Book-Chapter 4: **Transport and ATP synthesis in mitochondria** (1978-1984)

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

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Abbreviations: NSPM, N'-[N''-n-nonyl-4-sulfamoylphenyl]-maleimide, MNT, n-mono-nonyl-thiourea; NTU, 6-nonyl-2-thiouracil; NPTU, N-phenyl-N'-n-nonyl-thiourea; NPA, 2-azido-4-nitrophenol; DNP, 2,4-dinitrophenol; GSH, reduced glutathione; GSSG, oxidized glutathione; RCR, respiratory control ratio

Summary

Incubation of well-coupled beef heart mitochondria with [^{35}S] n-nonylthiouracil results in the isolation of [^{35}S] thiosulfenic acid of glutathione. Calculation to 100 % inhibiting concentrations for State 4 → State 3 transition, and to 100 % inhibiting concentrations for 2.4-dinitrophenol uncoupling by n-nonylthiouracil demonstrates that almost the whole glutathione pool is involved. Under our conditions, most of the glutathione is associated with the mitochondrial membrane and no free reduced glutathione could be found. Importantly, phosphate modulates the bound and free glutathione concentrations. A mechanism suggesting glutathione as an endogenous regulatory factor (presumably at a suitable receptor site) for the mitochondrial phosphate/proton symport (and possibly for the other carriers, including transhydrogenase and H^+-pumping ATPase) is presented. The effects of the sulfenyl-and thiol trapping compounds n-nonylthiouracil, thiophosphate, 2-azido-4-nitrophenol, diamide, cadmium and N'-[N''-n-nonyl-4-sulfamoylphenyl]-maleimide, as well as the high energy compound picrylacetate in this mechanism are discussed. The results are linked to cellular events and their importance for medicine (pharmacology and toxicology) is emphasized.
Key words: n-nonylthiouracil, inhibition of RCR and uncoupling, glutathione, regulatory factor for P_i/H^+-symport, regulatory model.

Zusammenfassung

Inkubation von gut gekoppelten Rinderherz- Mitochondrien mit [^{35}S] n-Nonylthiouracil resultiert im Endeffekt in der Isolierung von [^{35}S]Thiosulfensäure von Glutathion. Kalkulation zu 100 % inhibierenden Konzentrationen für State 4 → State 3- Übergang und zu 100 % inhibierenden Konzentrationen für 2,4-Dinitrophenol-Entkopplung durch n-Nonylthiouracil zeigt, daß fast der gesamte Glutathion-Pool beteiligt ist. Das meiste Glutathion ist unter unseren Bedingungen mit der mitochondrialen Membran assoziiert und kein freies reduziertes Glutathion konnte gefunden werden. Wichtig ist, daß Phosphat die freien und gebundenen Glutathion-Konzentrationen moduliert. Ein Mechanismus, der Glutathion als endogenen Regulationsfaktor (wahrscheinlich an einer entsprechenden Rezeptorbindungsstelle) für den mitochondrialen Phosphat/Proton-Symport (und möglicherweise auch für die anderen Transporter, einschließlich Transhydrogenase und H^+-pumpender ATPase) zeigt, wird beschrieben. Die Effekte von Sulfenyl- und Thiolgruppen spezifischen Reagentien wie n-Nonylthiouracil, Thiophosphat, 2-Azido-4-Nitrophenol, Diamid, Cadmium und N'[N''-n-nonyl-4-sulfamoylphenyl]-Maleimid als auch der hochenergetischen Verbindung Picrylacetat in diesem Mechanismus werden diskutiert. Die Resultate werden mit

dem zellulären Geschehen verbunden und ihre Wichtigkeit für die Medizin (Pharmakologie und Toxikologie) hervorgehoben.
Schlüsselwörter: n-Nonylthiouracil, Inhibierung von RCR und Entkopplung, Glutathion, Regulationsfaktor für P_i/H^+-Symport, Regulationsmodell.

Introduction

The lipophilic thiol reagent NSPM was introduced as a trapping agent for thiol groups in a hydrophobic environment [1-3]. These groups may be free or liberated from disulfides by a nucleophilic attack, or by protonation [2-4]. NSPM inhibits State 4 → State 3 transition at Site I in mitochondria, whereas Site II respiration was stimulated [2, 3]. NSPM almost completely blocks the thiol- dependent transport activities for P_i/H^+- and DNP/H^+-symport by covalent modification, for Ca^{2+}-transport [5, 6] and ADP/ATP-antiport [7], and stimulates K^+/H^+-antiport [in preparation] thereby acting as a chemically active uncoupler [2, 8].

[^{14}C]NSPM binding to various membrane proteins (2 to 2,5 nmoles/mg at 30, 3 to 3,4 nmoles/mg at 28 and 2 to 2,5 nmoles/mg at 7,5 kDa), including the carriers or uncoupler binding protein, respectively, as well as small molecular weight compounds [2, 6, 8, 9], could be shown. More than 50 % of the applied NSPM reacts with thiol groups in the membrane, and most of the remaining molecules are hydrolyzed and without effect [see also 6]. One of the radioactive-labelled proteins has been isolated [2, 9]; however its function and amino acid composition has not been clarified until now.

On the other hand, the lipophilic sulfenyl group reagents MNT, NTU and NPTU were applied as trapping agents for sulfenyl groups in a lipophilic environment [1, 2, 4, 10]. These groups should be generated from the activated disulfides described above [2-4]. MNT and NTU (as well as the analog compounds, unpublished) inhibit in mitochondria State 4 → State 3 transition at Site I, whereas Site II respiration is stimulated [2, 4], this reaction resembles NSPM on mitochondria. In this study we isolated the reaction partner of NTU and discuss its relevance for membrane-associated functions (incl. transport and coupling) in relation to medicine. Preliminary results were presented [11].

Material and methods

Beef heart (obtained from the slaughterhouse) and rat liver (from 2-3 week old male wistar rats) mitochondria were isolated as described [2, 9, 12]. Respiration was measured according to [2-4]. The respiratory control ratio (RCR) was >4 with succinate as the substrate.

NTU, [^{14}C]NTU and [^{35}S]NTU were synthesized [1,2, 13]. A rapid polyacrylamide gel electrophoresis at pH 5.0 without dithiothreitol was performed [2, 9].

The incubation of beef heart mitochondria with [^{14}C]NTU or [^{35}S]NTU and isolation of the radioactive products has been performed as follows: the incubation of mitochondria with radioactive NTU and isolation of the radioactive products was essentially identical to the [^{14}C]NSPM procedure [2, 9]. Incubated mitochondria were extracted at 4^0C with ethanol (absolute), the crude lipidic and peptidic residue of the evaporated ethanol supernatant with ether (absolute), the crude residue of the evaporated ether supernatant with $CHCl_3$/MeOH (2:1)

(absolute), the final crude residue of the evaporated $CHCl_3$/MeOH was disolved in H_2O and separated on Sephadex LH20 with water as eluant. Only one radioactive peak (elution volume 120 to 142 ml) could be isolated, part of the applied radioactivity has been found at the head of the column (radioactive NTU).

The fractions obtained were analyzed by t.l.c. on Silicagel (Merck F254) with $CHCl_3$/MeOH/H_2O/AcOH (50/30/4/8) [1, 2, 9]. This system was designed to separate lipidic materials. Lipoic acid could therefore be excluded at this stage as a possible reaction-partner of [^{35}S]NTU. Amino acid analysis of the water soluble fraction was done [2, 9].

Sample preparation for glutathione titration [14, 15]: 1 ml assay mixture (220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 20 mM KCl, 5 mM $MgCl_2$, 12 mM succinate, pH 7.4), plus or minus 10 mM phosphate, plus 2.5 mg mitochondria and 5 µM Rotenone were incubated for 4 minutes at room temperature and protected from light without NSPM, or after 2 minutes with 25 nmoles NSPM/mg mitochondria and incubated for a further 2 minutes. The samples were frozen at -20^0C for 1 to 4 weeks (the controls showed no significant reduction or oxidation of glutathione during this time), thawed to room temperature, peroxide-free Triton X100 (fresh batches or purified samples) was added to a final concentration of 0.03 % and shaken for 1 minute, then perchloric acid to a final concentration of 5.6 % was added, shortly shaken and spun for 10 minutes at 14 000 rpm with an Eppendorf bench centrifuge, the cooled (0^0C) supernatant was carefully adjusted to pH 7.5 with 5 N KOH, and after respin the supernatant was taken for glutathione determination.

Transport activities were measured as described [5, 6]. Active swelling rates, indicative of cation movement [16], were determined by the absorbance variations at 750 nm [5, 6].

K^+-transport rates were monitored continuously at 30^0C by means of a Beckmann K^+-electrode. Calibration of the K^+-electrode was achieved in each experiment by adding a known amount of KCl solution (17).

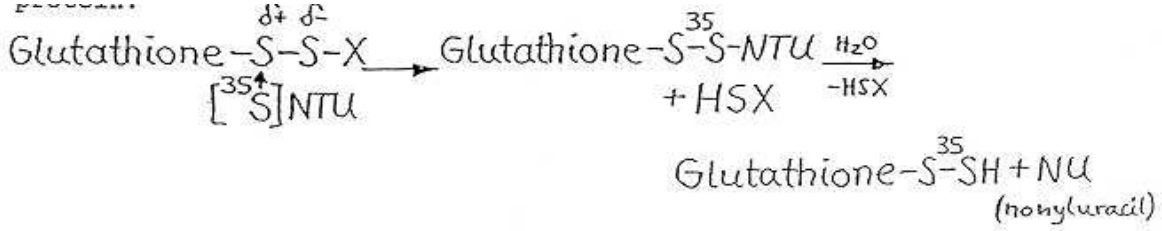
The [^{3}H]NPA binding experiments were carried out as described in (6, 18). The additional materials and methods are described in [1-4, 7-9, 17, 18]. Due to radioactivity, special care has to be taken when repeating any of the above experiments.

Results

A quite stable ß-lactoglobulin-sulfenyl iodide, RSJ, reacted very rapidly with thiourea and thiouracil to form the corresponding mixed disulfides. These mixed disulfides could be isolated with Sephadex G-25 at acidic pH and low temperature [19]. Our first attempt to isolate a mixed disulfide between [^{14}C]NTU and a mitochondrial component was not successful, although we used no disulfide breaking reagents (like dithiothreitol) and acidic pH (pH 5):

If beef heart mitochondria were incubated overnight with 8.5 nmoles [^{14}C]NTU/mg protein, at concentrations for 50 % inhibition of Site I coupling and 50 % inhibition of DNP uncoupling [2], one radioactive peak at the dye front could be detected by analyzing the incubated mitochondria in sodium-dodecylsulfate-gel-electrophoresis. This radioactivity could be totally extracted with ethanol and moved identically to [^{14}C]NTU on t.l.c. (R_f =0.95).

We solved this problem after reading the work of Maloof and Spector [20]. These authors found desulfuration of thiourea to urea after reaction with an activated disulfide; the second product was thiosulfenic acid. If our assumed mixed disulfide with [^{14}C]NTU followed the same reaction pathway, we would expect to find [^{35}S] labelled thiosulfenic acid by using [^{35}S]NTU. Based on this assumption, the same procedure applied to [^{14}C]NTU, including the whole isolation process described in the methods section, was carried out with [^{35}S]NTU-labelled mitochondria. We obtained a water-soluble fraction not moving in t.l.c. Amino acid analyses resulted in glutaminic acid, glycin and cysteic acid (1:1: ~1.2) and some minor contaminants. Thin layer chromatography, together with reduced glutathione for comparison, showed no migration of the compounds (R_F: glutathione=0; isolated compound=0), in contrast to [^{35}S]NTU (R_F =0.95). The radioactivity balance results in 3.5 nmol isolated [^{35}S] glutathione (thiosulfenic acid?) out of 8.5 nmol applied [^{35}S] NTU per mg mitochondrial protein:



The mixed disulfide between glutathione and [^{35}S]NTU was presumably cleaved during dissolving of the final crude residue in water.

In the model system of Cunningham [19], the ß-lactoglobulin sulfenyl iodide reacted rapidly with our synthesized thioureas and thiouracils to form the corresponding mixed disulfides [1, 2]. The oxidized thiol group of ß-lactoglobulin is located in a lipophilic environment, which leads initially to the stable sulfenyl iodide, and secondly to a drastic increase in the level of hydrophobic compounds [1, 2, 4]. The real target, mitochondria, showed similar behavior: only the most lipophilic compounds abolished RCR [2, 4].

Furthermore, the lipophilic compounds are not soluble in the media used for the experiments, but the mitochondrial membrane acts like a sponge for these compounds. The effects of NTU (MNT and similar compounds) is therefore expressed on activated glutathione molecules in the lipophilic membrane (presumably at corresponding receptor sites). X in the reaction equation has to be protein, which will be substantiated during discussion. However, it will be searched for a direct proof of a mixed glutathione protein disulfide in future experiments. The glutathione sulfur in the activated disulfide has to be positively charged, otherwise there would be no label dilution in the glutathione pool.

7-9 nmoles total glutathione (GSH + GSSG) per mg protein were found in rat heart [21] and liver mitochondria [22]. Assuming that beef heart mitochondria have a similar total concentration of glutathione, the value for 100 % inhibition of Site I coupling, which is 2 times 3.5 nmoles=7 nmoles glutathione/mg protein (assuming a linear binding/inhibition relationship), represents almost the total glutathione pool of these mitochondria. It should be mentioned that changing from short time (minutes) incubation of mitochondria with higher concentrations of NTU to long time (hours) incubation with lower concentrations of NTU switches stimulation at Site II to inhibition of State 4 → State 3 transition, as well as to inhibition of DNP uncoupling at Site II This is very likely due to a slow build-up of the thiosulfenic acid and inhibition of the phosphate/ proton symport [6], which would be a further indication of glutathione binding to protein.

Our well-coupled respiring mitochondria contain not only no free reduced glutathione molecules, but also low concentrations of oxidized glutathione. First titrations with 5,5'-dithio-bis (2-nitrobenzoic acid) and glutathione reductase [14, 15] of free GSH or GSSG in well-coupled rat liver mitochondria under succinate respiration resulted in 1.05 ± 0.23 nmoles GSSG/mg mitochondrial protein (n = 3) but in no free GSH (Table I). Remarkably, these oxidized glutathione levels disappear (probably by inducing formation of mixed disulfides) after 2 minutes preincubation of respiring mitochondria with uncoupling concentrations of NSPM (25 nmoles/mg protein). By omitting phosphate from the Site II, State 4 respiration mixture, 0.44 ± 0.14 nmoles additional NSPM-sensitive GSSG appears equal to about 1 nmol GSH/mg mitochondrial protein (n=3) (Table I):

Table I. **Free glutathione content in rat liver mitochondria**

	GSSG (nmol/mg)	GSH (nmol/mg)
active = succ. resp./plus phosphate	1.05 ± 0.23 (3)	0 (3)
active = succ. resp./no phosphate	additional = 0.44± 0.14 (3)	0 (3)
together	~ 1.1 - 1.9	0
passive no substrate (Vignais)[a]	0.1 - 0.4 (3)	4 - 5 (3)
- plus 2.6-Dichloroindo-phenol (Vignais)[a]	2.2	0
spec. NPA-binding (Hanstein)[b]	0.56 ± 0.13	
conc. phosphate carrier (Wohlrab)[c]	0.45	

a) Vignais, P.M. and Vignais, P.V. (1973) Biochim Biophys Acta 325, 357-374.
b) Hanstein, W.G., Hatefi, Y., Kiefer, H. (1979) Biochemistry 18, 1019-1025.
c) Wohlrab, H. and Greaney, J. (1978) Biochim Biophys Acta 503, 425-436.

Analytical methods:

Sample preparation for glutathione titration: 1 ml assay mixture (220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 20 mM KCL, 5 mM $MgC1_2$, 12 mM succinate, pH 7.4) plus or minus 10 mM phosphate, plus 2.5 mg mitochondria (from 2-3 weeks old normal fedded male wistar rats) and 5 µM Rotenone were incubated for 4 minutes at room-temperature and protected from light without NSPM or after 2 minutes with 25 nmol NSPM/mg mitochondria for further 2 minutes. The samples were kept frozen at $-2O^0C$ (for 1 to 4 weeks, the controls showed no signifikant reduction or oxidation of glutathione during this time), thawed to room-temperature, peroxide-free Triton X100 (fresh batches or purified samples) to a final concentration of 0.03 % was added and shaken for 1 minute, than perchloric acid to a final concentration of 5.6 % was added, shortly shaken and spun for 10 minutes at 14000 rpm with an Eppendorf bench Centrifuge the cooled (O^0 C) supernatant has been carefully adjusted to pH 7.5 with 5 N KOH, the supernatant after respin has been taken for glutathione determination (Ellmann G, 1959, Arch. Biochem. Biophys. 82, 70-77, and Tietze F, 1969, Anal. Biochem. 27, 502-522).

At this point, the work of Jocelyn [23] should be mentioned who did numerous measurements on mitochondrial GSH content. However, his results are questionable, first of all based on the general treatment of mitochondria, and secondly due to the methodology used, which was inferior to our isolation and assay conditions for well-coupled (energized or deenergized) mitochondria. Nevertheless, he found inhibition of phosphate-dependent glutathione loss (passive swelling) and little permeability of mitochondria to GSH (with or without phosphate) in the presence of succinate/rotenone. For oxidation of GSH, he found part of the formed GSSG lost as mixed disulfides with protein SH groups. These results agree with and support our findings, even if the mode of endogenous GSH - oxidation is not clear.

In this context, the free and oxidized glutathione levels in mitochondria vary considerably based on the methods and media used for isolation, as well as on the media used for assay. The above-mentioned characteristics of titration will be further explored by using varying concentrations of cations (protons) and anions.

Our results demonstrate P_i as modulating anion for free and bound glutathione concentrations. They implicate glutathione as a regulating factor for P_i/H^+- and DNP/H^+-symport [6], they suggest P_i^- or DNP^- and a proton as activating ions for the involved disulfides [2-4], and finally, they are one more indicator for a mixed glutathione-protein disulfide (which can be seen in the discussion of Fig. 3).

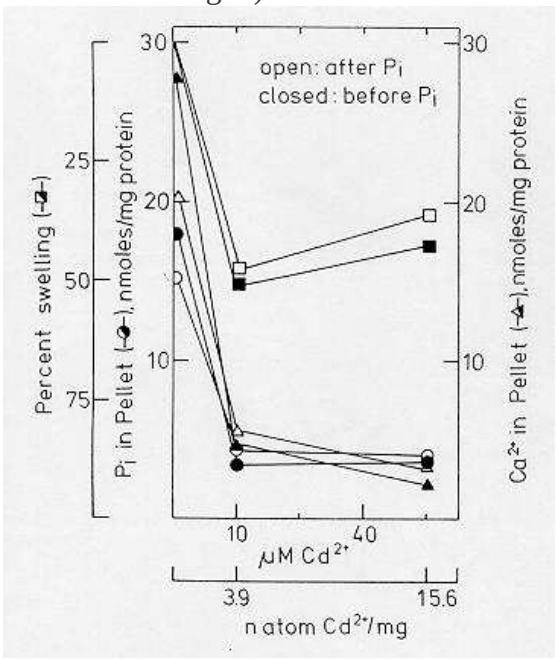


Fig. 1. Phosphate-, Ca^{2+}-transport and swelling of rat liver mitochondria in the presence of $CdCl_2$. Conditions as described in Methods and Materials. A) $CdCl_2$ added 2 min. after 2 mM phosphate plus ^{33}phosphate, 2 min. incubation with $CdCl_2$ before stopping reaction. Percent active swelling after 2 min. reaction with $CdCl_2$. B) 2 mM phosphate plus ^{33}phosphate added 2 min. after $CdCl_2$, further 2 min. incubation with phosphate plus ^{33}phosphate before stopping reaction. Percent active swelling after 2 min. reaction with $CdCl_2$ in the absence of phosphate. Noteworthy: The behaviour of NSPM on phosphate transport[6] is not seen using the indicated concentrations of $CdCl_2$. Mersalyl very much resembles $CdCl_2$ action in the phosphate-binding site.

Fig. 1 depicts active phosphate- and calcium-transport and swelling in the presence of varying concentrations of the site-specific uncoupler Cd^{2+} [5, 26, 31]. As can be seen, even such low concentrations as 4 natom Cd^{2+}/mg mitochondria strongly inhibit Ca^{2+}/P_i-transport and lead to about 50 % swelling. But it should be added: the kind of inhibition curves as obtained by the use of low concentrations of NSPM, in the presence or absence of phosphate [6], are under similar conditions, plus or minus phosphate, in the case of Cd^{2+} not visible; and 4 natom Cd^{2+}/mg mitochondria are near the concentration range of the P_i/H^+-symporter (Table I).

Fig. 2 shows the specific (h) and unspecific (1) binding of [^{3}H]NPA in the presence and absence of 69 nmoles NPTU/mg mitochondrial protein [6, 16]. NPTU was chosen in the competition experiments as one example of a lipophilic sulfenyl group trapping compound. There is competition between NPA and NPTU on the specific site, K_i of NPA_h changes from 3 to 15.4 μM

in the presence of NPTU. There is no change in NPA_l by NPTU and, therefore, no inhibition of NPA accumulation [6].

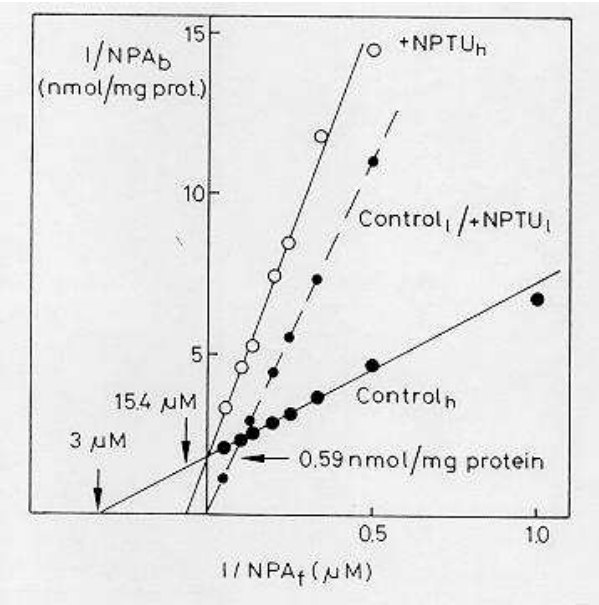


Fig. 2. Double reciprocal (Benesi-Hildebrand) plot of the specific (h) and unspecific (l) binding of [^{3}H]NPA in the presence and absence of 69 nmoles NPTU/mg heavy beef heart mitochondria.

Discussion

1. A relay mechanism with glutathione as essential factor explains the effects of many energy transfer inhibitors and uncouplers

In order to find a possible explanation for the presented and previous reactions, we combine in our suggestions [2-6] the involved membrane carriers, especially P_i/H^+-symport, with oxidized and reduced glutathione. We do not intend to review the immense amount of work done on mitochondrial energy-linked functions, therefore we only give a few references explaining our results. The participation of glutathione in mitochondrial carrier functions has not been demonstrated until now. Proving the direct participation of glutathione on cellular events is a difficult task [24] which we are aware of. For this reason, we concentrate at first on the phosphate-transport system [6]. This system has been described as a dimer with vicinal thiols which could be reversibly oxidized and inactivated with cupric di(1,10-phenanthroline) [25]. Our postulated disulfides activated by nucleophilic attack or by protonation [2-4] resemble this system[6]. The following "hypothetical" scheme (Fig. 3) presents then, in our opinion, the most reliable summation of our results.

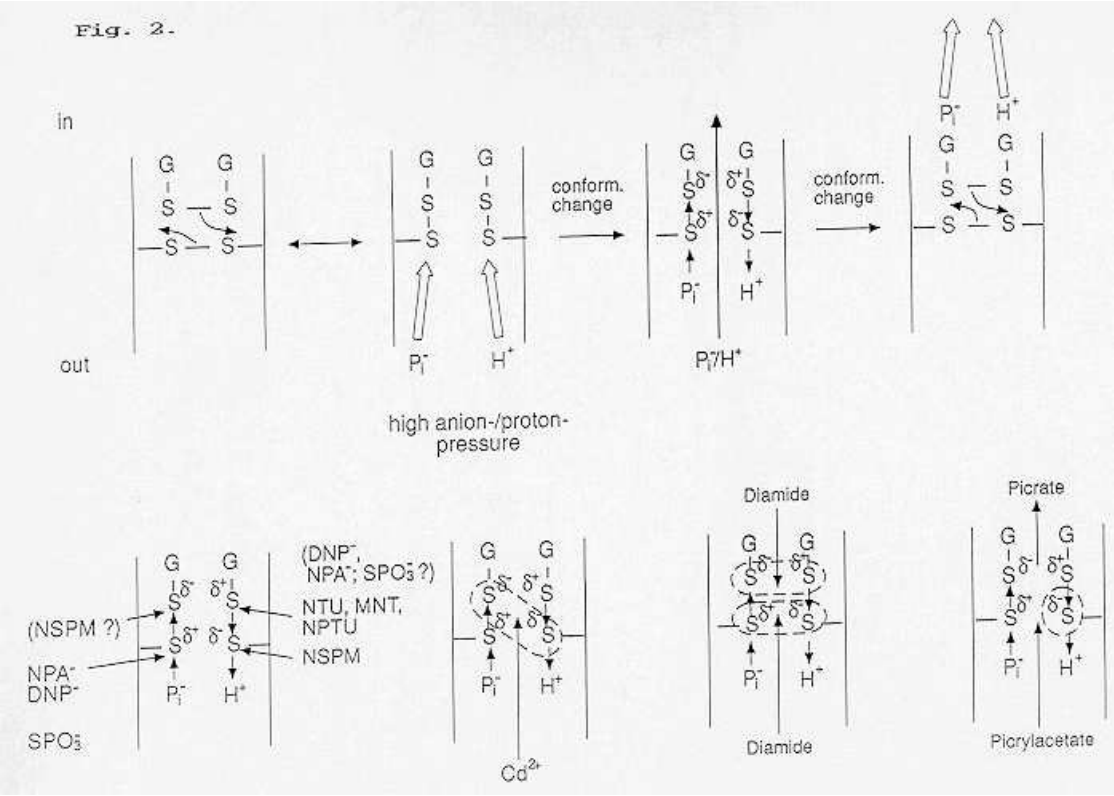


Fig. 3. Glutathione dependent P_i/H^+-symport system and the effects of various thiol- and sulfenyl-group reagents.

Fig. 3 shows the P_i/H^+-symport system dependent upon endogenous oxidized GSH. Phosphate and protons from outside the mitochondria activate the two neighboring disulfide bridges. During P_i/H^+ movement across the channel and release into the mitochondria, oxidized glutathione may be briefly liberated from it's receptor NSPM reacts with the formed protein (and glutathione?) thiol group and the uncoupler cadmium [26] complexes the two negatively charged sulfer atoms, thereby inhibiting phosphate transport (Fig. 1) [5]. Mersalyl probably acts similarly to Cd^{2+} [6]. NTU, MNT and NPTU trap the glutathione sulfenyl group and the uncoupler 2-azido-4-nitrophenol (NPA) [27], as well as DNP, possibly the protein (and glutathione?) sulfenyl group. SPO_3^- , in contrast to nonylthiophospahte, inhibits phosphate transport [2] presumably by sulfur-sulfur exchange in the activated disulfides. There is competition between NPA and NSPM (K_i of NPA_h changes from 3 to 8.4 μM in the presence of NSPM)[6, 8, 16], as well as between NPA and NPTU (the K_i of NPA_h changes from 3 to 15.4 μM in the presence of NPTU, Fig. 2) [16]. NPTU (as well as the other sulfenyl group reagents) does not influence the phosphate transport activities [see 6: NSPM-NPA competition experiments]. It should be mentioned that the specific NPA-binding, 0.56 $\pm$ 0.14 nmol/mg mitochondrial protein [28] (Table I), is in the concentration range of the phosphate-sensitive GSSG and the phosphate carrier [29] (Table I). Diamide [30] cross-links the activated disulfides (dithiothreitol sensitive) and blocks NSPM reaction [7].

Low amounts (13 to 15 μM) of the high-energy compound picrylacetate [31] inhibit State 4 $\rightarrow$ State 3 transition dithiothreitol and DNP sensitively, but P_i/H^+-symport is only marginally affected. The behaviour of picrylacetate resembles than very much NTU. NSPM abolishes RCR and P_i/H^+-symport equally in contrast to N-ethylmaleimide, which inhibits phosphate transport much easier [6]. Labelling of well-coupled mitochondria (RCR > 4, succinate) with ^{3}H-picrylacetate results in 0.10 to 0.15 nmol ^{3}H-acetyl/mg mitochondria divided into 30 kDa polypeptides and the proteolipid, respectively. The capacity of both bands is about 2.10 to 2.25 nmol ^{3}H-acetyl/mg mitochondria (electrophoresis at pH5, dithiothreitol, Methods) and is therefore as high as that for NSPM (30 kDa) or dicyclohexylcarbodiimide (proteolipid). Labelling of decoupled mitochondria (RCR < 2, succinate) results in normal ^{3}H-acetyl incorporation into the proteolipid, but diminished incorporation into the 30 kDa polypeptides. We conclude that decoupled mitochondria either lack GSSG itself, or the ability to bind available GSSG to the 30 kDa polypeptides (incl. ca. 0.5 nmoles/mg ATPsynthase/phosphate carrier), and that only mitochondria with activated bound glutathione will be acetylated [Fig. 3]. A rough calculation for well-coupled mitochondria would then lead to at least 3.2 (2.2 bound + 1 free) nmol GSSG/mg, which represents a large part of the glutathione pool.

In summary, NTU reacts in the membrane with an activated mixed glutathione-protein disulfide, thereby abolishing RCR. Involved is the 30 kDa P_i/H^+-symport system. PA acetylates as a function of RCR and sensitively to DTE and DNP 30 kDa membrane proteins, including the P_i/H^+-symport system, thereby abolishing RCR. Our conclusion: RCR is dependent upon glutathione bound to 30 kDa membrane proteins. DNP, its analogs, lipophilic NTU and its nucleophilic analog compounds, as well as lipophilic NSPM and most probably PA share the same reaction site on these proteins (e.g. ~ 0.5 nmoles/mg ATPsynthase/phosphate carrier). This could be shown for most compounds by performing competition experiments.

We think the described "relay" mechanism is, with small modifications, valid for signal transduction not only in cell organelles, but also in cells and between cells. This mechanism would imply new pharmacological treatments of diseases, may help in understanding the

functions of our brain, and could even provide a possible explanation for the known effects of acupuncture. Most of the results summarized by Ziegler [24] should be viewed with regard to our supposed mechanism.

2. Participation of glutathione in other mitochondrial functions, including glutathione transport and its relation to medicine.

The other carriers presumably function similarly under the influence of glutathione as the proposed P_i/H^+-symport system. Coupling between the carriers and the H^+-pumping ATPase could be shown [5]. Factor B and similar preparations (47, 42, 30, 11-12 kDa) [32] most probably contain glutathione as the active principle (glutathione binding proteins?), which suggests glutathione involvement not only in transport and ATPase activities, but also in transhydrogenation from NADH to NADP. On the other hand, isolated charged monothiols, such as reduced glutathione and cysteine, were shown to be poorly effective in restoring submitochondrial particle-supported, energy-linked functions [34]. Results favoring our suggestions. The cation (proton)/anion pressure accompanied by membrane transitions (swelling and contraction) [5, 6] regulates, therefore, the activity of the respective carriers, of transhydrogenase, and of H^+-pumping ATPase. Also, direct synthesis of ATP (out of ADP and P_i?) by GSH/GSSG, using a model system has been demonstrated [34]. A reinvestigation of the results [35] using incorrect conclusions did not provide sufficient evidence to disprove the direct synthesis of ATP in the used model system.

However, glutathione is the endogenous regulatory factor for mitochondrial energy-linked functions, as could be demonstrated for State 4 $\rightarrow$ State 3 transition (respiratory control ratio, coupling and associated transport). The implications for ATP synthesis are evident [see 2] and will be discussed. Under our conditions, there is no detectable free reduced glutathione inside mitochondria, but in contrast, there exists a large cytosolic pool of reduced glutathione [21, 22]. The known glutathione functions in mitochondria will be summarized and also related to chemiosmosis at a later point. The known glutathione functions in cells were reviewed by Sies [36]. Our results can be applied very well to these functions. But the important question remains, by what mechanism do mitochondria acquire their glutathione? In our opinion, there should be a connection beween the cytosolic- and mitochondrial-glutathione pools, either by a carrier or a pump (which includes glutathione itself). This assumption most probably explains the different concentrations of free oxidized and reduced glutathione obtained in mitochondria with the methods described in [21, 22] as well as used by us (incl. well coupled energized mitochondria) and make than an investigation of the mitochondrial glutathione transport activities necessary. Mitochondrial glutathione synthesis would be another possibility, but this option has been excluded [37].

The above results are of great importance for medicine as already mentioned. The functions of the human body are dependent on a certain "sulfer status" (e.g. thiol/disulfide balance), and any disturbance of this balance is accompanied by serious dysfunctions of the body. For instance, heavy metal intoxication (Cd^{2+}, Hg^{2+})[5, 38] may be the triggering factor for immunological dysfunctions, autoimmune diseases, collagen disorders, neurological disturbances, general metabolic disorders and cancer, among others. Our mechanism implies the same conclusion for other environmental chemicals.

Finally, we should emphasize the fact that only a few heavy metal ions or chemicals are able to

totally change body functions: impairment of the functions in one mitochondrion by a few heavy metal ions or chemicals [5, 6, 27, 38] is possible, which results in the dysfunction of the whole cell, which in turn may lead to certain diseases. Considering this fact, we must realize that the inability to detect low amounts of Hg, Cd-ions or chemicals possibly responsible for a disease, make it therefore impossible to diagnose the cause of the disease. Consequently, we need new biological test systems to evaluate the toxic limits of these compounds. A potentially reliable system for this purpose has been presented [39].

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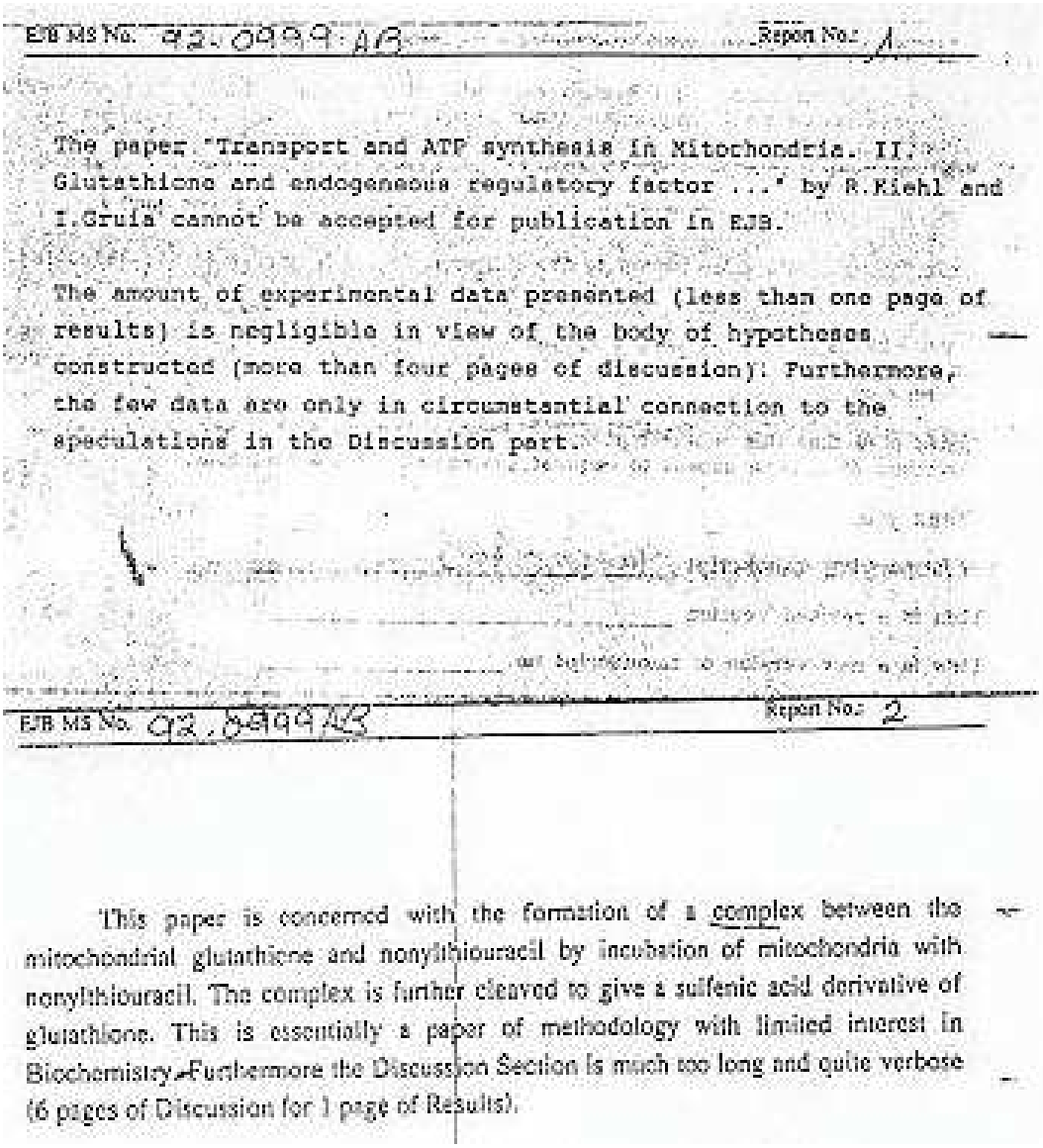

EJB MS No. 92.0999.AB Report No: 1

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

The amount of experimental data presented (less than one page of 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. 92.0999.AB 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).