1.Evidence for mitochondrial 2,4-dinitrophenol accumulation across the P_i/H^+-symport system

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Abbreviations: NSPM, N'-[N"-n-nonyl-4-sulfamoylphenyl]-maleimide; NEM, N-ethylmaleimide; DNP, 2,4-dinitrophenol; NPA, 2-azido-4-nitrophenol

Summary: N'-[N"-n-nonyl-4-sulfamoylphenyl]-maleimide (NSPM) has been shown to be a very potent and specific reagent for inhibition or stimulation of various mitochondrial membrane-associated functions: ß-hydroxybutyrate-dehydrogenase, ATP/ADP-carrier, calcium- and potassium transport. 20 nmol NSPM/mg mitochondria inhibit the phosphate(H^+) activated P_i/H^+-symport system, prevent uncoupling by the classical uncoupler 2,4-dinitrophenol (DNP), and compete with the DNP analog compound 2-azido-4-nitrophenol (NPA). Furthermore, in this investigation we found that 20 nmol NSPM/mg mitochondria prevent DNP (H^+)activated DNP accumulation. It is concluded that DNP uncouples these mitochondria only after being transported into mitochondria by the P_i/H^+-symport system. A further conclusion is that NSPM reacts at the phosphate binding site, thereby abolishing P_i/DNP-binding and transport.

Key words: Thiol-reagent, P_i/H^+-symport, uncoupling, 2,4-dinitrophenol binding, 2,4-dinitrophenol-accumulation.

Zusammenfassung: N'-[N"-n-nonyl-4-sulfamoylphenyl]-Maleimid (NSPM) ist ein sehr potentes und spezifisches Reagenz für die Inhibierung oder Stimulierung verschiedener mitochondrialer Membran-assoziierter Funktionen: Beta-Hydroxybutyrat-Dehydrogenase, ATP/ADP-Carrier, Calcium- und Kalium-Transport. 20 nmol NSPM/mg Mitochondrien inhibieren den Phospat-aktivierten P_i/H^+-Symport, verhindern die Entkopplung durch den klassischen Entkoppler 2,4-Dinitrophenol (DNP) und zeigen Kompetition mit dem DNP analogen 2-Azido-4-Nitrophenol (NPA). In dieser Kommunikation finden wir weiterhin, daß 20 nmol NSPM/mg Mitochondrien die DNP/H^+ aktivierte DNP-Akkumulierung verhindern. Wir schließen daraus, daß DNP die Mitochondrien erst nach seinem Transport in die Mitochondrien durch den P_i/H^+-Symporter entkoppelt. Ein weiterer Schluß ist, daß NSPM mit der Phosphat-Bindungsstelle reagiert und hier P_i/2,4-Dinitrophenol-Bindung und Transport verhindert.

Schlüsselwörter: Thiol-Reagenz, P_i/H^+-Symport, Entkopplung, 2,4-Dinitrophenol-Bindung, 2,4-Dinitrophenol-Akkumulierung.

Introduction

In our studies as early as 1976 [1, 2], we found that the lipophilic thiol group trapping reagent NSPM inhibited the uncoupling normally seen with DNP in mitochondria. About 15 to 25 nmol NSPM/mg mitochondria blocked this uncoupling, but not the uncoupling by dicoumarol. Since then, we have examined NSPM action on the mitochondrial membrane [3-6, part II and III]. NSPM is a very potent and specific reagent (10 or 50 times more than, for instance, mersalyl or NEM) for membrane-associated functions: ß-hydroxybutyrate-dehydrogenase, the nucleotide-carrier, calcium- and potassium-transport (part IV), and phosphate/proton-symport are inhibited or stimulated. The NSPM competition experiments with the DNP analog compound NPA [5] clearly demonstrate the effect of NSPM on DNP interaction with mitochondria (binding and possibly accumulation), and suggested to us the necessity of measurement of a possible DNP-accumulation and the influence of NSPM on this

accumulation. Preliminary results were presented [7].

Material and Methods

Rat liver mitochondria were prepared according to Kaschnitz et al [8]. Oxygen uptake of mitochondria was measured with an oxygen electrode. All mitochondrial preparations were checked for structural integrity using the criterion of respiratory control [9].

Transport activities were detected as follows [6]: For assay of endogenous calcium and phosphate concentrations, 2.5 mg mitochondria were incubated at 22^0C for two minutes in 1 ml of a mixture containing 10 mM K-succinate, 5 mM $MgCl_2$, 20 mM KCl, 0.22 M mannitol, 70 mM sucrose, 2 mM K-Hepes, 6 µM Rotenone, pH 7.4, with appropriate concentrations of the various compounds used either before or after addition of 2 mM phosphate including ^{33}phosphate. The mixture was then rapidly cooled in an ice-salt bath at -10^0C to stop the reaction, and centrifuged for 5 minutes in an Eppendorf bench centrifuge. The pellet was washed 3 times with 0.22 M mannitol, 70 mM sucrose, 2 mM K-Hepes, pH 7.4, 0^0 C and dissolved with 200 µl 10 % sodium-dodecylsulfate to yield a final volume of about 230 µl. Aliquots were taken for calcium determination by atomic absorption spectrophotometry, and phosphate determination in a liquid scintillation counter. Active swelling rates (turbidity changes), indicative of K^+(Na^+)- movement [10, 11], were determined by the absorbance variations at 750 nm in 1 ml of a solution containing 10 mM K-succinate, 5 µM Rotenone, 2 mg mitochondria, plus or minus 10 mM K-phosphate, 5 mM $MgCl_2$, 20 mM KCl, 0.22 M mannitol, 70 mM sucrose, 2 mM K-Hepes, pH 7.4, 30^0C. K^+-transport rates were monitored continously at 30^0 by means of a Beckman K^+-elektrode. Calibration of the K^+-electrode was made in each experiment by adding a known amount of a KCl solution [11].

^{14}C-DNP transport was measured similarly to phosphate transport: 1.22 mg mitochondria were incubated at 22^0C for one minute with 20 nmoles NSPM/mg mitochondrial protein in 1 ml of a mixture containing 5 mM Na-succinate, 10 µM Rotenone, 0.22 M mannitol, 70 mM sucrose, 2 mM K-Hepes, pH 7.4, either bevore or after ^{14}C-DNP-addition. ^{14}C-DNP was allowed to equilibrate for 1 minute before or after NSPM addition. In the experiments with Triton X100, Triton was added one minute after ^{14}C-DNP and allowed to react for one minute. The reaction was stopped by rapid cooling in an ice-salt bath at -10^0C and centrifuged for 5 minutes (in the Triton experiments for up to 10 minutes) in an Eppendorf bench centrifuge. The pellet was washed 3 times with 0.22 M mannitol, 70 mM sucrose, 2 mM K-Hepes, pH 7.4, 0^0C and dissolved in 200 µl 10 % sodium-dodecylsulfate to yield a final volume of about 230 µl. After Triton treatment, the pellet could not be washed and was dissolved directly in 200 µl 10 % sodium-dodecylsulfate. Aliquots were taken for ^{14}C-DNP determination in a liquid scintillation counter.

The [^{3}H]NPA binding experiments were carried out as in [12].

^{3}H-NPA and ^{14}C-DNP were gifts from Prof. Dr. W.G. Hanstein, Bochum, dicoumarol was obtained from Schuchardt, NSPM was synthesized as described [1].

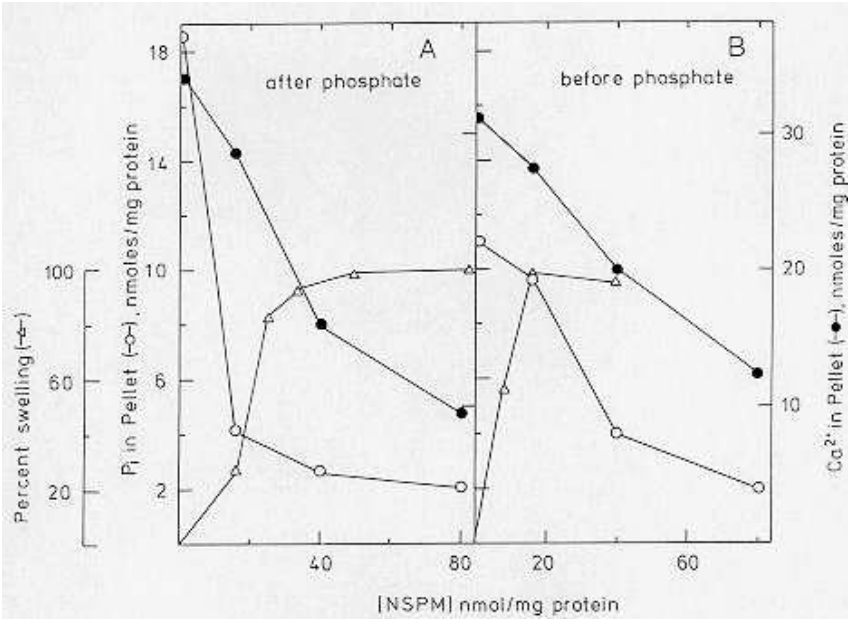


Fig.1. Phosphate-, Ca^{2+}-transport and swelling of rat liver mitochondria in the presence of NSPM. Conditions as described in Methods and Materials. A) NSPM added 2 min. after 2 mM phosphate plus ^{33}phosphate, 2 min. Incubation with NSPM before stopping reaction. Percent active swelling after 2 min. reaction with NSPM. B) 2 mM phosphate plus ^{33}phosphate added 2 min. after NSPM, further 2 min. incubation with phosphate plus ^{33}phosphate before stopping reaction. Percent active swelling after 2 min. reaction with NSPM in the absence of phosphate.

Results

Fig. 1 describes active phosphate- and calcium-transport, as well as swelling (K^+/Na^+-movement; 10, part IV) in the presence of varying concentrations of NSPM. There is no relation in inhibition by NSPM and either one of former activities. At a concentration of 20 nmoles NSPM/mg mitochondrial protein in the presence of phosphate (1 A), there is almost 90 % inhibition of phosphate transport, but $K^+(Na^+)$-movement (swelling by these osmotic active cations) and Ca-transport are not much affected. On the other hand, concentrations of about 20 nmoles NSPM/mg added before phosphate leads to maximal swelling of mitochondria (K^+, Na^+-uptake), but phosphate- and Ca-transport are only slightly affected (1 B). Important to note is that these 20 nmoles NSPM/mg added in the presence of phosphate led to about 80 % inhibition of phosphate transport, whereas only 14 % inhibition was obtained when the same concentration of NSPM was added before phosphate. Furthermore, NSPM itself is not a protonophoric compound [5]; if so, both transport activities would have been equally affected.

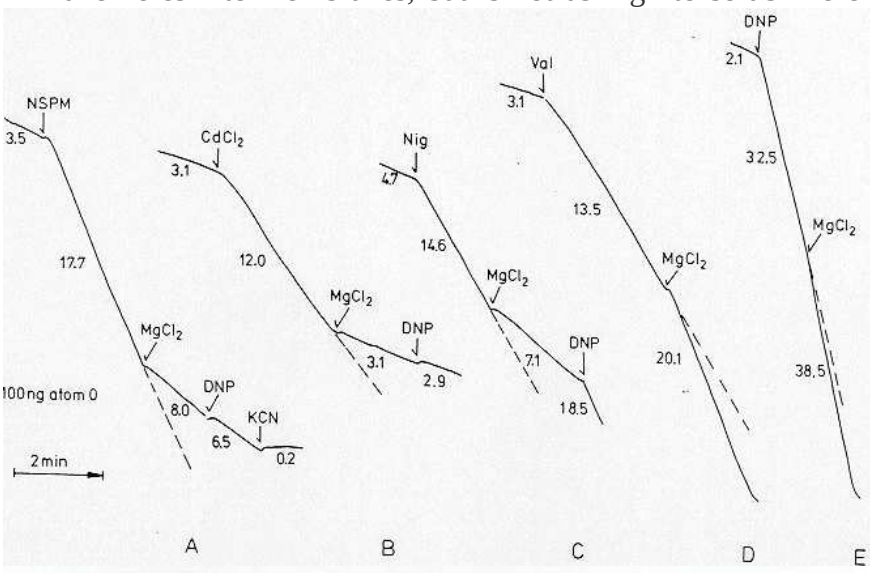
Table I. **^{14}C-DNP-accumulation in the presence of NSPM**

Conditions		^{14}C-DNP, Pellet Accumulation		
		nmol/mg	nmol/mg	%
95 µM	14C-DNP	13.30 ± 0.33 (3)	6.63	100
95 µM	14C-DNP, + 223 nmoles Triton X 100/mg [a)]	6.67 ± 0.10 (3)	0	0
95 µM	14C-DNP, + 20 nmoles NSPM/mg	8.75 ± 0.15 (3)	2.08	31.4
20 nmoles NSPM/mg, + 95 µM	^{14}C-DNP	11.60 t 0.10 (3)	4.93	74.4

a) amount of Triton resulting in uncoupling; inhibition of RCR at 90 nmoles/mg

Analytical methods: 14C-DNP Transport was measured using the same procedures as for phosphate transport: 1.22 mg mitochondria (from 2-3 weeks old normal feeded male wistar rats) had been incubated at 22^0 C for one minute with 20 nmoles NSPM/mg mitochondrial protein in 1 ml of a mixture containing 5 mM Na-succinate, 10 µM Rotenone, 0.22 M mannitol, 70 mM sucrose, 2 mM K-Hepes, pH 7.4, either before or after ^{14}C-DNP addition. ^{14}C-DNP was allowed to equilibrate for 1 minute before or after NSPM addition. In the experiments with Triton X1OO, Triton was added one minute after ^{14}C-DNP and allowed to react for one minute. The reaction was stopped by rapid cooling in an ice-salt bath of -10^0C and then centrifuged for 5 minutes (in the Triton experiments for up to 10 minutes) in an Eppendorf bench centrifuge. The pellet was washed 3 times with 0.22 M mannitol, 70 mM sucrose, 2 mM K-Hepes, pH 7.4, at O^0C and dissolved in 200 µl 10 % sodiumdodecylsulfate to yield a final volume of about 230 µl. After Triton treatment the pellet could not be washed and was dissolved directly in 200 µl 10 % sodium-dodecylsulfate. Aliquots were taken for ^{14}C- DNP determination in a liquid scintillation counter.

Table 1 shows the energy-dependent ^{14}C-DNP-accumulation in the presence of NSPM. The concentration of NSPM used (20 nmoles/mg) at the applied incubation time (1 minute) does not significantly stimulate respiration by itself. To distinguish between membrane-bound and movable DNP, the membrane had to be disintegrated. For this purpose we decided to use the well-known uncoupler Triton X100. The effects of Triton are very carefully described by Helenius and Simons [13]. We titrated mitochondria with Triton and looked at the minimal concentrations necessary in our system to uncouple oxidative phosphorylation, which were in the range of 220 nmoles/mg mitochondria; concentrations far above the levels (ca. 90 nmoles/mg) inhibiting State 4 → State 3 transitions [14]. This concentration is supposed to make holes into membranes, but is not as high to solubilize entirely the membranes: a fact which we needed for our experiments.



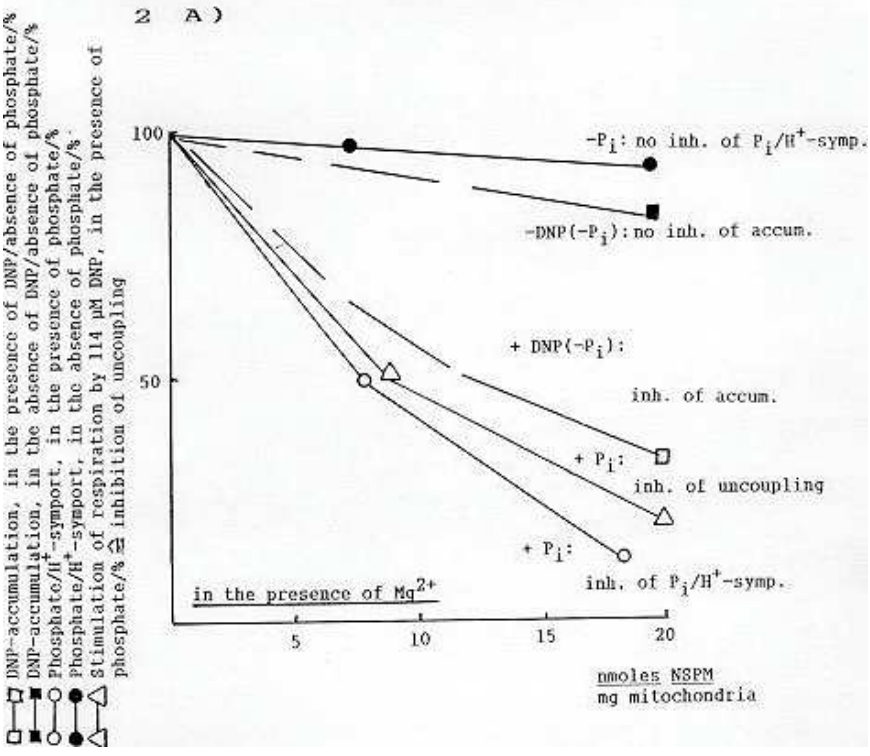


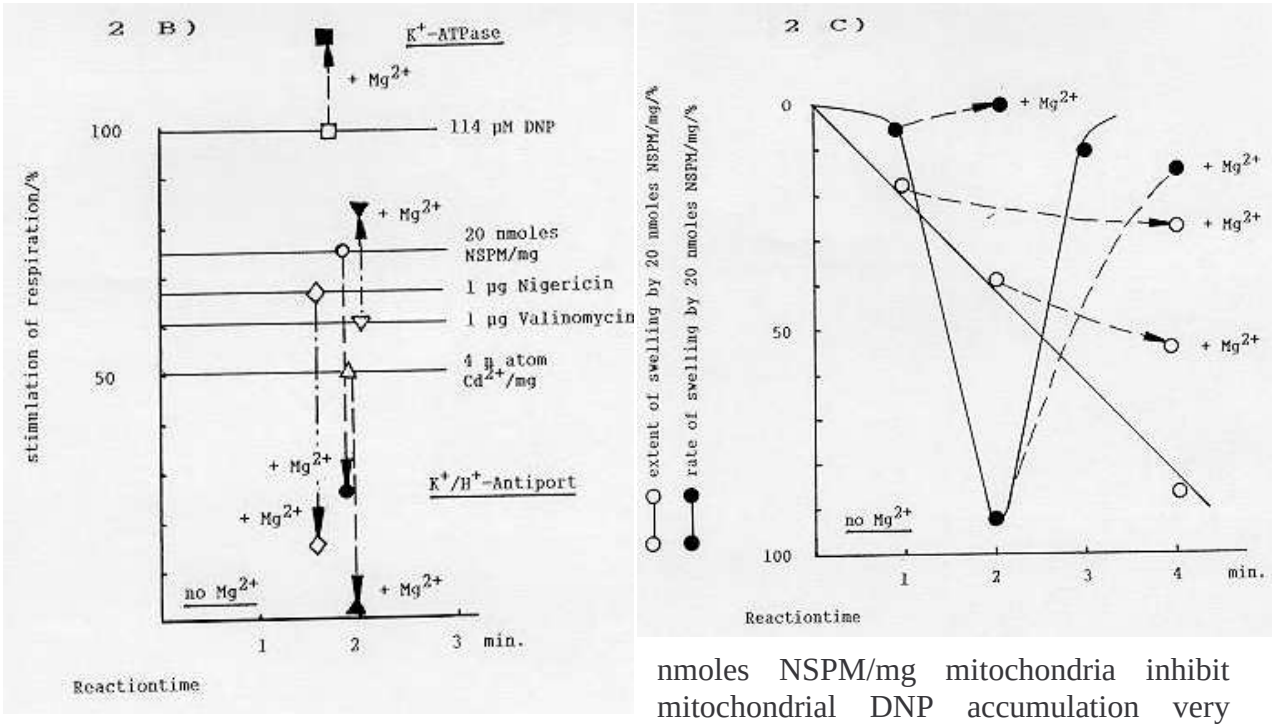
Fig. 2. Effect of various uncouplers on the coupled respiration of rat liver mitochondria. Reaction medium containing 0.22 M mannitol, 70 mM sucrose, 2 mM K-hepes, pH 7.4, 1.2 mg mitochondria, 5 µM rotenone, 10 mM Na-succinate (NSPM, Cd^{2+}, DNP) or 10 mM K-succinate (Nigericin, Valinomycin) and 30 mM KCl (Nigericin). Additions: 20 nmoles NSPM/mg protein, 5.7 mM $MgCl_2$, 114 µM DNP, 4 nA $CdCl_2$/mg protein, 1 µg Nigericin, 1 µg Valinomycin; RCR> 4.

Fig.2A. Effect of phosphate and/or DNP on NSPM-dependent phosphate/H^+-symport, DNP-accumulation and DNP-stimulated respiration (uncoupling) of rat liver mitochondria.Conditionsas described in Methods and Materials; RCR= 4.

Fig. 2B. Effect of various uncouplers on the coupled respiration of rat liver Mitochondria. Reaction medium containing 0.22 M mannitol, 5 mM sucrose, 2 mM K-hepes, pH 7.4, 1.2 mg mitochondria, 5 µM rotenone, 10 mM Na-succinate (NSPM,Cd^{2+},DNP) or 10 mM K-succinate (Nigericin, Valinomycin) and 30 mM KCl (Nigericin). Additions: 20

nmoles NSPM/mg protein, 5.7 mM $MgCl_2$, 114 µM DNP, 4 nA $CdCl_2$/mg protein, 1 µg Nigericin, 1µg Valinomycin; RCR>4.

95 µM DNP completely uncouple oxidative phosphorylation, but with no effect on respiratory chain activity) lead to the uptake of about 13 nmoles DNP/mg mitochondria. 50 % of this concentration can be released by Triton X100 and represents therefore a net accumulation concentration of 6 to 7 mM DNP, using a volume of 1 µl/mitochondrion for calculation. The remaining 6 nmoles DNP/mg are most likely bound to membrane components [15] and do not influence our activity measurements. In our experiment, 20



nmoles NSPM/mg mitochondria inhibit mitochondrial DNP accumulation very effectively and DNP- dependently, like the phospate-dependent NSPM inhibition of the P_i/H^+-symport [6, above]. As already suggested in the introduction, NSPM inhibition of DNP uncoupling is correlated to inhibition of DNP accumulation. This is best resolved by modulation of the P_i and Mg^{2+}-concentrations in the assay medium, as well as by changing the time intervals of Mg^{2+} addition to the assay medium (Fig. 2A - C): 8-9 nmoles NSPM/mg mitochondria added after P_i or DNP in the presence of Mg^{2+} inhibit about 50 % DNP stimulation of respiration and 20 nmoles/mg about 80 %; in correlation 8 nmoles NSPM/mg mitochondria inhibit about 50 % P_i/H^+ symport, 18-19 nmoles NSPM/mg mitochondria about 85 %, and 20 nmoles NSPM/mg mitochondria about 70 % DNP-accumulation (+P_i); NSPM addition after phosphate but before Mg^{2+}, results in immediate stimulation of site II respiration rates, which is immediately inhibited on Mg^{2+} addition. Essentially the same is valid for Cd^{2+} or nigericin as for NSPM. In contrast, DNP or valinomycin induced respiratory rates increase even more on Mg^{2+} addition (Fig. 2D and E). Extent and rate of swelling in the presence of NSPM behave similarly and correlate (at least during the first 2 to 3 minutes) with the increased respiratory rates (Fig. 2A) [part IV]. Important to remember: 1). Mg^{2+} is required for ATPase activity but not for ATP synthesis and 2). Mg^{2+} regulates the respiratory rate by interplay with Ca^{2+} [part IV]. The dithiol reagent Cd^{2+} [16] inhibits Ca^{2+} and P_i/H^+-symport with less than 4 nA/mg

Fig.2C.Swelling of rat liver mitochondria in the presence of NSPM. Reaction medium containing 0.22 M mannitol, 70 mM sucrose, 2 mM K-hepes, pH 7.4, 1.2 mg mitochondria, 5 µMrotenone, 5 mM Na-succinate. Additions: 20 nmoles NSPM/mg protein, 10 mM $MgCl_2$; RCR4.

mitochondria (a level near the concentration range of the P_i/H^+-symport system; part II), and shows essentially the same behaviour as NSPM (part IV). Inhibition of DNP-uncoupling by these quite different and highly specific thiol reagents is sensitive to Triton (not shown), as is DNP accumulation (Table I). Other specific effects exerted by the reagents (as described in this article series), besides the inhibition of P_i/H^+-symport, do not mutually interfere with each other. It should be noted that the accumulation of DNP without succinate is limited to about 50 % and most likely due to endogenously present substrates.

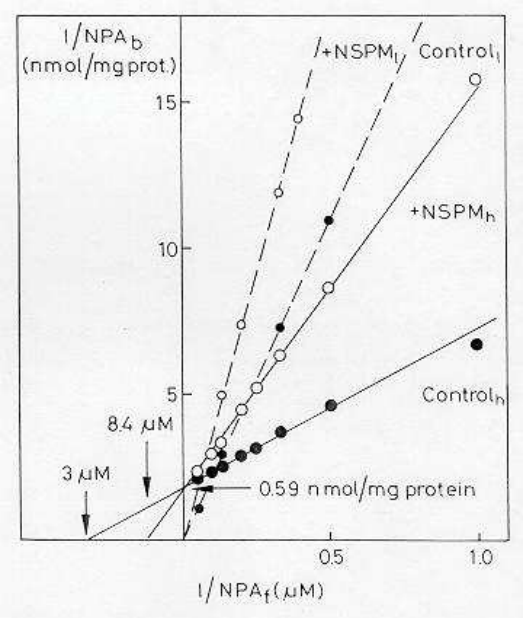


Fig.3. Double reciprocal (Benesi-Hildebrand) plot of the specific (h) and unspecific (l) binding of [^{3}H]NPA in the presence and absence of 58 nmoles NSPM/mg heavy beef heart mitochondria.

Fig. 3 shows the specific (h) and unspecific (l) binding of [^{3}H]NPA in the presence and absence of 58 nmoles NSPM/mg mitochondrial protein [5]. The concentration of NSPM is slightly raised (but still in the concentration range used for the other measurements) in order to achieve optimal conditions for "competition" with NPA [12]. The original NPA-binding curves obtained for mitochondria [12] are divided into specific and unspecific binding data in our study. There is "competition" between NPA and NSPM on the specific site; the K_i of NPA_h changes from 3 to 8.4 μM in the presence of NSPM. As may be seen in part II, NPA (DNP) and NSPM bind at the same site to different residues. NPA (DNP) presumably traps a sulfenyl group, but NSPM a thiol group. NSPM is able to block DNP reaction by its sulfamoylphenyl residue. The change in NPA_l by NSPM presumably represents inhibition of NPA accumulation based on the above DNP results.

Discussion

The results presented demonstrate that the classical uncoupler DNP is not a simple protonophoric compound: Hanstein and Drosdat et al [15] showed binding of the analog compound NPA to 30 kDa-proteins, the α-subunit of F_1, as well as the proteolipid (in F_0) and phospholipids. One of these 30 kDa-proteins represents the P_i/H^+-symport system [part I-III], the identity of the other 30 kDa-proteins will be described elsewhere. Our experiments indicate that phosphate, DNP, and its analogs are bound to [Fig. 3] and transported by this system [Table I] in using up the proton gradient, but without uncoupling mitochondria: The P_i/H^+-symport-system requires the presence of P_i or DNP/H^+ for activation of its symport (TableI, Figs.1 and 2). NSPM blocks P_i and DNP accumulation in the presence of P_i or DNP anions (TableI, Figs.1 and 2). NSPM blocks stimulation of respiration by DNP in the presence of P_i and H^+ (Fig. 2). The pH-gradient under these conditions is still present (part IV). The dissociation constant of NPA (the analog compound of DNP) increases in the presence of NSPM, but the "high affinity binding" of NPA remains unchanged (Fig. 3). The conclusion is that NSPM blocks accumulation of these anions by binding to the P_i or DNP (H^+) liberated thiol group (Fig. 4). This means DNP first needs to be accumulated in order to exert its uncoupling effect on mitochondrial respiratory-driven ATP synthesis (part III). P_i accumulation, although behaving identically to DNP, does not uncouple mitochondria! Another point determined from these results is that DNP is not a protonophoric uncoupling agent (part III, e.g. DNP phosporylation).

It should be added that the reaction of NSPM with the phosphate carrier or the nucleotide carrier in itself, suggests that these carriers are the only possible candidates for active accumulation of DNP^-/H^+. Since the nucleotide carrier works electrophilically, Cd^{2+}, which at a very low concentration (near the concentration range of the p_i-carrier) inhibits the only possible candidate: the P_i/H^+-symport system, in accordance with the other presented data.

At high internal concentrations of DNP (6 to 7 mM_i at 95 μM_0) it is difficult to determine where and how uncoupling takes place (F_1, F_0, phospholipids, etc.). However, one should consider that at high internal steady-state concentrations of DNP, the membrane potential may be abolished by release of DNP anions [17]. But the important point is that DNP, besides P_i, is actively transported into mitochondria before its uncoupling action takes place (by replacement of ADP), and this transport can therefore be prevented by certain compounds. This is new and important information for pharmacologists, toxicologists and biochemists.

Equlibrium of DNP across the membrane by diffusion, or even just change in binding to the membrane, is not at all possible with the data presented (Fig. 4). Also the possible argument that DNP may bind hydrophobically to a Triton accessible site and may be released there by this detergent, can be easily rejected. DNP is bound presumably as an anion, Triton as a lipophilic compound. The compounds are therefore bound at different sites. Triton replaces lipids from their binding sites. DNP^- may bind at the basic head groups of lipids. The different properties of these compounds are reason enough to exclude competition between them. For instance, protonated DNP would only bind by charge transfer interactions with other aromatic systems.

Preceding results could not be found by Hanstein and Hatefi [12] because they performed their experiments under unfavorable conditions (high protein, low ATP-plus succinate, as well as limited O_2 -concentrations) at high endogenous substrate respiration, which means on rapidly deenergized mitochondria and consequently release of the anions already taken up. In this context it would be very interesting to compare results obtained on mitochondria with transport activities in cell membranes.

Hüther and Kadenbach [18] detected in mitochondria (in the presence of 10 mM phosphate) a lag phase in the mersalyl inhibition curve for phosphate transport, but not in the case of the reconstituted carrier. Also, the dithiothreitol reversible oxidation of vicinal thiols by cupric di(1,10-phenanthroline) to intermolecular disulfide bridges on the carrier in mitochondria, with accompanied reversible inactivation, could not be seen in the reconstituted carrier. The authors arrived at a model of the carrier involving two identical subunits in cooperative reactivity, with the conclusion that the reconstituted carrier had lost its differential reactivity to the two thiol groups. Mersalyl and NSPM obviously react with different thiol groups or in a different manner in the phosphate binding site: The lag phase with mersalyl is obtained in the presence of 10 mM phosphate, in contrast to NSPM where a lag phase is seen without phosphate (Figs. 1 and 2).

Alkylation of Cys^{42} by NEM or dithiothreitol sensitive formation of a disulfide bond between Cys^{42} and a neighboring thiol upon auto-oxidation blocks purified beef heart phosphate transport [19, 20]. Cys^{42} has been suggested to be at, or very close to, the intramolecular transport path of P_i, probably directly in the phosphate-binding site(s) because of the extreme homology to the ADP/ATP-carrier along the primary structure [20]. We obtained NEM inhibition of P_i-transport in energized mitochondria at much higher concentrations than with the specific reacting NSPM. In the case of NSPM, phosphate transport and respiratory control ratio are equally abolished, but not so in the case of NEM: inhibition of respiratory control ratio lags far behind the inhibition of phosphate transport [1, 2, 6]. We conclude, therefore,

that NEM either binds outside the phosphate-binding site or at a "modified" binding site (a conclusion holding also for the reconstituted carrier), in contrast to NSPM which reacts with the"normal"binding site.Our conclusion is supported by Guerin et al[21].$Cys^{42}$is not preserved in mitochondria; their reconstituted active S. cerevisiae transport protein lacking Cys^{42} is sensitive to mersalyl, but insensitive to NEM. In future experiments we will isolate the membrane proteins containing bound NSPM molecules, locate the trapped cysteines in order to clarify described points, characterize the reaction centers and finally compare sequences.

The behaviour of phosphate- or DNP-transport against inhibition by NSPM suggests to us the involvement of a regulatory factor in these transport activities. The results described by Hüther and Kadenbach favor our suggestion. The modulation of the mitochondrial glutathione concentrations (among others) by active phosphate-transport relates to glutathione as a regulatory factor (Fig. 4, description under Fig. 4) [part II].

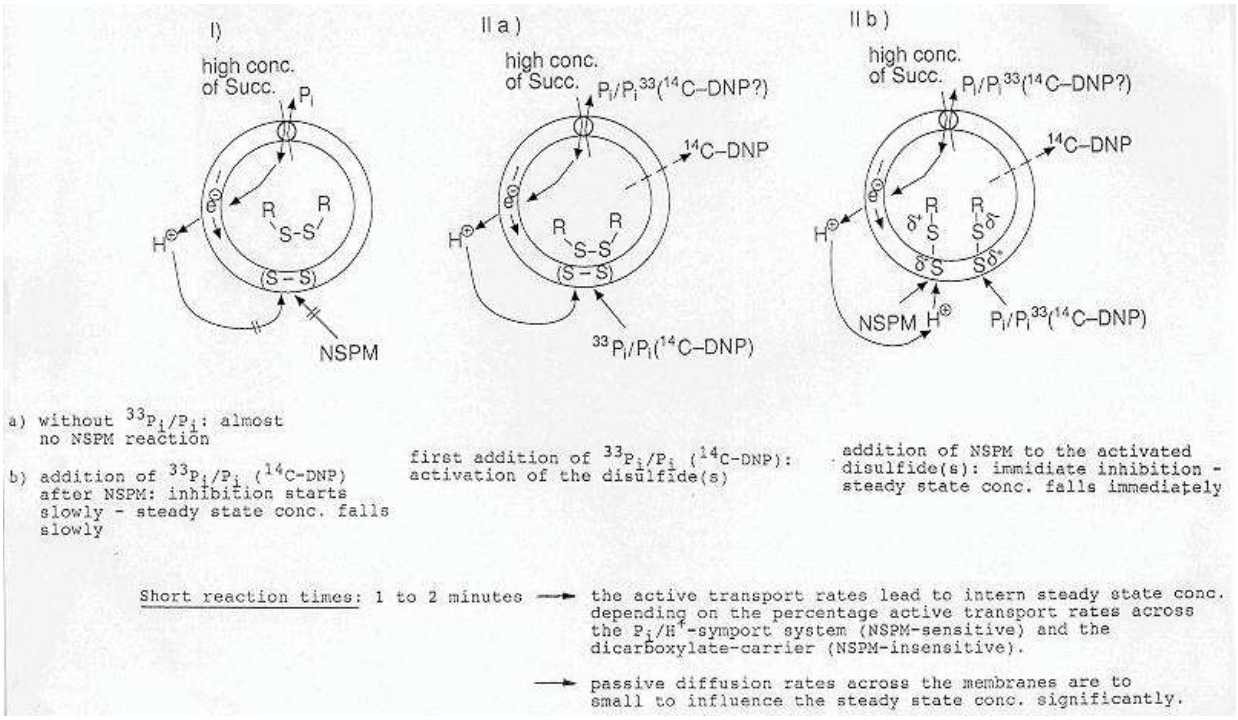


Fig. 4. Involvement of a regulatory factor in the P_i/H^+-symport system and DNP-accumulation as demonstrated with the thiol reagent NSPM

Another old, but still important conclusion is that there is more than one kind of uncoupler action on mitochondria. As just outlined, DNP may uncouple by reaction on F_0. NSPM (and the dithiol reagent Cd^{2+}) itself uncouples by reaction on the K (Ca)-transport system [part IV], and Triton makes holes in the membrane which leads to uncoupling. Dicoumarol derivates are not only important as anticoagulant agents, but their method of action on blood cell energy metabolism and as vitamin K-antagonists is of eminent importance, and therefore these compounds play a significant role in antithrombolytic prophylaxis. Taking into account the numerous functions involved in mitochondrial energy transfer and utilization, the former conclusion is logical and compelling [see also 22].

Our results make it possible to investigate various mitochondrial carrier functions without isolation and reconstitution of the carriers into artificial systems - systems which are in many cases totally unrelated to reality [see for example 18, 19, 23]. We like to consider, for instance, that mitochondria contain only a small percentage of phospholipids in contrast to phospholipid vesicles, and data obtained from vesicles are, therefore, for this reason not comparable to data obtained from mitochondria as has been done by Pressman [24].

Finally, one point should be stressed, their is absolute requirement of the lipophilic alkyl chain and the aromatic ring to induce all described membrane functions by our compound NSPM [see also 1, 2, as well as part II and III]. NEM action obtained at extremely high concentrations (up to 50 times higher) is different from, and even contrary to, NSPM effects [6, mersalyl discussion]. NSPM is designed solely for elucidation of unknown functions in membranes, while NEM and some other compounds are not.

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Reference no.: 92-0998/92-0999AB

Transport and ATP synthesis in mitochondria 1
I. Evidence for mitochondrial 2,4-dinitrophenol accumulation across the Pi/H+-symport system

II. Glutathione and endogenous regulatory factor for mitochondrial phosphate/proton symport

by

Kiehl Reinhold

Editor: Böck

Dear Dr. Kiehl,

Thank you for submitting your manuscripts.

I regret to inform you that the Editor responsible for your manuscripts has advised me that they cannot be accepted for publication in the Journal. The referees' reports are enclosed for your information.

The top copy of your manuscripts are being returned to you by separate printed-matter mail.

Yours sincerely,

Philipp Christen

Chairman of the
Editorial Board

Encl.: 4 reports
Copy: Editor

EUB n° 920998/ 0999/ 92 1073

Report 2

The refered manuscripts E.J.B. n° 92/0998, 0999 and 92/1073 are very poor, and sometimes non sense.

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Report 1

thank you for your FAX from Sept. 14. It is admittedly always disappointing to realise the rejection of a paper, and thus the author clearly argues against this decision. However, the authors arguments in this particular case are not solid both with respect to scientific reasons and to the style of his argumentation. In the following I will answer to the main arguments of the author criticizing the rejection of the two papers.

It is in fact obvious that it is not clear to anybody how transport and ATP synthesis in mitochondria works (at least on a molecular level). That is a good reason not to spoil the field with another unproven hypothesis. The general disappointment of the author about the current state of bioenergetics is to some extend justified, however, this is not the point to discuss here, and it will - in the opinion of the reviewer - not be improved by publishing these two papers.

Furthermore, it is in fact the general procedure in reviewing a scientific paper to "pick out one data of a set of connected data", as the author complains, if that particular result seems to be not correctly obtained and methodically doubtful. In his reply (MS 92.0998), the author gives some explanation to criticized point No 2, but no answer at all to point 1, 3 and 4. In his reply to the criticism of MS 92.0999 the author obviously overlooks that a paper to be published in a Journal of the level of EJB should include a sound presentation of experimental facts, on which only then a hypothesis may be constructed in the discussion part. This order is clearly turned round here: very limited amount of results and a lot of hypotheses. This fact is only expressed by the number of corresponding pages and the page numbers are of course not the basic reason for the rejection.

A last word to the English which was criticized by another reviewer. The author should not emphasize the point too much that the other reviewers have not criticized the poor English. In general, papers which are rejected on the basis of severe lacks in methodical aspects or regarding content are not criticized in addition with respect to poor English, since that is estimated as a comparatively minor point. Also in this case, absence of evidence does not mean evidene for absence.

EUB n° 920998/ 0999/ 92 1073

Report 2

The refered manuscripts E.J.B. n° 92/0998, 0999 and 92/1073 are very poor, and sometimes non sense.

The paper "Transport and ATP synthesis in Mitochondria. I. Evidence for mitiochondrial 2,4-dinitropohnol ..." by R.Kiehl cannot be accepted for publication in EJB.

1. There is not even circumstantial evidence that DNP is in fact transported by the phosphate carrier. The inhibiting agents are unspecific and have a lot of other effects on mitochondria besides inhibition of the phosphate carrier.

2. I do not understand what the souther means by the term "inhibition" of phosphate transport in Fig. 1. Phosphate was added before the inhibitor, how can NSPM inhibit transport under these circumstances.

3. Uncoupling by Triton clearly changes the membrane, it may thus also change the binding properties of the amphiphilic compounds like DNP. Addition of Triton is no proof at all for DNP to be transported to the matrix space instead of being equilibrated by diffusion of even being only bound to the membrane.

4. The context of the NPA-data is completely unclear and not explained in the paper.

EJB MS No. 92.0999AC3 Report No: 2

This paper is concerned with the problem of the mechanism of action of 2,4 dinitrophenol. The experiments have focused on the role of Pi/H^+ symport system in this mechanism, and it is concluded that the Pi/H^+ carrier is involved in the entry of 2,4 DNP into mitochondria. The experimental basis leading to this demonstration is poor and not convincing. It is partly based on the old fashioned swelling approach which is not really appropriate in the present work, due to uncontrolled artifacts. The experiments carried out with nonyl sulfamoylphenyl maleimide are not either convincing. Maleimide derivatives react with a number of protein components in mitochondria and the conclusion that maleimide derivative tested acts on the 2,4 dinitrophenol binding is not founded.

EJB MS No. 92.0999AC3 Report No: 1

The paper "Transport and ATP synthesis in Mitochondria. II. Glutathione and endogeneous regulatory factor ..." by R.Kiehl a I.Gruia cannot be accepted for publication in EJB.

The amount of experimental data presented (less than one page o results) is negligible in view of the body of hypotheses constructed (more than four pages of discussion). Furthermore, the few data are only in circumstantial connection to the speculations in the Discussion part.

EJB MS No. 92.0999AC3 Report No: 2

This paper is concerned with the formation of a complex between the mitochondrial glutathione and nonylthiouracil by incubation of mitochondria with nonylthiouracil. The complex is further cleaved to give a sulfenic acid derivative of glutathione. This is essentially a paper of methodology with limited interest in Biochemistry. Furthermore the Discussion Section is much too long and quite verbose (6 pages of Discussion for 1 page of Results).