

## INVOLVEMENT OF N<sub>1</sub>-OXIDE DERIVATIVES OF ADENINE NUCLEOTIDES IN THE REACTIONS OF THE OXIDATIVE PHOSPHORYLATION

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### 1. Introduction

Adenine nucleotide analogs with modifications in the phosphate side chain, in the adenine ring, in the sugar moiety or in the base-sugar linkage [1–5], are interesting in view of the central role of adenine nucleotides, both as energy source and as metabolic control agents [6, 7].

In view of testing such compounds which may be used as effective antimetabolites for normal nucleotides or have significance in biological oxidation–reduction reaction systems we prepared and investigated a number of modified adenine nucleotides. For the present investigation we have synthesized the modified adenine nucleotides ADP-NO\* and ATP-NO, which were tested as potential substrate and/or inhibitors of mitochondrial processes in intact organelles.

### 2. Materials and methods

The following commercially available chemicals were used: crystalline BSA, G6PDH (BDH Chemicals,

Ltd.), yeast HK (Nutritional Biochemicals, Cleveland), ATP and ADP (Sigma Chemical Co., St. Louis). ATP-NO and ADP-NO were obtained by gentle oxidation of ATP and ADP with permaleic acid in aqueous solutions at neutral pH. Details regarding the synthesis and chemical reactivity of these modified nucleotides shall be given elsewhere. All chemicals were checked for purity by spectroscopic and chromatographic procedures.

Rat and human liver mitochondria were prepared by the procedures previously described [8, 9]. The proteins were determined by the method of Lowry et al. [10]. The mitochondrial respiration was monitored spectrophotometrically at 430 nm [11], and the rate of ATP synthesis measured from the amount of glucose-6-phosphate obtained in the presence of HK + glucose. The ATPase activity was evaluated colorimetrically by the release of P<sub>i</sub> from ATP or ATP-NO. The method of Lowry and Lopez [12] was used for measuring the inorganic phosphate. Energized accumulation of Ca<sup>2+</sup> was assayed essentially as described by Bielawski and Lehninger [13]. ATP-supported contraction of swollen mitochondria was measured by using the changes of the absorbance at 548 nm.

#### \* Abbreviation:

ADP-NO: adenosine diphosphate N<sub>1</sub>-oxide; ATP-NO: adenosine diphosphate N<sub>1</sub>-oxide; BSA: bovine serum albumin; G6PDH: glucose-6-phosphate dehydrogenase (EC 1.1.1.49); HK: hexokinase (EC 2.7.1.1.); HbO<sub>2</sub>: oxyhemoglobin; DNP: 2,4-dinitrophenol.

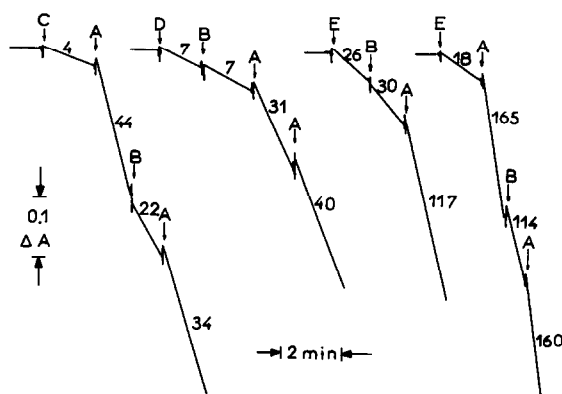


Fig. 1. Effect of ADP-NO on the respiratory activity of human liver mitochondria. The basic respiratory medium contained in 1 ml final volume and at 37°C: 180 mM sucrose, 50 mM KCl, 15 mM Tris-HCl, pH 7.4, 2.5 mM MgCl<sub>2</sub>, 2 mM EDTA, 5 mM phosphate buffer, pH 7.4, 10 mg BSA and 0.04 mEq HbO<sub>2</sub>/liter. After addition of the reagents, the spectrophotometric cell (0.3 cm path length) was bubbled for 2 min with N<sub>2</sub> (the fractional saturation of hemoglobin with oxygen decrease is about 1/2) and human liver mitochondria (0.54 mg of protein in experiments with glutamate and pyruvate + malate, and 0.27 mg of protein in experiments with succinate) were injected. The sample was covered with paraffin oil and the recording started. At the point indicated, 0.12  $\mu$ moles ADP (A), 0.09  $\mu$ moles ADP-NO (B), 10  $\mu$ moles glutamate (C), 10  $\mu$ moles pyruvate + 5  $\mu$ moles malate (D), or 10  $\mu$ moles succinate (E) were added. At 430 nm, an absorption increase of 0.1 correspond to an oxygen uptake of 16 ngAtoms. The numbers besides the traces indicate the oxygen consumption expressed as ngAtoms/min/mg protein.

### 3. Results

The comparative effect of ADP and ADP-NO on the respiration and the oxidative phosphorylation of rat and human liver mitochondria is shown in fig. 1 and table 1. ADP-NO is a poor phosphate acceptor in the oxidative phosphorylation when glutamate, pyruvate + malate or succinate is oxidized by intact mitochondria. The ADP-stimulated respiration is reduced after addition of ADP-NO to the respiratory medium showing a competition between these nucleotides at the level of the translocase system. A new addition of ADP again increased the respiratory rate. In order to make certain that the reaction rate of ATP synthesis (experiments in table 1) is not determined by the activity of the HK-trapping system, we measured in a separate experiment the  $V_m$  and  $K_m$  of HK with ATP and ATP-NO. The affinity of the yeast enzyme for these compounds is quite similar, but the  $V_m$  is 4.5 times lower with ATP-NO as compared to ATP. 7.4 EU of HK were used per assay, to ensure that an excess was present during the experiments. Table 2 shows that DNP greatly stimulates ATP hydrolysis in intact mitochondria, whereas ATP-NO is hydrolysed at a rate which is 15–20 times lower, in the presence

Table 1

Phosphorylation of ADP and ADP-NO by rat liver mitochondria.

ADP (mM)	ADP-NO (mM)	Phosphorylation rate (nmoles/min/mg protein)
—	—	9.3
0.03	—	99
0.10	—	144
0.30	—	152
—	0.03	10.1
—	0.10	12.8
—	0.30	21.9
0.30	0.30	110

The incubation medium (1 ml final volume) contained essentially the same reagents as described in fig. 1. It was supplemented with 10 mM NaF, 20 mM glucose, 2 mg yeast HK (3.7 EU/mg), 10 mM glutamate and varying concentrations of ADP and ADP-NO. No BSA and HbO<sub>2</sub> was present. The reaction was initiated by addition of 0.23 mg of mitochondrial protein. After 10 min of incubation at 37°, 0.5 ml of 1.5 N perchloric acid was added. The glucose-6-phosphate was measured enzymatically on the neutralized extract, by examining the reduction of NADP with G6PDH at 340 nm.

Table 2  
Hydrolysis of ATP and ATP-NO by rat liver mitochondria.

Addition	Intact mitochondria			Osmotically shocked mitochondria*		
	ATP	ATP-NO	ATP + ATP-NO	ATP	ATP-NO	ATP + ATP-NO
None	18	12	18	37	22	38
Mg <sup>2+</sup>	36	38	39	113	118	120
DNP	313	18	249	160	25	155

\* After the second washing the mitochondrial pellet was resuspended in distilled water, at a protein concentration of 5 mg/ml.

The test medium (0.2 ml final volume) contained 100 mM KCl, 25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 5 mM ATP or ATP-NO and 0.14–0.18 mg of protein. Mg<sup>2+</sup> (5 mM) or DNP (0.1 mM) were added to this basic medium. After 10 min of incubation at 37° the reaction was terminated by addition of 0.1 ml 20% trichloroacetic acid and the inorganic phosphate in the protein-free filtrate was determined. The ATPase activity (a mean value of four experiments) was expressed as nmoles P<sub>i</sub> released/min/mg protein.

of the uncoupler. Osmotically shocked mitochondria, on the other hand, exhibit an important increment of Mg<sup>2+</sup>-stimulated ATPase both using ATP or ATP-NO as substrate. Energy-dependent reactions in mitochondria such as the reversal of electron flow in the respiratory chain [14], the accumulation of Ca<sup>2+</sup> [13] or the reversal of mitochondrial swelling induced by different agents [15] indicate that ATP appears to have

Table 3  
Ca<sup>2+</sup> accumulation by rat liver mitochondria in the presence of ATP or ATP-NO.

Addition	Ca <sup>2+</sup> accumulation (nmoles/mg protein)
None	0
ATP	88
ATP-NO	4
ATP + ATP-NO	69

The incubation medium (1.5 ml final volume) contained 10 mM Tris-HCl, pH 7.4, 80 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM NaF and 5.3 mg of mitochondrial protein. After 4 min at 25°, the medium was supplemented with ATP (0.7 mM) or ATP-NO (0.7 mM) and CaCl<sub>2</sub> (0.4 mM). After 3 min, the samples were centrifuged at 15,000 g for 5 min and 0°, and the Ca<sup>2+</sup> content in the supernatant was measured by atomic absorption spectrophotometry.

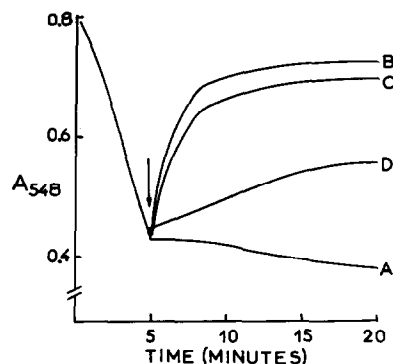


Fig. 2. Phosphate and ATP (or ATP-NO) induced volume modifications of rat liver mitochondria. The standard "swelling" medium contained in 1 ml final volume and at 25°: 125 mM KCl, 20 mM Tris-HCl, pH 7.4, 4 mM phosphate buffer, pH 7.4 and 0.8 mg of protein. At the time indicated by the arrow, BSA (1.5 mg), Mg<sup>2+</sup> (5 mM), ATP or ATP-NO were added in small volumes, and the absorbance changes were measured at 548 nm, using a 0.5 cm cell. A) Without nucleotide; B) 4.8 mM ATP; C) 2.4 mM ATP; D) 4.5 mM ATP-NO.

an absolute specificity. Therefore, we also tested the comparative effect of ATP and its N<sub>1</sub>-oxide analogue on such reactions. As presented in table 3 and fig. 2, ATP-NO was not able to stimulate the Ca<sup>2+</sup> accumulation, while the contraction of swollen mitochondria was much slower (both its rate and amplitude) with ATP-NO as compared to ATP.

#### 4. Discussion

The primary products of the oxidation of adenine and its derivatives were shown previously to be mono-N<sub>1</sub>-oxides [16, 17]. Since the conversion of ADP and ATP to the corresponding N-oxides proved to be more difficult, we first worked out a gentle one-step oxidation method which yielded no secondary products. The biological effects of adenine-N<sub>1</sub>-oxides, tested on pure and crude enzymatic systems [18–20] generally proved to be similar to those of their natural analogs.

The present experiments, in which we tested the modified nucleotides ADP-NO and ATP-NO as phosphate acceptor in the oxidative phosphorylation, respectively, as energy donor in energy dependent mitochondrial processes, showed marked differences as

compared to their natural analogs ADP and ATP.

The oxidative phosphorylation and associated processes are highly specific for ADP and ATP. This specificity in intact mitochondria reflects the properties of the adenylate translocation system [21]. Taking advantage of the evident spectral differences between ADP and ADP-NO, we investigated the exchange between extramitochondrial ADP-NO (0.01–0.08 mM) and mitochondrial ADP at 0° in a medium containing 110 mM KCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA and mitochondria. No measurable exchange in intact organelles was observed. Our data are in good agreement with the results obtained with mitochondrial ATPases. While DNP-stimulated enzyme, reflecting the structural integrity of mitochondria [22] is active with ATP as substrate only, Mg<sup>2+</sup>-stimulated ATPase in intact or in osmotically shocked mitochondria is active with both ATP or ATP-NO. The lack of nucleotide specificity in this case indicates that in contrast to intact mitochondria, ATP-NO has direct access to the ATPase molecule in damaged organelles, i.e. without having to cross an atractyloside-sensitive barrier [23]. On the other hand we observed that ADP-NO and ATP-NO do retain the property to function as substrate or as effectors for the soluble cytoplasmic enzymes (L. Mureşan et al., in preparation) which renders them particularly suitable for studying the relationship between mitochondrial and cytoplasmic processes involving adenine nucleotides.

We may conclude that the mono-N<sub>1</sub>-oxides of ADP and ATP deserve a special place among the structural analogs of adenine nucleotides, since the apparently minor structural modification at the N<sub>1</sub> group involves the immediate vicinity of the 6-amino residue, which plays an important role in the substrate specificity of oxidative phosphorylation and related reactions.

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