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Specific Inhibition of Phosphate Transport in Mitochondria by N-Ethylmaleimide

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Abstract

N-ethylmaleimide (NEM), a reagent that alkylates free sulfhydryl groups, was shown to be a highly effective inhibitor of the following coupled mitochondrial processes: oxidative phosphorylation, ATP-^{32}Pi exchange, Pi-induced light scattering and configurational changes, State III respiration, valinomycin-induced translocation of potassium with Pi as the anion, and calcium accumulation in presence of Pi. However, NEM was less effective or ineffective in inhibiting some processes that do not require inorganic Pi, namely electron transfer and ATPase activity, ADP binding, energized light scattering changes induced by arsenate and nonenergized light scattering changes induced by acetate. The rate of oxidative phosphorylation and of ATP-^{32}Pi exchange was normal in ETP_H particles prepared from NEM-treated mitochondria. Also NEM, even at levels 2-3 times greater than those required to inhibit oxidative phosphorylation in intact mitochondria, did not inhibit coupled processes in submitochondrial particles. We are proposing that NEM alkylates sulfhydryl groups in the mitochondrion that modulate Pi translocation, and that the suppression of Pi translocation blocks oxidative phosphorylation, the Pi-dependent energized configurational change in mitochondria and Pi-dependent transport processes.

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Introduction

Mitochondria have been shown to accumulate inorganic phosphate (Pi)* when carrying out electron transfer [1-8]. This energized accumulation of phosphate has been shown to be blocked by the addition of a number of sulfhydryl reagents, e.g. mersalyl [3-6], *p*-hydroxymercuribenzoate [2], 5,5′dithiobis (2-nitrobenzoic acid) [7], and fluorescein mercuric acetate [8]. These observations have been the basis for the postulate that mitochondria contain a carrier system that facilitates the transmembrane transport of Pi, and that this carrier system contains a sulfhydryl group or groups that modulate Pi transport.

Since N-ethylmaleimide (NEM) is an effective reagent for binding sulfhydryl groups covalently,† it appeared to us that NEM would be an ideal reagent for evaluating the postulate of a Pi carrier system. In the present communication we shall be presenting evidence: (a) that NEM is bound to a significant degree of both mitochondria and ETP_H; (b) that NEM inhibits all Pi-dependent processes whether energized or non-energized; (c) that in comparable concentrations NEM has little or no effect on several processes which are not Pi-dependent; and finally (d) that NEM has no inhibitory effect on Pi-requiring processes in a submitochondrial particle. On the basis of this evidence, we have invoked a transmembrane phosphate carrier system (Pi translocase) in the inner membrane, the activity of which is modulated by sulfhydryl groups.

Methods

Preparation of beef heart mitochondria and submitochondrial particles. Heavy beef heart mitochondria were prepared by the method of Crane *et al.* [9], and Hatefi and Lester [10], except that in the medium used for

* *Abbreviations*:

EDTA	ethylene diamine tetraacetate
EGTA	ethylene-glycol-bis-(amino ethyl)-tetraacetate
ETP_H	phosphorylating electron transfer particles prepared from HBHM
HBHM	heavy beef heart mitochondria
m-ClCCP	carbonyl cyanide-*m*-chlorophenyl hydrazone
NEM	N-ethylmaleimide
Pi	inorganic phosphate
SH	sulfhydryl
TMPD	tetramethylphenylenediamine
DNP	2,4-dinitrophenol

† Although NEM reacts with histidine and the ϵ amino group of lysine, the rate of these interactions is an order of magnitude slower than the rate of interaction with sulfhydryl groups [see C. F. Brewer and J. P. Rhiem, *Anal. Biochem.*, 18, 248 (1967)].

isolation and washing, EGTA (1 mM) and Tris-Cl (10 mM) were used in place of Pi.

Phosphorylating submitochondrial particles (ETP_H) were prepared from beef heart mitochondria (untreated or exposed to 20 nmoles NEM per mg protein) by the method of Hansen and Smith [11]. Protein concentrations were determined by the biuret method applied to cholate-solubilized mitochondrial suspensions [12].

Assay of oxidative phosphorylation. Oxidative phosphorylation was assayed at 30° by the conversion of inorganic to esterified phosphate (glucose-6-phosphate). The assay system contained 1000 μmoles of sucrose (calcium-free), 40 μmoles of ^{32}Pi labelled potassium phosphate, pH 7.4 (specific activity of 500-1000 cpm per nmole of Pi), 20 μmoles of potassium succinate, 4.0 μmoles of ADP, 12 μmoles of magnesium chloride, 20 μmoles of glucose, 12 units of hexokinase* and 4.0 mg protein of either mitochondria or ETP_H in a total volume of 4.0 ml. The standard medium contained 2 μg of rotenone per ml of medium. The reaction was started by the addition of mitochondria or ETP_H to a cell in which oxygen consumption was continuously measured with a Clark-type electrode. When the oxygen in the cell was consumed, 1.0 ml of the mixture was removed and added to 1 ml of a 1.2 N solution of perchloric acid. Organic phosphate was separated from inorganic phosphate by the method of Lindberg and Ernster [13]. The aqueous phase was washed three times with water saturated isobutanol and assayed for radioactivity in a Tri-Carb liquid scintillation spectrometer in Brays scintillation fluid [14].

The ADP-induced state III respiration was assayed in a medium identical with the one described above except that hexokinase and glucose were omitted from the medium, and the ADP concentration was reduced to 0.2-0.4 mM.

Assay of ATP-^{32}Pi exchange activity and of ATPase activity. ATPase activity was assayed at 30° for 5 min in a system (1 ml volume) containing 250 μmoles of sucrose (calcium-free), 10 μmoles of Tris-Cl, pH 7.4, 3 μmoles of magnesium chloride, 10 μmoles of ATP, and 1 mg protein either of mitochondria or ETP_H. The reaction was terminated by the addition of 0.1 ml of a 6 N solution of perchloric acid. After centrifugation of the suspension, the Pi in the supernatant fluid was determined by the method of Takahashi [15].

The ATP-^{32}Pi exchange activity was assayed at 30° for 5 min in a system (1 ml volume) containing 250 μmoles of sucrose (calcium-free), 10 μmoles of Tris-Cl, pH 7.4, 3 μmoles of magnesium chloride, 10 μmoles of ATP and 10 μmoles of ^{32}Pi labelled potassium phosphate, pH 7.4 (specific activity of 500-1200 cpm per nmole Pi). The reaction

* One unit will catalyze the phosphorylation by ATP of one μmole of glucose per minute at 25° and at pH 8.5.

was terminated by addition of 0.1 ml of a 6 N solution of perchloric acid. The organic phosphate was measured by the method of Lindberg and Ernster [14].

Measurement of respiration. Oxygen consumption was measured with a Clark-type electrode at 30°. The mitochondria were treated with 20 nmoles of NEM per mg protein at 0° for 20 min before the respiration was measured. The concentration of the substrates (pyruvate + malate, succinate, and ascorbate + TMPD) as well as the composition of the media used are specified in the appropriate tables.

90° Light scattering. A Brice-Phoenix light scattering photometer and recorder was used to follow the changes in light scattering at 30°. The assay system (3 ml volume) contained 750 μmoles of sucrose (calcium-free), 30 μmoles of Tris-Cl, pH 7.4, 15 μmoles of magnesium chloride, 6 μg of rotenone, and 3 mg of mitochondrial protein. The light scattering response was followed after the addition of 15 μmoles of potassium succinate and 30 μmoles of either potassium phosphate or sodium arsenate, both at pH 7.4.

Potassium translocation. Valinomycin-induced potassium translocation was assayed as described by Blondin *et al.* [16].

Determination of the energized accumulation of Pi and the binding of ADP. The basic assay system contained 500 μmoles of sucrose (calcium-free), 20 μmoles of Tris-Cl, pH 7.4, 10 μmoles of potassium succinate, 6 μmoles of magnesium chloride, 4 μg of rotenone and 2 mg of mitochondrial protein in a volume of 2 ml. When the energized accumulation of Pi was determined, 20 μmoles of ^{32}Pi labelled potassium phosphate, pH 7.4 (specific activity 500-1200 cpm per nmole Pi) were added; when binding and/or exchange of ADP was being determined, 4.2 μmoles of ^{14}C-labelled ADP (specific activity 300 cpm per nmole) were added in place of the Pi. The reaction was initiated by addition of mitochondria (2 mg protein) and the suspension was then incubated for a given period (usually 60 sec) in a tube inserted in a Misco high speed centrifuge (22,000 rpm). After incubation, the centrifuge was turned on and the mitochondria were sedimented. The surface of the mitochondrial pellet was rinsed three times with a chilled solution which was 0.25 M in sucrose and 10 mM in Tris-Cl, pH 7.4. The pellet was exposed to 2 ml of a 1% solution of sodium dodecyl sulfate for 18 h. The control samples contained all the reactants except succinate. The radioactivity of aliquots of the dissolved pellet was counted in a Tri-Carb scintillation spectrometer.

Assay of energized translocation of calcium and Pi. The reaction was initiated by addition of mitochondria (0.5 mg protein) to 2.5 ml of a reaction medium which contained 625 μmoles of sucrose, 25 μmoles of Tris-Cl, pH 7.4, 5 μg of rotenone, 7.5 μmoles of magnesium chloride, 25 μmoles of potassium phosphate, pH 7.4, 500 nmoles of ^{45}Ca-labelled calcium chloride, 12.5 μmoles of ascorbate and 125 nmoles of TNPD.

The reaction was carried out at 30° for 60 sec and was terminated by the rapid filtration of the reaction mixture through a millipore filter (with a pore size of 0.8 μ). Filtration was complete within 5 sec. The filter was washed once with 5 ml of the reaction mixture from which magnesium chloride, phosphate, ^{45}Ca-labelled calcium chloride, and substrates were deleted. The washing procedure was completed within 10 sec. The millipore filters were dissolved in Brays scintillation solution with Cab-O-Sil, and the radioactivity of the solution was determined in a Tri-Carb scintillation spectrometer. The interaction of the mitochondria with NEM (20 nmoles per mg protein) was carried out for 20 min at 0°. Blank determinations without mitochondria were carried out under conditions identical with those described above.

Determination of free sulfhydryl groups. Free sulfhydryl groups were determined by the method of Sedlak and Lindsay [17] and Butterworth *et al.* [18] as modified below. A 0.5 ml aliquot (containing 0.5 mg protein) of the mitochondrial or ETP_H suspension was incubated for 3 min in the standard solution which was 0.25 M in sucrose, 10 mM in Tris-Cl, pH 7.4, 3 mM in magnesium chloride and contained 2 μg of rotenone. To this suspension 0.5 ml of a solution which was 0.2 M in Tris-Cl, pH 8.3, 0.2 mM in DTNB and 1.0 mM in EDTA, was added, and the mixture was incubated for 15 min. Then 4 ml of absolute methanol was added to precipitate the protein. The absorption of TNB in the clarified supernatant was determined at 412 nm. The amount of TNB in the supernatant was equated with the amount of DTNB that reacted with the particle. Several processes may contribute to TNB formation: (a) interaction of DTNB with free sulfhydryl groups; (b) interaction of DTNB with acid labile sulfur; and (c) interaction of DTNB with bound free sulfur groups. In order to determine the bound free sulfur groups by this method, the pellet of precipitated protein was suspended in 4 ml of 90% methanol solution which was 0.1 M in Tris-Cl, pH 8.3 and 0.5 mM in EDTA, and then centrifuged to wash out any unreacted DTNB or trapped TNB. The pellet was then suspended at room temperature in 2.5 ml of 90% methanol containing 5 μmoles of DTT, 250 μmoles of Tris-Cl, pH 8.3 and 1.25 μmoles EDTA; and after 15 min the suspension was centrifuged. The TNB content in the supernatant was estimated by measuring the absorption at 412 nm. The TNB formed by interaction of the DTNB with acid labile free sulfur was taken to be the difference between total TNB released into the first supernatant and TNB released from the mitochondrial pellet. A molar extinction coefficient of 11,800 $M^{-1}\,cm^{-1}$ for TNB was determined with known amounts of reduced gluthathione as the source of sulfhydryl group in the standard medium.

Free sulfhydryl groups were also determined by the following procedure with ^{14}C-NEM. The samples, mitochondria or ETP_H, were incubated with graded amounts of ^{14}C-NEM in the standard medium at

0° for 20 min. The particles were centrifuged in a Misco centrifuge for 30 sec and were washed again with the standard medium to remove unreacted ^{14}C-NEM. ETP_H was centrifuged in a Spinco L-2 at 198,000 × *g*, for 30 min and was washed again with the standard medium to remove unreacted ^{14}C-NEM. The surface of the pellets was washed three times with a chilled solution which was 0.25 M in sucrose, and 10 mM in Tris-Cl, pH 7.4. The samples were mixed with absolute methanol to a final concentration of 80%, to remove products formed by the interaction of NEM with non-protein bound free sulfur groups [17, 18]. The mixture was kept at 30° for 30 min, and centrifuged for 15 min; the pellet was washed with absolute methanol. The first supernatant contained solubilizable sulfur or sulfhydryl groups which had reacted with ^{14}C-NEM. The final pellet was completely dissolved with 1% sodium dodecyl sulfate and the radioactivity of the solution was counted in Brays scintillation fluid. This gave an estimate of tightly bound sulfhydryl groups.

Electron microscopy. Mitochondrial suspensions were fixed by mixing with an equal volume of a solution which was 4% in glutaraldehyde, 0.05 M in potassium cacodylate, pH 7.4, and 0.2 M in sucrose. The samples were further treated with osmium tetroxide and uranyl acetate, dehydrated, and then embedded as described previously [19].

Sources of chemicals. The sources of the following chemicals are indicated in parentheses: ATP, N-ethylmaleimide, ADP, catalase and hexokinase (Sigma Chemical Co., St. Louis, Missouri); rotenone (Aldrich Chemical Co., Milwaukee, Wisconsin); ^{32}Pi, ^{14}C-ADP (International Chemical and Nuclear Co., City of Industry, California).

Results

Binding of NEM to Sulfhydryl Groups of Mitochondria and ETP_H

The determination of the sulfhydryl groups which can react with NEM as a fraction of the total sulfhydryl content of the mitochondrion requires a method which measures all titratable sulfhydryl groups and distinguishes between sulfhydryl groups and the sulfur of nonheme iron. There have been several published methods for determining SH groups in mitochondria. Riley and Lehninger [20] used the amperometric method of Benesch *et al.* [21], and reported about 100 nmoles of $AgNO_3$ reactive sites per mg protein. Brierley *et al.* [22] used radioactive *p*-CMB also and found about 100 nmoles of *p*-CMB-reacting sites per mg protein. Klouwen [23] used diphenylpicrylphenylhydrazine as the probing reagent and found about 40 nmoles of reactive sulfhydryl groups per mg protein. Using DTNB, we found that about 100 nmoles of TNB were released per mg protein (Table I). An explanation for this variability in

TABLE I. Titration of SH groups in Mitochondria and ETP_H with DTNB

Titer of SH groups in	Mitochondria (nmoles TNB per mg protein)	ETP_H (nmoles TNB per mg protein)
Protein bound SH groups	36 ± 1	27 ± 2
Acid labile sulfur or solubilizable SH	62 ± 2	27 ± 2

SH titer came with the recognition that DTNB reacts with acid labile sulfur as well as with sulfhydryl groups. Malkin and Rabinowitz [25] demonstrated that DTNB would react readily with the acid labile sulfur atoms of ferrodoxin. The stoichiometry for this former reaction was found to be 2 moles TNB released per mole of acid labile sulfur. However, if there there any easily solubilized sulfhydryl groups, these would be included in the acid labile sulfur fraction by this assay.

Titration of intact heart mitochondria with ^{14}C-NEM results in a progressive increase in the amount of NEM bound to protein (Table II).

TABLE II. Binding of ^{14}C-NEM to SH groups of mitochondria and ETP_H

nmoles NEM added per mg protein	Mitochondria		ETP_H	
	^{14}C-NEM bound (nmoles per mg protein)		^{14}C-NEM bound (nmoles per mg protein)	
	Protein bound SH groups	Acid labile S or solubilizable SH	Protein bound SH groups	Acid labile S or solubilizable SH
5	3.5	1.9	2.5	0.1
10	6.5	2.0	3.4	0.1
20	9.8	2.8	3.8	0.1
40	13.5	2.7	7.0	0.2
100	16.4	2.8	9.0	0.6

At levels of 10-20 nmoles of NEM added per mg protein (a concentration which inhibits phosphate-requiring reactions by 80-90%) 6.5-9.8 nmoles of NEM were bound per mg of heart mitochondria. It would appear from the data to follow that when NEM interacts to the point that 6-10 nmoles of bound SH per mg protein are alkylated, inorganic phosphate-requiring reactions are inhibited. When ETP_H was exposed to NEM at concentrations equivalent to those used in the mitochondrial titrations, the amount of NEM bound per mg protein was about 50% of that bound by mitochondria.

^{14}C-NEM was used to determine the distribution of bound sulfhydryl groups in beef heart mitochondria which interact with NEM. In the

original mitochondrion, 7.3 nmoles of bound sulfhydryl per mg protein could be titrated with excess NEM (after incubation of mitochondria with 20 nmoles of NEM per mg protein for 20 min at 0°). This value for the titration has already been corrected for the nonheme iron sulfur. When the mitochondria thus reacted with ^{14}C-NEM were fractionated after sonication, the fraction corresponding to the innner membrane was found to contain 4.2 nmoles of bound sulfhydryl per mg protein as measured by ^{14}C radioactivity. It was thus established that the inner membrane contains a substantial number of sulfhydryl groups capable of interacting with NEM.

Modulation of Phosphate-requiring Mitochondrial Functions by NEM

Table III provides a summary of the key mitochondrial processes that are inhibited by NEM. At a concentration of 20 nmoles per mg protein, NEM essentially abolishes oxidative phosphorylation as measured by P/O ratio or ATP-^{32}Pi exchange, as well as active transport of potassium with Pi as the anion. Although this table does not summarize all the processes which we will be considering in this communication, it points up the invariant correlation between the inhibition by NEM and the participation of Pi in the coupled process which is inhibited.

TABLE III. Inhibition of Pi-requiring mitochondrial functions by NEM

NEM[a]	0	10	20	40
P/O ratio	1.7	1.0	0.15	0
ATP-^{32}Pi exchange[b]	61.7	34.6	6.2	1.2
K^+ translocation (Pi)[c]	2.0	0.10	0.10	0.10

[a] nmoles NEM per mg protein.
[b] nmoles Pi exchanged per min per mg protein.
[c] μmoles K^+ translocated per mg protein with Pi as anion.

Effect of NEM on respiratory control. When mitochondria carry out electron transfer in presence of Pi, the rate of oxygen uptake is increased several fold on addition of ADP [24]. The ratio of the rate of respiration in presence and in absence of added ADP is a measure of respiratory control. Figure 1 shows that the ADP-induced increase in respiration is progressively reduced as the concentration of NEM is increased. At a concentration of 20 nmoles of NEM per mg protein, respiratory control is essentially abolished. This suppression of respiratory control by NEM could be a consequence of interference with the translocation either of Pi or of ADP.

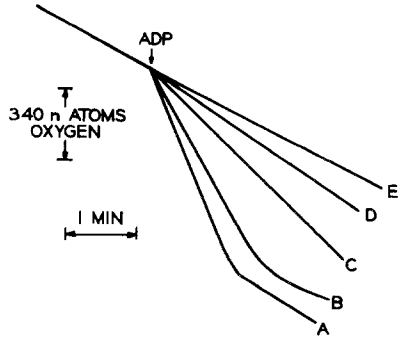

Figure 1. Effect of NEM on State III respiration of mitochondria. The assay medium was described in the methods section: A, 1.6 μmoles of ADP; B, 1.6 μmoles of ADP and 16 nmoles of NEM; C, 0.8 μmoles of ADP and 32 nmoles of NEM; D, 0.8 μmoles of ADP and 64 nmoles of NEM; E, 0.8 μmoles of ADP and 96 nmoles of NEM. Each assay contained 4 mg mitochondrial protein.

Effect of NEM on Accumulation of Pi, ADP and CA^{++}. Figure 2 shows that NEM at 20 nmoles per mg protein had no effect on the binding and/or exchange of ADP but completely suppressed the energized accumulation of Pi. The time course of Pi accumulation in mitochondria is shown graphically in Fig. 3. The phosphate-dependent accumulation of CA was also strongly inhibited by NEM at the same concentration (see Table IV). The data in this section strongly suggest that the inhibitory effect of NEM on mitochondrial functions which require Pi depends on its inhibitory effect on Pi translocation.

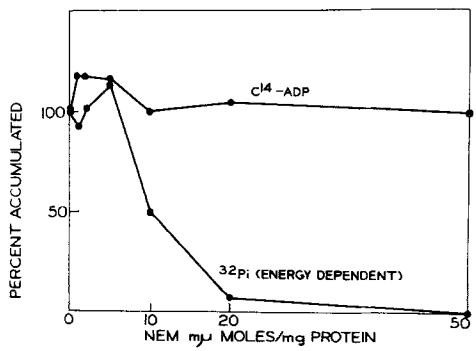

Figure 2. Effect of NEM on energized uptake of phosphate and binding of ADP. The 100% values for phosphate uptake and ADP binding (values in absence of added NEM) were 33.4 or 9.2 nmoles per mg protein respectively.

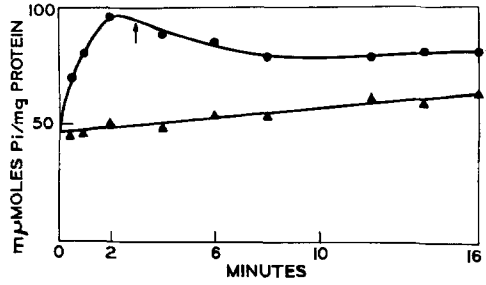

Figure 3. Time sequence of Pi accumulation in mitochondria. Two milligrams of mitochondria were incubated at 25° in 2 ml of the standard medium in presence of ^{32}Pi (10 mM) with (●) or without (▲) 5 mM K succinate. The pellets were solubilized by overnight exposure to 1% sodium dodecyl sulfate, and the radioactivity was measured by a Tri-Carb liquid scintillation counter in Brays solution. The arrow mark represents the beginning of anaerobiosis as measured by a Clark-type oxygen electrode in the same reaction mixture. Phosphate was estimated by the procedure described in the methods section.

TABLE IV. Effect of NEM on energized translocation of Ca^{++} plus Pi

System	Rate of translocation of Ca^{++} (nmoles Ca^{++}/min/mg protein)		% inhibition
	−NEM	+NEM	
Complete	226	62	73
Pi omitted	33	38	0

The experiments were carried out at 30° for 60 sec. The mitochondrial suspensions were exposed to NEM (20 nmoles per mg protein) for 10-20 min at 0° before initiation of the experiment. The (Ascorbate + TMPD) system was used as substrate at the same concentration as specified in the legend of Table VI.

Effect of NEM on anion-induced energized light scattering changes. The configurational transition from the aggregated (fused) to twisted mode can be followed by 90° light scattering changes. Figure 4 shows that NEM at a concentration of 10 nmoles per mg protein abolishes the Pi-induced but not the arsenate-induced light scattering change. The specificity of NEM for phosphate-dependent coupled processes apparently does not extend to weak acid anions generally.

Effect of NEM on the Pi-dependent formation of the twisted configuration. It has been demonstrated that under energizing conditions, Pi induces the transition of beef heart mitochondria from the aggregated (fused) configuration to the twisted configuration [25]. The electron micrograph in Fig. 5 establishes that NEM essentially completely suppresses the above mentioned Pi-dependent configurational transition.

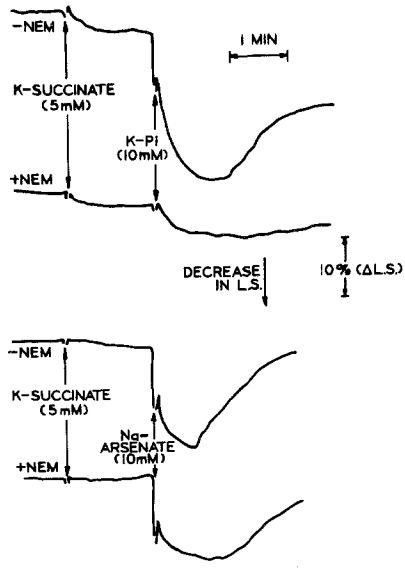

Figure 4. Effect of NEM on 90° light scattering. The reaction medium has been described in the text. The concentration of NEM was 10 nmoles per mg protein.

Modulation of Phosphate-independent Mitochondrial Functions by NEM

We have examined the effect of NEM on a set of mitochondrial processes that do not require the presence of Pi. These processes were respectively ATPase activity (see Table V), and electron transfer activity in presence of DPNH, succinate and ascorbate + TMPD (see Table VI). The endogenous ATPase activity is stimulated by NEM at the concentration levels required to suppress transphosphorylase activity, whereas the dinitrophenol-induced ATPase activity is partially inhibited at these same concentration levels. Electron transfer activity shows variable sensitivity to NEM depending upon the substrate, and no inhibition by NEM is observed on electron transfer in the cytochrome oxidase region of the respiratory chain. Thus, inhibition of electron transfer by NEM is a negligible factor in the causation of NEM-induced inhibition of Pi-requiring coupled processes.

Although it is clear from the data presented above that NEM at the concentration levels tested is not a general inhibitor of mitochondrial processes, nonetheless, the data are insufficient to exclude the possibility that coupled processes other than those which are Pi-requiring, may be found to be sensitive to NEM at the critical range of concentration. We raise this possibility because in fact we are aware from some unpublished studies of J. Southard in our laboratory of a high degree of sensitivity to

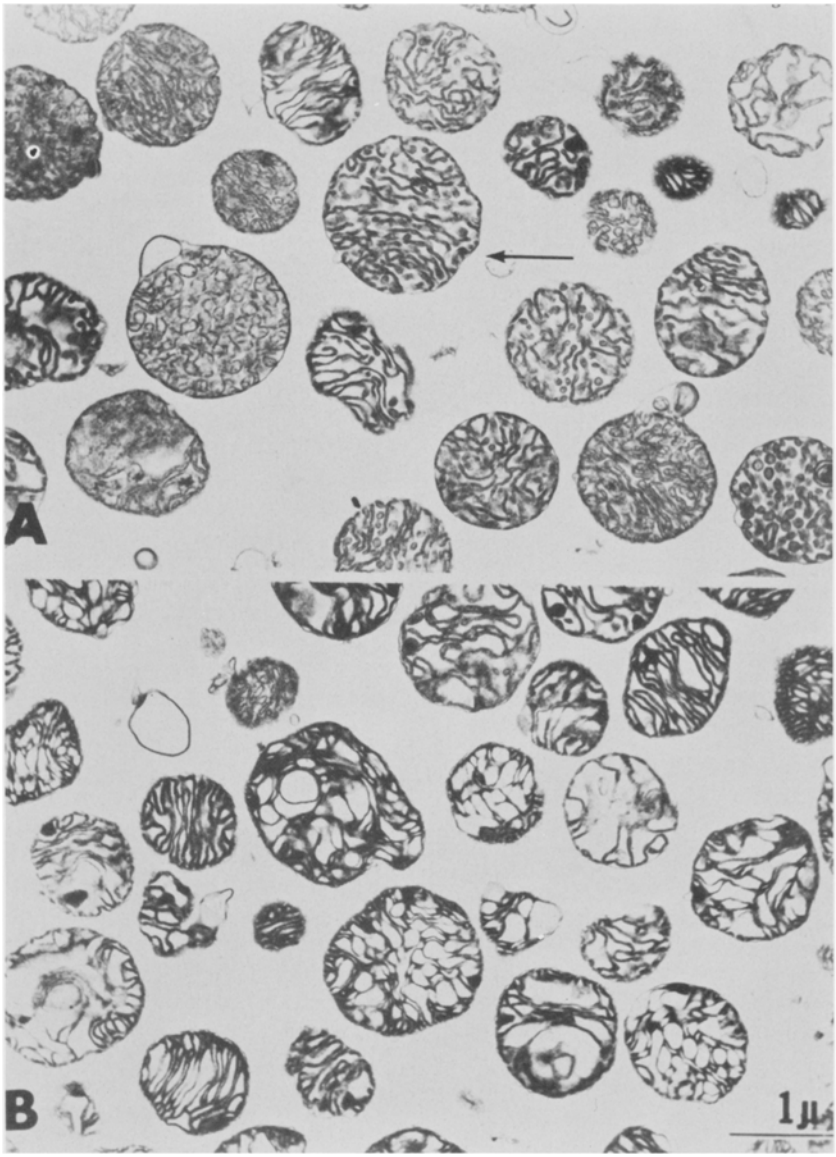

Figure 5. Effect of NEM on the phosphate-dependent aggregated to twisted configurational transition. A, mitochondria exposed to substrate, oxygen and inorganic phosphate (10 mM); B, conditions as in B except for addition of NEM (20 nmoles per mg protein); the arrow points to a mitochondrion in the characteristic configurational state; x30,000.

TABLE V. Effect of NEM on mitochondrial ATPase activity

NEM[a]	0	10	20	40
ATPase activity[b]	78	102	105	99
ATPase activity induced by 2,4-dinitrophenol[c]	168	94	118	106

[a] nmoles NEM per mg protein (heavy beef heart mitochondria).
[b] nmoles Pi released per mg protein per min at 30°.
[c] The concentration of dinitrophenol was 5×10^{-4}.

TABLE VI. Inhibition by NEM of electron transfer in mitochondria and ETP_H

Particle	Substrate	Rate of respiration (natoms O_2/min/mg protein) −NEM	+NEM	% inhibition
Mitochondria	Pyruvate + malate	29.0	20.0	38
Mitochondria	Succinate	110.0	83.0	25
Mitochondria	Ascorbate + TMPD	100.0	106.0	0
ETP_H	NADH	156.0	69.0	49
ETP_H	Succinate	94.0	79.0	16
ETP_H	Ascorbate + TMPD	92.0	95.0	0

The measurement of respiratory rates was carried out at 30°. Samples were preincubated with 20 nmoles of NEM per mg protein at 0° for 10-20 min. The concentration of the substrates used was: pyruvate, succinate and ascorbate (5 mM); NADH (2.5 mM); malate (0.5 mM); and TMPD (0.1 mM).

NEM of active transport processes which do not involve Pi. In our screening studies, we failed to find this inhibition, but then we were unaware of the kinetic factors which J. Southard subsequently has found to influence profoundly the degree of sensitivity of these active transport processes to NEM.

Effect of NEM on Pi-dependent Coupled Processes in ETP_H

NEM at a concentration of 20 nmoles per mg protein – a level that inhibits Pi-requiring reactions in intact mitochondria – neither lowers the P/O ratio of ETP_H nor reduces the ATP-^{32}Pi exchange activity (Table VII). These processes are in fact not affected even when the level of NEM is 2.5-5 times higher.

The inhibition by NEM of oxidative phosphorylation and ATP-^{32}Pi exchange activity in intact mitochondria can be completely relieved by sonication of the treated suspension. In Table VII, data are also

TABLE VII. Inhibition of oxidative phosphorylation and ATP-Pi exchange in particles prepared by sonication of NEM-exposed mitochondria

Preparation	P/O ratio with		ATP-^{32}Pi exchange[a & b]
	Succinate[d]	Ascorbate-TMPD[a]	
HBHM	1.8	0.90	61.7
NEM-HBHM	0.19	0.09	4.1
ETP_H from HBHM	1.4	0.35	62.0
ETP_H from NEM-HBHM	1.3	0.30	59.1
ETP_H + NEM[c]	1.4	0.37	60.5

[a] The assay conditions were same shown in Table VI in the concentration and temperature.
[b] mμmoles Pi exchanged per min per mg protein.
[c] ETP_H from HBHM were assayed in presence of 20 mμmoles NEM per mg protein.

presented which show that a submitochondrial particle (ETP_H) prepared from NEM-treated mitochondria, has coupled activities which were no longer demonstrable in the starting preparation before sonication. Moreover, ETP_H derived from NEM-treated mitochondria is no less coupled than ETP_H derived from untreated mitochondria.

Discussion

The experimental data now available on the selective suppression of Pi-dependent mitochondrial functions by NEM provide considerable support for the notion of a system in the inner mitochondrial membrane which facilitates the transmembrane transport of inorganic phosphate. We have elected to name this sytem, Pi-translocase [26].

Green and Brucker [27] have developed the concept of membrane complexes with internal cavities and with channels leading into the cavity from both sides of the membrane and the concept of the conformational entry and exit of the transport molecule into and from the internal cavity. The notion of membrane complexes as structural devices for implementing transmembrane transport of polar molecules is very much in line with our present knowledge of membrane structure [27]. Each such complex would be highly specific for the molecule to be transported. We are postulating that Pi-translocase would be a complex selective for transport of inorganic phosphate.

The form of inorganic phosphate which is transported by Pi-translocase is a critical consideration. If we assume that it would be the fully protonated form, then one could expect some interrelation between the mitochondrial proton transfer system and Pi-translocase. This possible interrelation is not negated by any of the experiments we

have reported in the present communication and is still a viable possibility.

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References

1. R. G. Hansford and J. B. Chappell, *Biochem. Biophys. Res. Commun.*, **30** (1968) 643.
2. A. Fonyo, *Biochem. Biophys. Res. Commun.*, 32 (1968) 624.
3. D. D. Tyler, *Biochem. J.*, **107** (1968) 121.
4. S. Papa, N. W. Lofrumento, M. Loglisci and E. Quagliarello, *Biochem. Biophys. Acta*, **189** (1969) 311.
5. A. J. Meyer and J. M. Tager, *Biochim. Biophys. Acta* **189** (1969) 136.
6. A. Tulp and K. Van Dam, *Biochim. Biophys. Acta*, **189** (1969) 337.
7. N. Haugaard, N. M. Lee, R. Kostrzewa, R. S. Harris and E. S. Haugaard, *Biochim. Biophys. Acta*, **172** (1969) 198.
8. M. J. Lee, R. A. Harris, T. Wakabayashi and D. E. Green, *Bioenergetics*, 2 (1971) 13.
9. F. L. Crane, J. L. Glenn and D. E. Green, *Biochim. Biophys. Acta*, 22 (1956) 475.
10. Y. Hatefi and R. L. Lester, *Biochim. Biophys. Acta*, 27 (1958) 83.
11. M. Hansen and A. L. Smith, *Biochim. Biophys. Acta*, **81** (1964) 214.
12. A. G. Gornall, C. H. Bardawill and M. M. David, *J. Biol. Chem.*, 177 (1949) 751.
13. O. Lindberg and L. Ernster, in: *Methods in Biochemical Analysis*, Glick (ed.), Interscience Publishing Co., Inc., New York, 1956, Vol. III, p. 1.
14. Packard Technical Bulletin, no. 3 (1961).
15. H. Takahashi, *Japanese Biochem. Soc.*, **26** (1955) 690.
16. G. A. Blondin and D. E. Green, *Arch. Biochem. Biophys.*, **132** (1969) 509.
17. J. Sedlak and R. H. Lindsey, *Anal. Chem.*, 25 (1968) 192.
18. P. H. W. Butterworth, H. Baum and J. W. Porter, *Arch. Biochem. Biophys.*, **118** (1967) 716.
19. D. W. Allmann, T. Wakabayashi, E. F. Korman and D. E. Green, *Bioenergetics*, 1 (1970) 73.
20. M. V. Riley and A. L. Lehninger, *J. Biol. Chem.*, 239 (1964).
21. R. E. Benesch, H. A. Lardy and R. Benesch, *J. Biol. Chem.*, **216** (1955) 663.
22. G. P. Brierley, V. A. Knight and G. T. Settlemire, *J. Biol. Chem.*, **243** (1968) 5035.
23. H. M. Klouwen, *Arch. Biochem. Biophys.*, 99 (1962) 116.
24. H. A. Lardy and H. Wellman, *J. Biol. Chem.*, **195** (1952) 215.
25. D. E. Green, J. Asai, R. A. Harris and J. T. Penniston, *Arch. Biochem. Biophys.*, 125 (1968) 684.
26. J. D. McGivan and J. B. Chappell, *Biochem. J.*, 127 (1972) 3.
27. D. E. Green and R. F. Brucker, *Bio Science*, 22 (1972) 13.