Biochemical Pharmacology, Vol. 31, No. 23, pp. 3823–3830, 1982.
Printed in Great Britain.

0006-2952/82/233823-08 $03.00/0

INHIBITION BY NITROSO-CHLORAMPHENICOL OF THE PROTON TRANSLOCATION IN MITOCHONDRIA

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(*Received* 12 *January* 1982; *accepted* 19 *April* 1982)

Abstract—We have found recently that, unlike chloramphenicol (CAP), its nitroreduction derivative nitroso-chloramphenicol (NO-CAP) behaved as a potent inhibitor of the energy-conserving mechanism in mitochondria [Abou-Khalil *et al. Biochem. Pharmac.* **29**, 2605 (1980)]. Concentrations of 75 and 250 μM NO-CAP were required to inhibit ATP formation with glutamate and succinate, respectively, whereas similar CAP concentrations were without effect. Testing several key reactions associated with the biosynthesis of ATP, inhibitory concentrations of NO-CAP were found to interfere as follows: (a) the transport of an NAD-linked substrate (e.g. glutamate) into mitochondria was only partially inhibited, whereas that of an FAD-linked substrate (e.g. succinate) was not inhibited but was rather slightly activated; (b) the transport of P_i was only inhibited at about 50%; (c) mitochondrial ADP transport was not affected at all; (d) the ATPase activity, measured either by P_i release in the presence of an uncoupler or by H^+ ejection, was only slightly affected; and (e) under either phosphorylation or no phosphorylation conditions and in the absence of P_i, NO-CAP was found to completely block mitochondrial H^+ extrusion resulting from the oxidation of either succinate or glutamate; however, under similar conditions the oxidation of the two substrates was not totally inhibited. The possibility of interference by NO-CAP with reactive mitochondrial thiols groups is discussed in the light of previous data and current experiments showing protection by P_i against NO-CAP effects on respiration. Moreover, NO-CAP as compared to conventional inhibitors of oxidative phosphorylation (e.g. rotenone, antimycin A, oligomycin, mersalyl and others) appeared to have a distinct mode of action on that process. The results demonstrate that the inhibitory effect of NO-CAP is primarily located at the respiratory chain level where the proton translocation activity is fully blocked.

Micromolar levels of the antibiotic chloramphenicol (CAP)† inhibit specifically mitochondrial protein synthesis in eukaryotes (for reviews, see Refs. 1 and 2), whereas millimolar levels are required to block the energy-transducing mechanism in mitochondria [3–5]. In CAP therapy, the serum drug level is usually in the micromolar range (e.g. 10–30 μg/ml); those levels were shown to block protein synthesis in hematopoietic mitochondria, but proved to be ineffective on their oxidative phosphorylation activity [6, 7]. Moreover, it is known that nitro-compounds may undergo bioreduction in various tissues [8–10] to yield the amino form via the nitroso and hydroxylamino derivatives. As a nitro-compound (cf. Fig. 1), CAP has been shown recently to be reduced by human liver extracts [11]. Such reduction suggests that the drug may provide additional toxic effects *in vivo* through its nitro-reduction intermediates.

In a recent study [5], we have compared the effects of CAP and its nitroso-derivative nitroso-chloramphenicol (NO-CAP) on protein synthesis and oxidative phosphorylation in mitochondria. The study clearly indicated a difference between the action of CAP and that of NO-CAP; while the latter was considerably less effective than CAP as an inhibitor of protein synthesis, it was found to be a much more potent inhibitor of the energy-conserving mechanism. Only micromolar concentrations of NO-CAP were needed to inhibit both ATP formation and the uncoupled state with either glutamate or succinate as substrate; furthermore, the inhibition was prevented or relased by cysteine, but not by washing [5].

In the present work, we have studied the mode of action of NO-CAP on the energy-transduction mechanism in mitochondria. Its effects on respiratory substrates, phosphate and ADP transport, and also on ATPase activity and proton translocation were compared with those of conventional inhibitors of oxidative phosphorylation, as well as with chloramphenicol and another nitroso-compound, nitroso-benezene (NO-Benz). The results indicate that NO-CAP primarily inhibits respiratory-chain components associated with the translocation of generated protons by respiring mitochondria.

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† Abbreviations: CAP, chloramphenicol; NO-CAP, nitroso-chloramphenicol; NO-Benz, nitroso-benzene; GKMS, glycylglycine–KCl–$MgCl_2$–sucrose; NEM, *N*-ethylmaleimide; Val, valinomycin; Oligo, oligomycin; Rot, rotenone; Anti A, antimycin A; P_i, inorganic phosphate; Glu, glutamate; Succ, succinate; M, mitochondria; DNP, 2,4-dinitrophenol; Hepes, N-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate.

METHODS AND MATERIALS

Preparation of mitochondria. Mitochondria were isolated as described in Ref. 5 from the liver of 5- to 8-week-old Charles River (CD) male rats fasted overnight.

Mitochondrial swelling. Swelling was measured in the presence of isotonic ammonium phosphate, glutamate, or succinate according to methods in Ref. 12. Mitochondria were preincubated at room temperature in a GKMS reaction medium consisting of 24 mM glycylglycine, 60 mM KCl, 9 mM $MgCl_2$, and 87 mM sucrose, adjusted to pH 7.4 with KOH. Other additions were as indicated in the legends. After 2.5 min of incubation, aliquots of 1 mg mitochondrial protein were added to a spectrophotometric cell containing 3.5 ml isotonic solution of the studied substrate at room temperature. The increases of percentage transmittance at 520 nm were recorded immediately with a Linear-255 recorder connected to a Bausch and Lomb Spectronic 70 spectrophotometer, with full scale transmittance set from 20 to 70% as in Ref. 13.

Translocation of ADP. The Millipore filtration procedure described in Ref. 14 was used to study [^{3}H]ADP transport in mitochondria. The latter were incubated at 2° for 1 min with [^{3}H]ADP (sp. act. 0.097 mCi/mmole) in the GKMS medium with other specified additions as indicated. The reaction was terminated by collecting mitochondria (aliquots of 1 mg protein) on the Millipore filter (pore size 0.64 μm), and the radioactivity was assayed in a Tri-Carb Packard liquid scintillation counter.

ATPase activity and phosphate release. This activity was measured by incubating mitochondria in the GKMS medium with ATP and 2,4-dinitrophenol as previously described [15]. Inorganic phosphate was determined according to the method of Fiske and Subbarrow [16].

H^+ ejection due to ATP hydrolysis. This activity was measured according to Ref. 17 with minor modification. The reaction medium (7.5 ml) contained 125 mM sucrose, 40 mM LiCl, 20 mM KCl, 3 mM Hepes (adjusted to pH 7.0 with KOH), 0.5 mM EGTA, 8 μg rotenone, and 40 nmoles *N*-ethylmaleimide/mg protein; other inhibitors were added as indicated in the legends. Mitochondria (10 mg protein) were added at time zero, valinomycin (0.3 μg/mg protein) at 2.5 min, and 400 μM ATP at 3.5 min. All reactions were carried out at room temperature. H^+ ejection in the reaction medium was followed by using a Beckman Futura pH glass electrode connected to a Beckman Expandomatic IV pH meter. The saturated KCl solution of the glass electrode was replaced by 0.1 M KCl as described previously [18] to diminish considerably the noise due to stirring of the medium without changing the response of the pH meter. Simultaneously, proton release was recorded by a Linear-255 recorder connected to the pH meter. The pH electrode was calibrated with internal standards by injecting standardized amounts of HCl solution at the end of each assay.

H^+ movement due to respiration and ATP formation. The reaction medium (4 ml) used in these experiments consisted of 3 mM glycylglycine, 40 mM KCl, 9 mM $MgCl_2$, and 150 mM sucrose adjusted to pH 7.4 with KOH. To this medium the following additions were made: 10 mM glutamate or 10 mM succinate, ± 5 mM phosphate, and ± other inhibitors. Mitochondria (6 mg protein) were added at time zero at room temperature, and proton variations in the medium were followed and recorded as described above.

Measurement of oxidative phosphorylation. Mitochondrial oxidative phosphorylation was measured by oxypolarography as previously described [19]. The incubations were carried out at 30° in 2 ml of the GKMS medium described above under "Mitochondrial swelling".

Protein determination. Protein was determined in the mitochondrial suspension by the biuret method [20] using bovine serum albumin (fraction V) as a standard.

Materials. Crystalline chloramphenicol was provided by Parke, Davis & Co., Detroit, MI. Nitroso-chloramphenicol was provided by Dr. M. D. Corbett from the Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, FL, as prepared by chloramphenicol reduction according to Ref. 21. Other reagents were from the Sigma Chemical Co., St. Louis, MO, or were of analytical grade.

RESULTS

In a recent study [5], we have shown that, with glutamate as oxidizable substrate, 150 nmoles of NO-CAP/mg protein was able to inhibit completely ATP formation as measured by oxypolarography, whereas a similar concentration of CAP was without effect. With succinate as substrate, 500 nmoles of NO-CAP/mg protein was needed for total inhibition, but here again an equal amount of CAP was not inhibitory. In addition, since the only structural difference between CAP and NO-CAP lies at the *para*-nitro group level (see Fig. 1), the use of another nitroso-compound, nitroso-benzene (NO-Benz) was as inhibitory as NO-CAP. Therefore, to understand the NO-CAP inhibitory mechanism on the energy-conserving mechanism we have explored its mode of interference in parallel with that of CAP and NO-Benz on several important reactions associated with that mechanism.

Effect of NO-CAP on mitochondrial transport of respiratory substrates, P_i, and ADP. The existence

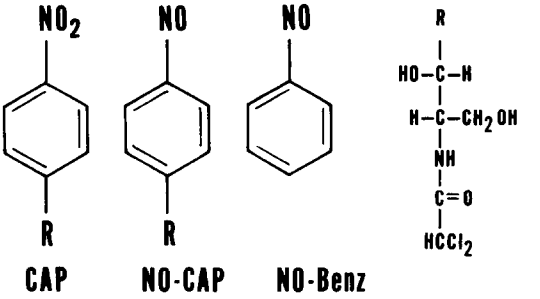

Fig. 1. Formulas of chloramphenicol (CAP), nitroso-chloramphenicol (NO-CAP), and nitroso-benzene (NO-Benz).

Table 1. Inhibition by CAP, NO-CAP, and NO-Benz of mitochondrial swelling in isoosmotic ammonium salt of glutamate, succinate, or phosphate*

Additions	% Inhibition		
	NH_4-Glutamate	NH_4-Succinate	NH_4-Phosphate
CAP	17	0	29
NO-CAP	56	NI†	47
NO-Benz	34	NI†	32
NEM	86		
Mersalyl		94	98

* Swelling was measured at 520 nm as described under Methods and Materials. Mitochondria were preincubated for 3 min in the GKMS medium in the absence (for control assays) or in the presence of reagent as indicated. The following concentrations in nmoles/mg mitochondrial protein were added: CAP, 400; NO-CAP, 200 (500 with succinate); NO-Benz, 200 (500 with succinate); NEM, 133; and mersalyl, 40. The swelling was initiated by adding aliquots of 1 mg mitochondrial protein (0.5 ml) to 3.5 ml of the ammonium salt studied (final concentration 110 mM). The percentage of inhibition was calculated after 2.5 min of swelling as compared to the respective control.

† No inhibitory effect was observed; the swelling was slightly activated.

of separate specific carriers for respiratory substrates, P_i and ADP in the inner mitochondrial membrane [22] allowed us to study the carriers activities in the presence of NO-CAP. Table 1 shows the different effects of CAP, NO-CAP, and NO-Benz on the transport of glutamate, succinate, and phosphate into mitochondria as compared with NEM and mersalyl, two known inhibitors of either glutamate or succinate and P_i carriers respectively [23–25]. At a concentration which totally inhibited ATP formation, NO-CAP blocked only 56% of the glutamate transport as measured in its isoosmotic ammonium salt; however, a similar level of NO-Benz was less effective. Moreover, while NO-CAP reacted with the phosphate carrier in a way similar to that of glutamate, it did not affect at all the succinate transporter. Total inhibition of swelling by NEM and mersalyl was recorded as anticipated (Table 1).

The effect of NO-CAP on ADP transport in mitochondria, another prerequisite reaction for ATP biosynthesis, is shown in Table 2. Clearly, 200–400 nmoles/mg protein of either NO-CAP or NO-Benz was without effect on ADP translocation; similar concentrations of CAP may have shown slight activation of that process. The addition of carboxyatractyloside, a known specific inhibitor of the translocase [26], resulted in almost total inhibition as predicted (Table 2).

Effect of NO-CAP on mitochondrial ATPase activity. The study of the ATPase activity as affected by NO-CAP was accomplished by measuring either P_i release in the presence of 2,4-dinitrophenol (DNP) or H^+ ejection in the presence of valinomycin. Table 3 depicts the comparative actions of CAP, NO-CAP, and NO-Benz. All three compounds showed similar effects on the DNP-dependent ATPase activity with only 20–30% inhibition observed when NO-CAP and NO-Benz were used at concentrations higher

Table 2. Effects of CAP, NO-CAP, NO-Benz and carboxyatractyloside on ADP transport in mitochondria*

Additions	[^{3}H]ADP transport (cpm/mg protein/min)	% Inhibition
None	26,000 ± 2,600	
CAP (200)	30,200 ± 3,000	NI†
CAP (400)	30,400 ± 2,900	NI
NO-CAP (200)	26,600 ± 1,800	NI
NO-CAP (400)	28,000 ± 2,100	NI
NO-Benz (200)	23,700 ± 2,600	9
NO-Benz (400)	27,600 ± 2,800	NI
Carboxyatractyloside	1,000 ± 60	96

* Mitochondria (1.25 mg protein) were added to the GKMS reaction medium (0.5 ml) with or without reagent as indicated. The addition of 100 μM [^{3}H]ADP (sp. act. 0.097 mCi/mmole) started the reaction at 2°. One minute later the reaction was terminated by collecting an aliquot of mitochondria (1 mg protein) on a Millipore filter (pore size 0.64 μm) as previously described [14]. Numbers in parentheses are nmoles/mg protein. Carboxyatractyloside concentration was 15 μM. Values are means of five assays ± S.E.

† No inhibitory effect.

Table 3. Effects of CAP, NO-CAP, NO-Benz and oligomycin on DNP-dependent ATPase activity*

Additions	P_i released (nmoles/mg protein)	% Inhibition
None	430 ± 16	
CAP (200)	330 ± 7	23
CAP (400)	300 ± 6	31
NO-CAP (200)	340 ± 17	21
NO-CAP (400)	310 ± 18	28
NO-Benz (200)	350 ± 7	19
NO-Benz (400)	340 ± 20	21
Oligomycin	130 ± 7	70

* Mitochondria (3.3 mg protein) were added to the GKMS medium (1.1 ml) with or without reagent as indicated. The simultaneous addition of 2.5 mM ATP and 0.25 mM DNP started the reaction at 30°. Five minutes later the reaction was stopped with 12.5% cold trichloroacetic acid. Numbers in parentheses are nmoles/mg protein. Oligomycin concentration was 1 μg/mg protein. Values are means of five to eight assays ± S.E.

than those needed to block ATP synthesis. Similarly, by measuring H^+ ejection triggered by ATP hydrolysis in respiration-inhibited mitochondria, NO-CAP was found to slightly affect this ATPase activity (Fig. 2), whereas CAP did not show any effect under these conditions. In both instances, the inhibitory action of oligomycin [27] was demonstrated (Table 3 and Fig. 2).

Effect of NO-CAP compared with those of rotenone, antimycin A, oligomycin, and CAP on oxidative phosphorylation. Figure 3B presents a typical oxypolarographic tracing showing the known effects of rotenone, antimycin A, and oligomycin. The first two compounds inhibited completely mitochondrial respiration, as expected, and oligomycin, added during state 3 where ATP is being synthesized, promptly inhibited that state without blocking the respiration. Subsequent addition of ADP did not

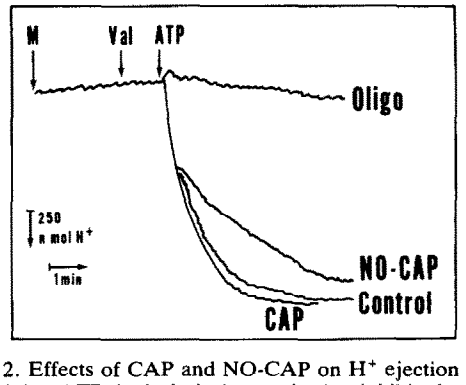

Fig. 2. Effects of CAP and NO-CAP on H^+ ejection triggered by ATP hydrolysis in respiration-inhibited mitochondria. The reaction medium (7.5 ml) contained 125 mM sucrose, 40 mM LiCl, 20 mM KCl, 3 mM Hepes (pH 7.0 with KOH), 0.5 mM EGTA, 8 μg rotenone, 40 nmoles NEM/mg protein, and, when present, CAP or NO-CAP (200 nmoles/mg protein of either) and oligomycin (1 μg/ml protein). Mitochondria (10 mg protein), valinomycin (0.3 μg/mg protein), and ATP (400 μM) were added as indicated. Other conditions were as described in Methods and Materials.

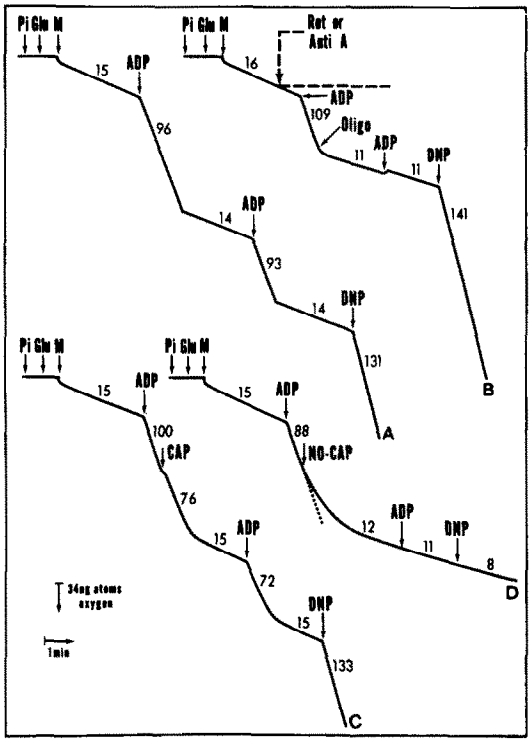

Fig. 3. Representative oxypolarographic tracings showing the effects of CAP and NO-CAP as compared to those of rotenone (Rot), antimycin A (Anti A), and oligomycin (Oligo) on oxidative phosphorylation. Mitochondria (M) (1 mg protein/2 ml) were incubated at 30° in the GKMS reaction medium [consisting of 24 mM glycylglycine, 60 mM KCl, 9 mM $MgCl_2$, and 87 mM sucrose (pH 7.4)] with 5 mM phosphate (P_i) and 10 mM glutamate (Glu) added to the medium. On each trace, the first addition of ADP was 360 nmoles and the second 180 nmoles. Other additions were as follows: 0.1 mM DNP; Rot, Anti A, or Oligo (all at 1 μg/mg protein); CAP or NO-CAP (200 nmoles/mg protein of either). Trace A is a control. The numbers along the traces give the initial rates of oxygen consumption expressed in ng atoms oxygen per min per mg protein.

give the normal respiratory jump (compare Fig. 3, A and B), whereas the addition of DNP resulted in uncoupling.

If CAP (200 nmoles/mg protein) was added during state 3 (Fig. 3C), or was added before mitochondria [5], neither state 3 nor the uncoupling by DNP was affected. However, as shown in Fig. 3D, the outcome of NO-CAP addition (200 nmoles/mg protein) was as follows: (a) under the conditions described in Fig. 3, NO-CAP, unlike rotenone and antimycin A, did not inhibit the respiration; (b) unlike oligomycin, NO-CAP did not promptly inhibit state 3, but it did inhibit the respiratory jump due to another ADP addition; and (c) in contrast to oligomycin, NO-CAP inhibited the uncoupling induced by DNP addition. It thus appears that NO-CAP reacts with mitochondria in a way distinct from that of the other inhibitors tested.

Protection by P_i against the NO-CAP effect on respiration. We have observed previously an increase of respiration with mitochondria preincubated in the presence of P_i and NO-CAP when succinate, but not

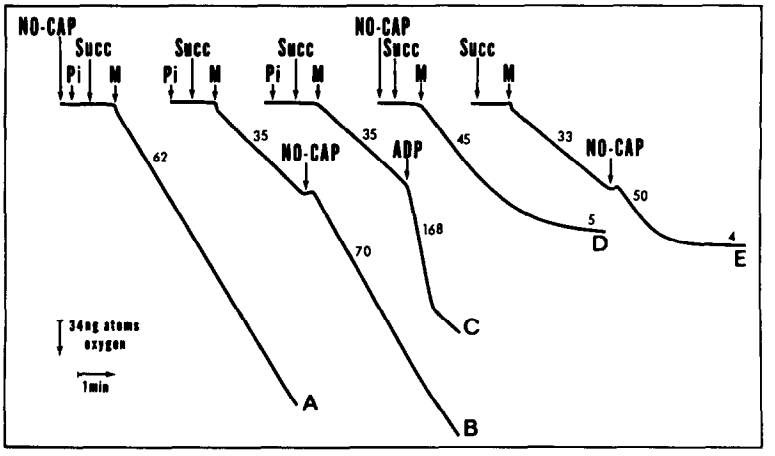

Fig. 4. Protection by phosphate against the effect of NO-CAP on mitochondria-oxidizing succinate. Arrows indicate the addition of NO-CAP (500 nmoles/mg protein), 5 mM P_i, 10 mM succinate (Succ), and 180 nmoles ADP. Other conditions were as in Fig. 3. Trace C is a control.

glutamate, was the substrate [5]. Traces A and B in Fig. 4 show similar respiratory increases in the presence of P_i + succinate and NO-CAP either preincubated with mitochondria or added to them during state 4 of respiration. However, in the absence of P_i, NO-CAP, added to the preincubation medium (Fig. 4D) or during state 4 (Fig. 4E), resulted in approximately 85% inhibition of respiration within 1–2 min of reaction. These results suggest that P_i interferes with NO-CAP action on mitochondrial membranes to protect against its inhibitory effect on respiration. Similar protection by some thiol group reagents has also been described [24, 28, 29].

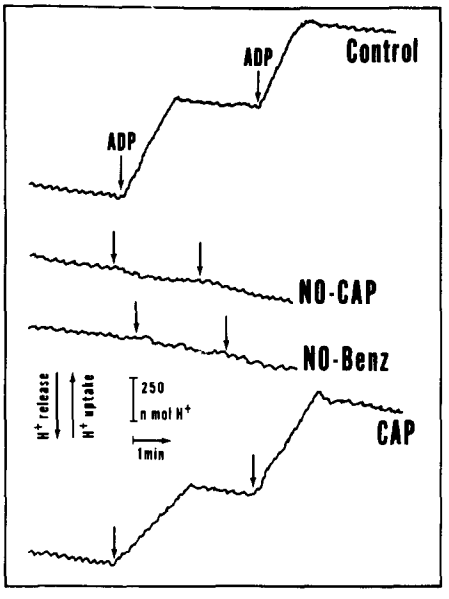

Fig. 5. Effects of NO-CAP, NO-Benz, and CAP on mitochondrial proton translocation under oxidative phosphorylation conditions with glutamate as substrate. Mitochondria (6 mg protein) were added to a medium (4 ml) [consisting of 3 mM glycylglycine, 40 mM KCl, 9 mM $MgCl_2$, and 150 mM sucrose (pH 7.4] with 5 mM P_i and 10 mM glutamate added to the medium. When present, NO-CAP, NO-Benz, or CAP (all at 200 nmoles/mg protein) was added prior to mitochondria. Arrows on traces indicate the addition of 240 μM ADP. Details of measurements are in Methods and Materials.

The protection by P_i against the effect of NO-CAP on succinate oxidation was also observed when non-energized mitochondria were preincubated for 2.5 min with the inhibitor before the addition of succinate. NO-CAP was about 74% inhibitory in the absence of P_i, whereas in its presence the respiration was increased as before. In both cases, further addition of ADP did not result in the normal respiratory jump, indicating inhibition of ATP formation (data not shown). Similarly, the protective effect of P_i was also observed with glutamate as substrate. About 67% inhibition by NO-CAP of mitochondrial respiration was seen in the absence of P_i, whereas no inhibition was observed in its presence.

Inhibition of proton translocation by NO-CAP. According to the chemiosmotic theory of oxidative phosphorylation as proposed by Mitchell [30], the respiratory chain translocates protons across the inner mitochondrial membrane which generate an electrochemical proton gradient that is used for ATP biosynthesis by the ATP-synthetase operating as a reversed proton pump. Because NO-CAP was found to inhibit ATP formation with only little effect on most reactions associated with oxidative phosphorylation, we have studied its effect on proton translocation under either phosphorylation or no phosphorylation conditions.

Figure 5 shows the effect of NO-CAP as compared with that of NO-Benz and CAP on proton translocation under conditions favoring oxidative phosphorylation in the presence of an NAD-linked substrate (e.g. glutamate). When either NO-CAP or NO-Benz was present in the incubation medium with P_i, the subsequent addition of ADP did not produce the expected proton uptake as shown in the control trace (Fig. 5), indicating that ATP formation did not occur. The presence of CAP, however, did not affect proton uptake. Moreover, under similar conditions but with an FAD-linked substrate (e.g. succinate), similar results were obtained (not shown).

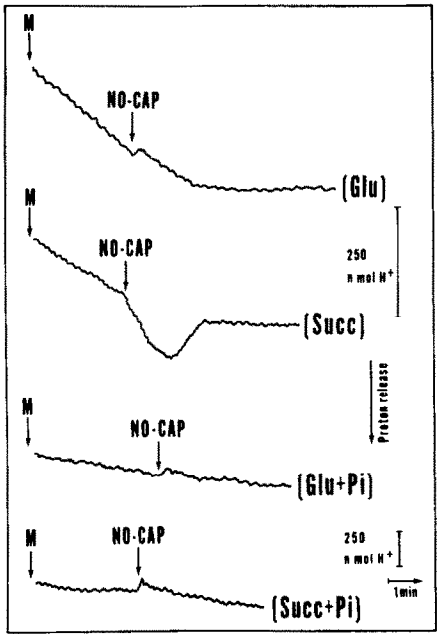

Fig. 6. Inhibition by NO-CAP of the proton release due to mitochondrial respiration under oxidative phosphorylation conditions. Mitochondria (6 mg protein) were added to the medium described in Fig. 5 with an oxidizable substrate and with or without phosphate. NO-CAP (200 and 500 nmoles/mg protein with glutamate and succinate respectively) was added as indicated. When present, 10 mM of either glutamate or succinate and 5 mM P_i were added prior to mitochondria. The difference between the higher scale for H^+ release made in the absence of P_i, and the lower one made in its presence, is related to the buffering capacity of P_i.

Due to the relatively high buffering power of P_i, which was present in the incubation medium (Fig. 5), the proton release due to respiration could not be detected. However, when P_i was omitted from the incubation medium, acidification by respiring mitochondria was readily recorded (Fig. 6). Under such conditions, the addition of NO-CAP to mitochondria-oxidizing glutamate resulted in total inhibition of proton release in about 2 min. Likewise, NO-CAP added to mitochondria-oxidizing succinate was totally inhibitory. The burst of proton release observed immediately upon its addition (Fig. 6) could be associated with the respiratory increase shown in Fig. 4E which was performed under similar conditions.

Under non-phosphorylating conditions (e.g. with oligomycin and NEM, and without P_i) the addition of NO-CAP or NO-Benz to the preincubation medium (Fig. 7A) produced complete inhibition of the protons generated by succinate oxidation, an inhibition identical to that produced by antimycin A (Fig. 7A). In contrast, CAP was without effect.

Figure 7B shows that NO-CAP inhibited succinate oxidation by 74%, as assayed by oxypolarography under the same conditions of Fig. 7A. Although proton release due to succinate oxidation was totally blocked by NO-CAP, respiration was not totally inhibited, as is evident in Fig. 7B.

DISCUSSION

We have shown recently [5] that NO-CAP at micromolar levels behaved as a potent inhibitor of oxidative phosphorylation in mitochondria; consequently, the present study was conducted with the aim of understanding the mechanism of the observed inhibition by NO-CAP. It was therefore important to address the question of whether one or more

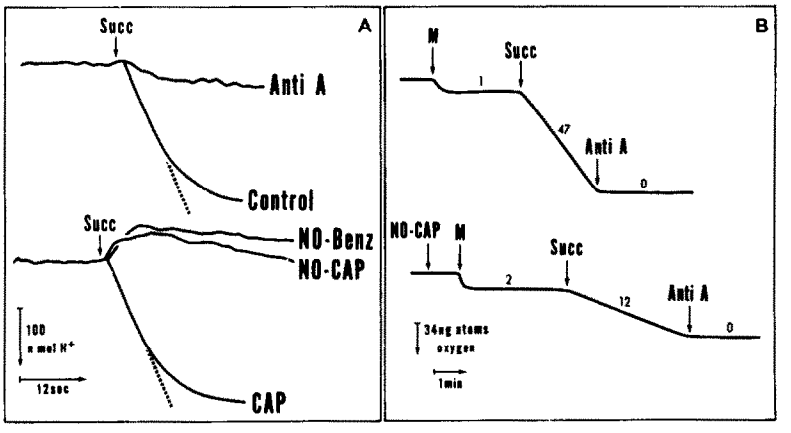

Fig. 7. (A) Inhibition by NO-CAP and NO-Benz of the proton release due to mitochondrial respiration under no phosphorylation conditions. Mitochondria (6 mg protein) were added to the medium described in Fig. 5, with 3 μg oligomycin, 6 μg rotenone, 240 nmoles NEM, and 1.2 μg valinomycin. After 3 min of incubation, the addition of 1 mM succinate started the reaction as indicated. When present, Anti A (1 μg/mg protein), NO-Benz, NO-CAP, and CAP (500 nmoles/mg protein of either) were added prior to mitochondria. (B) Effect of NO-CAP on succinate oxidation (under the no phosphorylation conditions of A) as measured by oxypolarography. Additions were as follows: mitochondria (M) (1 mg protein), NO-CAP (500 nmoles/mg protein), 1 mM succinate, and Anti A (1 μg/mg protein).

specific reaction(s) of those associated with the energy-transduction mechanism were directly affected by the NO-CAP.

Testing different mitochondrial transporters in the presence of NO-CAP (at concentrations inhibiting ATP formation), we found it to be only partially inhibitory on glutamate and P_i transport, whereas succinate and ADP translocation were not affected at all. The observed 47–56% inhibition by NO-CAP on P_i and glutamate transporters could only be partially, if at all, responsible for the total inhibition of ATP synthesis; that is because those substrates are usually added in excess to the medium and may not be a limiting factor. Therefore, additional mechanisms must be considered for the inhibition.

The recent finding that nitroso-benzene can form conjugates with glutathione [31], and our previous data [5] showing that the NO-CAP inhibitory effect on oxidative phosphorylation could be prevented or released by cysteine, strongly suggest that NO-CAP reacts with some mitochondrial enzymes having functional −SH groups. Candidate targets are glutamate and P_i carriers, known to bear such groups [23, 25], and they were indeed partially blocked by NO-CAP. The fact that only partial inhibition was observed may have been related to the permeability of the agent or more probably to the microenvironment of the reactive −SH groups [32]. Another indication of thiol group involvement comes from the experiments showing protection by P_i against the NO-CAP effect on respiration. This protection by P_i can be compared to that observed with other −SH group inhibitors [24, 28, 29].

Compared to conventional inhibitors of the respiratory chain, NO-CAP appeared to have a distinct mode of action. Thus, while rotenone and antimycin A totally inhibited respiratory chain sites 1 and 2, respectively, NO-CAP did not have any inhibitory effect on glutamate oxidation, but it did influence the oxidation of succinate by increasing substantially mitochondrial oxygen consumption. NO-CAP action appeared also to be different from that of oligomycin, the known inhibitor of mitochondrial ATP-synthetase-ATPase. The difference resides in the fact that oligomycin acts immediately on mitochondria to inhibit ATP formation without blocking uncoupling by DNP (Fig. 3B), whereas NO-CAP reacts rather slowly to block ATP formation after about 2 min and also to inhibit the uncoupling effect of DNP (Fig. 3D).

Assayed either in the absence of P_i, or under non-phosphorylating conditions, NO-CAP was found to completely inhibit proton extrusion in respiring mitochondria; however, under similar conditions it did not totally block oxygen consumption. These findings may be comparable to those obtained with both dicyclohexylcarbodiimide (DCCD) and fluorescamine. The former inhibited proton efflux but not electron transport of cytochrome oxidase [33], while the latter did not alter the rate of oxygen consumption in succinate oxidation but inhibited the related proton extrusion [34]. However, unlike DCCD and fluorescamine, NO-CAP did not inhibit proton ejection associated with ATPase activity. It thus appears that the inhibition observed in Fig. 5 was presumably the result of blocking proton movement through the respiratory chain which is required for the proton gradient formation to be used for ATP synthesis.

Because of its specific inhibitory action on proton ejection of the respiratory chain in mitochondria energized with respiratory substrates, NO-CAP as well as NO-Benz may serve as very interesting tools in exploring the controversial subject of the coupling between electron transport and vectorial proton movement in mitochondria [35–37].

Acknowledgement—This work was supported in part by USPHS Grant AM 26218.

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