH 7.0) in a total volume of 1 ml. The reaction with ferricyanide or cytochrome *c* as acceptors was also measured for comparison. The enzyme activity is expressed as nmol NADH oxidized/min per mg protein.

Test system, membrane preparation	Enzyme activity		Inhibition (%)
	–NA	+NA (10 μM)	
A			
Plasma membrane	476	29	94
Smooth endoplasmic reticulum	2413	1109	54
Rough endoplasmic reticulum	707	128	82
Golgi apparatus	138	5	96
Erythrocyte membrane	127	0	100
B			
Plasma membrane + $K_3Fe(CN)_6$ (0.1 mM)	245	181	26
+ cytochrome *c* (0.1 mg/ml)	10	8	20

ing vanadate effects, since the inhibition of $(Na^+ + K^+)$ATPase by vanadate was also reversed by noradrenalin [2,7].

Effect of adriamycin

Adriamycin, an anthracycline antibiotic now widely used against a range of neoplasms, is known to lead to cardiomyopathy [22] and damaged heart tissue with disrupted plasma membranes. Adriamycin gave significant inhibition of vanadate-stimulated NADH oxidase of plasma membranes (Fig. 8), with 50% inhibition at about 20 μM. Adriamycin also inhibited vanadate-stimulated NADH oxidation in other endomembranes (Table V). It appears that the cell membranes are more sensitive than the other endomembranes to adriamycin. In other experiments (data not given), adriamycin showed similar inhibition at pH values in the range of 4.5 to 8.0.

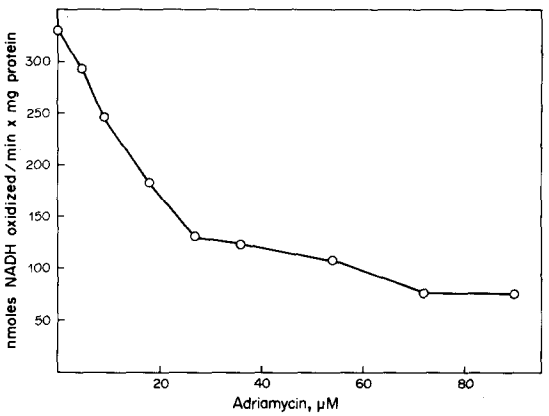

Fig. 8. The effect of various adriamycin concentrations on vanadate-stimulated NADH oxidation in plasma membrane. The standard assay conditions were used with varying concentrations of adriamycin.

Effect of superoxide dismutase

The specific inhibition of vanadate-stimulated NADH oxidase by Mn^{2+}, known to scavenge superoxide anions [23], prompted the study of the effect of superoxide dismutase. The results shown in Fig. 9 confirmed the involvement of superoxide anions in the process of vanadate-stimulated NADH oxidation by plasma membranes. The reaction was strongly inhibited by small amounts of superoxide dismutase, with 50% inhibition at about 0.1 μg/ml.

TABLE V

INHIBITION OF VANADATE-STIMULATED NADH OXIDATION BY ADRIAMYCIN (AD)

The assay system consisted of NADH (0.1 mM), vanadate (0.5 mM), membrane preparations (0.01–0.04 mg protein) and potassium phosphate buffer (0.05 M, pH 7.0) in a total volume of 1 ml. Where specified adriamycin (45 μM) was added. The enzyme activity is expressed as nmol NADH oxidized/min per mg protein.

Test system A membrane preparation	Enzyme activity		Inhibition (%)
	–AD	+AD	
A			
Plasma membrane	244	61	75
Smooth endoplasmic reticulum	1428	815	43
Rough endoplasmic reticulum	670	349	48
Erythrocyte membrane	248	72	71

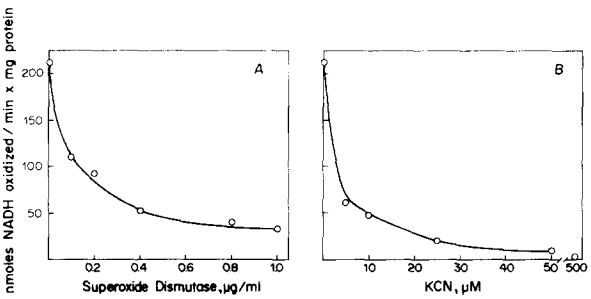

Fig. 9. The effect of superoxide dismutase and KCN concentrations on vanadate-stimulated NADH oxidation in plasma membrane. The standard assay conditions were used with varying concentrations of superoxide dismutase (A) or KCN (B).

Superoxide dismutase had no effect on NADH oxidation, when ferricyanide or cytochrome *c* was used as the acceptor, again showing the distinctive nature of vanadate-stimulated NADH oxidation. One other feature that distinguishes this activity is its sensitivity to cyanide, with 50% inhibition obtained at about 2 µM concentration (Fig. 9B).

Discussion

Vanadium is now recognized as an essential element in animal nutrition, and its metabolic implications are now being recognized (see Ref. 21 for a review). Vanadium compounds seem to have powerful regulatory effects on membranes, particularly plasma membranes. Vanadates affect cellular activities in a variety of ways: transport activity by inhibiting $(Na^+ + K^+)$-ATPase, metabolic regulation by activating adenyl cyclase, and energy transfer by activating NADH oxidase. It will be interesting to see whether a common mechanism emerges in these systems.

In the first two cases orthovanadate appears to be the active species. The present studies revealed the possibility that the active species of vanadate may be generated on acidification. Acid conditions are known to generate polymeric species of the type of decavanadate ($[HV_{10}O_{28}]^{5-}$, $[H_2V_{10}O_{29}]^{4-}$) [24]. Any of these types of vanadate species may be the active form. The present work showing that the yellow color or the absorbance at 340 nm remained unchanged during the NADH oxidation does not reveal any changes occurring in the small quantities of the active vanadate species. We consider that the active form of vanadate is an open question in all the cases, where effects are obtained with vanadium salts.

The present study confirms preliminary results that vanadate stimulates NADH oxidation [33,34]. This study showed that vanadate stimulated NADH oxidation required an oxygen atmosphere and was accompanied by oxygen uptake. The small intrinsic oxidase activity is increased 10- to 20-fold in different plasma membrane preparations by vanadate. Since Mn^{2+} and superoxide dismutase, which both scavenge superoxide anions, inhibit the reaction, it appears that superoxide anion may play some role in the mechanism of NADH oxidation. Adrenalin, noradrenalin, isoproterenol as well as caffeic acid inhibited the vanadate stimulated activity equally well. It appears that these compounds are working as a general class of catechols and not as hormones. They may either bind the vanadate and reduce it to an inactive species [30], or they may work as superoxide anion scavengers [31].

This study also showed, that vanadate stimulated NADH oxidation was an enzymatic reaction, because the activity increase observed when plasma membrane was added was not observed when boiled membrane was added. The NADH oxidation is not based on lactate dehydrogenase since it is still fully active after salt extraction of membranes to remove lactate dehydrogenase [29]. The enzymatic activity does not appear to require a membrane structure because soluble preparation from a 1% Triton X-100 extract also showed stimulation.

The vanadate-stimulated NADH oxidase of plasma membrane and the NADH-ferricyanide reductase can both be inhibited by quinacrine and azide, but there are differences in a number of other properties of activation and inhibition. The activation by vanadate and some of its properties are shared by microsomal membranes, but the distinctive nature of plasma membrane system is maintained in many respects discussed in the text.

The vanadate effect on plasma membrane NADH oxidase was inhibited by an array of compounds. The reversal of the effects on $(Na^+ + K^+)$-ATPase and NADH oxidase by noradrenaline prompts further comparisons. Further studies are needed to clarify, which of the effects are on the enzyme, and which on

the active species of vanadate. For example, inhibition by a reducing agent such as ascorbic acid may relate to reduction of vanadate. While Mn^{2+} and superoxide dismutase, which scavenge superoxide anions, inhibited the reaction, adriamycin, which increased superoxide anion-dependent cytochrome *c* reduction in the xanthine-xanthine oxidase system (Ramasarma, T. and Crane, F.L., unpublished data), also showed inhibition. The inhibition by superoxide dismutase, catalase, mannitol and inhibition of vanadate stimulation under an argon atmosphere point to the involvement of molecular oxygen, superoxide anions, H_2O_2 and hydroxyl radicals in the overall effect of vanadate, but the mechanisms involved in these reactions need further experimental clarification.

Vanadate also stimulated non-enzymatic oxidation. It is possible that the active form of vanadate may act as an intermediate electron carrier and thus account for acceleration of the reactions. In the case of $(Na^+ + K^+)$ATPase, the active species of vanadium was considered to be V^{5+}, and its reduction led to the loss of inhibition [32]. If this redox couple participates in the NADH oxidase, NADH would be able to regulate the transport processes.

The vanadate stimulation of oxidation of NADH is a general property of endomembranes, and the rates are among the highest known, especially under acidic conditions. Vanadate showed no stimulation of mitochondrial inner membrane NADH oxidation (Rau, M., unpublished data). This apparently wasteful release of energy in the form of heat would not be permitted under normal physiological conditions in view of the unfavorable pH and K_m. We would like to suggest that this system may, however, be used under the thermogenic conditions such as cold exposure [25,26] and malignant hyperthermia [27]. It should be emphasized that under conditions leading to the development of localized acidity in membranes, the combined action of these endomembrane systems can lead to high rates of NADH oxidation by the vanadate-stimulated pathway, resulting in the dissipation of cellular energy in the form of heat.

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