Book-Chapter 7: **REVIEW** (1978-1984)

Glutathione: The essential factor for mitochondrial energy-linked functions.

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Dedicated to the 80th birthday of Prof. Dr. Th. Wieland. Died at the 24th Nov.1995

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2,4-Dinitrophenol accumulation across the P_i/H^+-symport system

The thiol reagent N'-[N''-n-nonyl-4-sulfamyolphenyl]-maleimide (NSPM) reacts with adenine nucleotide binding sites because of its similarity to the adenine moiety of the corresponding nucleotides [l]. NSPM is therefore a very potent and specific reagent for the inhibition or stimulation of various mitochondrial membrane-associated functions: ß-hydroxy-butyrate-dehydrogenase and ATP/ADP-carrier activities, P_i/H^+- symport, 2,4-dinitrophenol/H^+ (DNP/H^+) -symport, accumulation, as well as uncoupling were eliminated, whereas calcium and potassium transport were stimulated by this compound. Based on our findings we concluded that NSPM competes with phosphate binding in some nucleotide binding sites, thereby suppressing, for example, P_i and DNP binding and transport. The effect of NSPM on phosphate- or DNP-transport suggested the involvement of a regulatory factor in these transport activities (Table I):

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

Conditions	^{14}C-DNP, Pellet nmol/mg	Accumulation nmol/mg	%
95 µM ^{14}C-DNP	13.30 ± 0.33 (3)	6.63	100
95 µM ^{14}C-DNP, + 223 nmoles Triton X 100/mg[a)]	6.67 ± 0.10 (3)	0	0
95 µM ^{14}C-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 ± 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 (Kiehl R. 1996, Eur.J.Biochem. submitted): 1.22 mg mitochondria (from 2-3 weeks old normal feeded male wistar rats) had been incubated at 220C 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 KHepes, pH 7.4, either before or after 14C-DNP addition. 14C-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 14C-DNP and allowed to react for one minute.

The reaction was stopped by rapid cooling in an ice-salt bath of -1O^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^0 C and dissolved in 200 µ1 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-dodecylsultate. Aliquots were taken for 14C-DNP determination in a liquid scintillation counter.

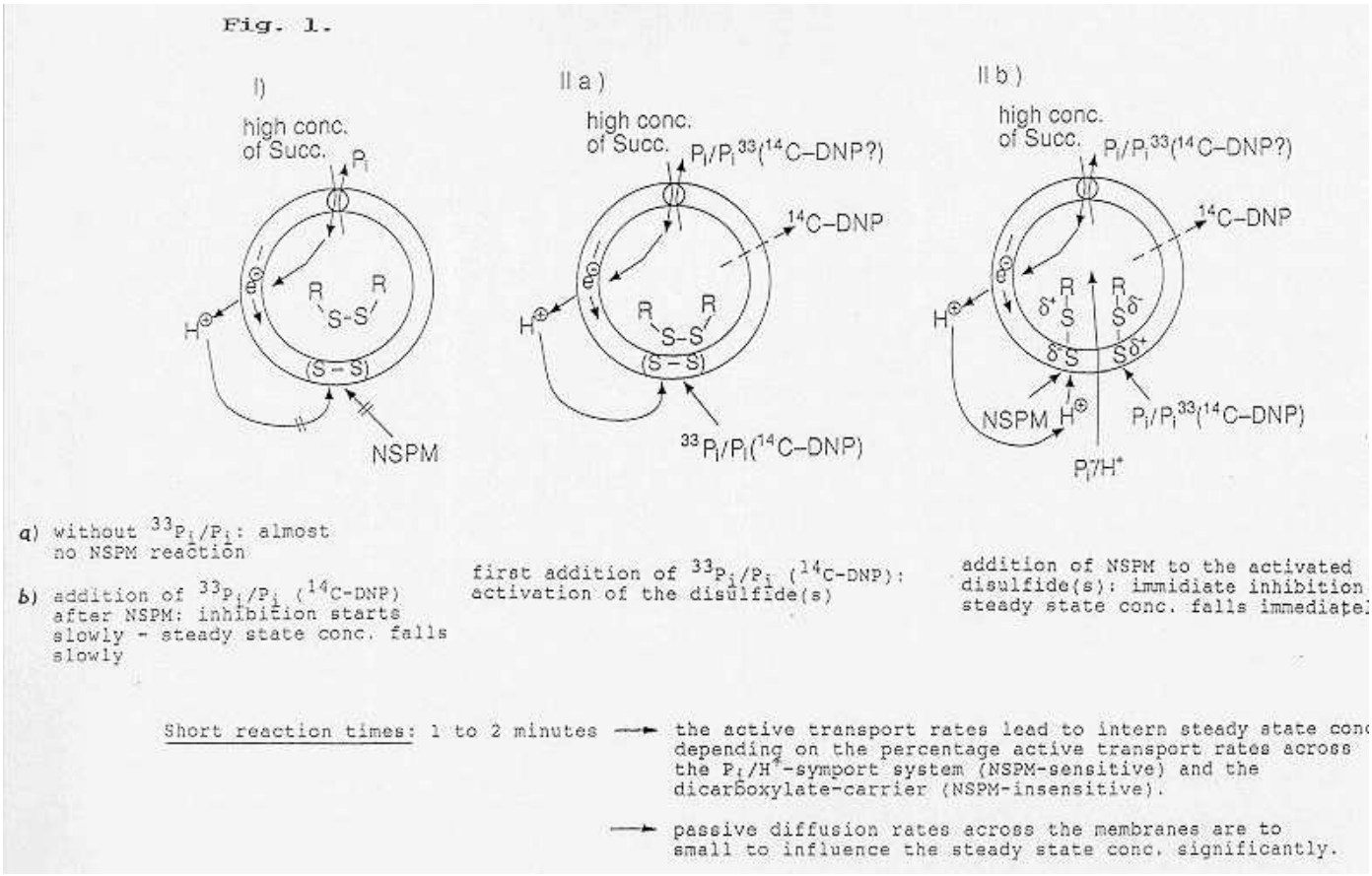


Fig. 1. Involvement of a regulatory factor in the P_i/H^+-symport system and DNP-accumulation as demonstrated with the thiol reagent NSPM.

[25]. The dithiol reagent Cd^{2+} shows essentially the same behavior as NSPM. The modulation of mitochondrial glutathione concentrations by active phosphate transport points to glutathione as a possible regulatory factor (Fig.1).

Another outdated but still importent explanation is that there is more than one kind of uncoupling action on mitochondria: DNP may uncouple by reaction to F_0, or NSPM (and Cd^{2+}) itself uncouples by reaction to the K^+ (Ca^{2+})-transport system and Triton makes holes in the membrane leading to uncoupling. CCCP (carbonylcyanide-m-chlorophenylhydrazone) and similar compounds presumably carry protons away from the membrane, thus collapsing the membrane potential. Uncoupling dicoumarol derivatives are important as anticoagulant agents. Their effect on blood cell energy metabolism and function as vitamin K-antagonists is therefore of eminent importance and these compounds play then not only a role in antithrombolytic prophylaxis, but also in other disfunctions.

On should keep in mind that mitochondria contain only a small percentage of phospholipids compared to phospholipid vesicles, and data obtained from vesicles are, for this reason, not comparable to data obtained from mitochondria as done by Pressman [6] and subsequently by others.

Glutathione: an endogenous regulatory factor for P_i/H^+-symport

The sulfenyl reagent n-nonylthiouracil reacts rapidly and specifically with sulfenyl groups in a lipophilic environment [7] and has therefore been used for detection of these groups in the mitochondrial membrane [8,9].

Incubation of well-coupled beef heart mitochondria with [35S]n-nonyl-thioracil ([35S]NTU) results in the isolation of [35S] thiosulfenic acid of glutathione. Calculation to 100 % inhibiting concentrations for State 4→State 3 transition and to 100 % inhibiting concentrations for DNP uncoupling by NTU demonstrates that almost the whole glutathione pool is involved (7-9 nmol total glutathione, GSH+GSSG, per mg protein) [10, 11]. Under these conditions, most of the glutathione was associated with the mitochondrial membrane and no free reduced glutathione could be found. It is important to note that phosphate modulates the bound and free concentrations of oxidized glutathione (Table II):

Table II. **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 -$2O^0$C (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).

A relay mechanism suggesting glutathione as an endogenous regulatory factor (presumably at a suitable receptor site) for the mitochondrial P_i/H^+-symport (and also possibly for other carriers) has been presented [2-5].

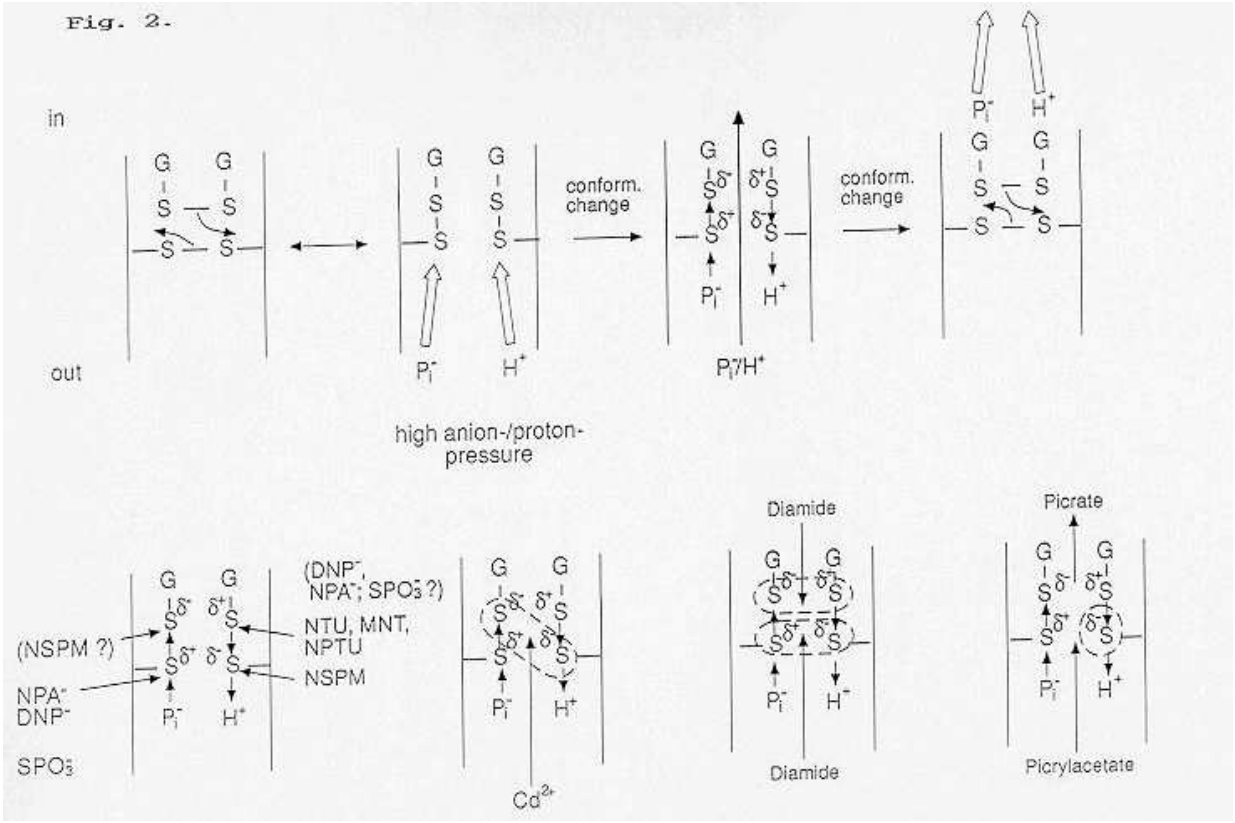


Fig. 2. Glutathione dependent P_i/H^+- symport system and the effects of various thiol- and sulfenyl group reagents.

The effects of various sulfenyl- and thiol-trapping compounds, as well as the high energy compound picrylacetate in this mechanism are discussed in Fig. 2. This figure shows that the P_i/H^+-symport system is dependent upon endogenous oxidized GSH. Phosphate and a proton from outside the mitochondria activate the two neighboring disulfide bridges in the carrier enzyme [12]. After P_i/H^+ movement across the channel and release into the mitochondria, oxidized glutathione may be liberated for a short time from its receptor site based on the reversibility of the proposed relay mechanism. Rebuilding of protein glutathione-disulfides, probably by conformational changes and activation of the disulfides by P_i/H^+ , complete the transport cycle. NSPM reacts with the activated formed protein thiol group (and possibly glutathione), and the uncoupler cadmium [13] complexes the two negatively charged sulfer atoms thereby inhibiting phosphate transport [14]. Mersalyl [12] probably acts similarly to Cd^{2+}. NTU, MNT and NPTU trap the glutathione sulfenyl group and the uncoupler 2-azido-4-nitrophenol (NPA) [15], as well as DNP, possibly trap the protein (and glutathione?) sulfenyl group. SPO_3^- , in contrast to nonylthiophosphate, inhibits phosphate transport [8] presumably by sulfer-sulfer exchange in the activated disulfides. There is competition between NPA and NSPM, as well as between NPA and NPTU [16] . NPTU (in addition to the other sulfenyl group reagents) does not influence the phosphate transport activities. It should be mentioned that the specific NPA-binding is in the concentration range of the phosphate sensitive GSSG and the

phosphate carrier (Table II). Diamide [17] crosslinks the activated disulfides (dithiothreitol sensitive) and blocks NSPM reaction [18] (Table IV).

Low amounts (20 to 25 μM) of the high energy compound picrylacetate [19] inhibit dithiothreitol and DNP sensitively State 4 → State 3 transition at sites II-III, but P_i/H^+- symport is only marginally affected. This effect of picrylacetate is very similar to that of NTU. NSPM suppresses RCR and P_i/H^+- symport equally well in contrast to N-ethylmaleimide, which inhibits phosphate transport much more easily. As in the case of NSPM interaction, acetylation by PA requires activation of the involved disulfide bridges by P_i/H^+ before any reaction can take place: phosphate accumulation or uptake is significantly inhibited (succinate/phosphate exchange is not affected) and, at the same time, picrate is released into the mitochondria in the presence of phosphate anions. Ca^{2+}- induced active swelling (indicative of Ca^{2+}- induced K^+ uptake and release) is eliminated, demonstrating a blockage of Ca^{2+} binding by acetylation of the involved functional groups (Table III a and b):

Table III a. **Effect of picrylacetate (PA) on some mitochondrial functions[1]**

function	PA/μM	addition	inhibition	stimulation
				respiration/%
1) state 4 → State 3 transition		--	85-95	
a) sites I-III: ß-hydroxybutyrate,		2,5 mg BSA	87-96	
1,8 mg mitochondria (RCR >5)	3-5	15 mM DTE	86-94	
		110 μM DNP	100	0
		6 μM CCCP	100	0
b) sites I-III: glutamate/malate,		--	86-95	25-30
2,9 mg mitochondria (RCR >10)	40-50	3 mg BSA	85-96	10
		25 mM DTE	45-55	
		110 μM DNP	86-95	13-21
c) sites II-III: succinate/rotenone.		--	90-95	
1,2 mg mitochondria (RCR> 4)	20-25	2,5 mg BSA	90-95	
		17 mM DTE	17-20	
		110 μM DNP	90-95	45-50
		1,7 mM arsenate	100	
		0,57 mM Ca^{2+}	100 (after a lag phase of ca.15 seconds), DTE insensitive	
	50-55	--	at State 4 arrested, insensitive toward DNP	
2) ATPase activity, 0,27 mg mitochondria	40-50	--	--	2-5 fold (oligomycin sensitive
t1/2 of H+-disappearance,				
02-Pulse, succinate,	40-50	--	60-70	
rotenone, 8 mg mitochondria	150-200	--	5	6-7 fold (time dependent)
3) Ca2+-induced active swelling, succinate,				
rotenone, 1,2 mg mitochondria	30	--	100 (DTE insensitive)	

Table III b

		Inhibition	Inhibition		
4) Ca2+-, Pi-uptake, picrate release to the inside and swelling, succinate, rotenone, 2,5 mg mitochondria	PA/µM	Ca/%	Pi/%	picrate/µM	swelling/%
a) PA addition after phosphate	30-40	no effect	53(36)	8	0
b) PA addition before phosphate	30-40	90	15(11)	0-1	1-2
5) acetvlation by [3H]PA: succinate, rotenone (RCR > 4)		(3H] acetyl bound/nmol/mg			
		30 kDA	10 kDa, extractable by acidified chloroform - methonol (2:1)		
	5	NS	NS		
	20	ca. 0.2	ca. 0.2		
	30	0.34	0.3		
	40	0.43	0.4		
	50	0.65	0.61		
	200	2.20	2.25		
decoupled (RCR < 2)	200	ca. 0.2	2.1		

RCR = respiratory control ratio, BSA = bovine serum albumin, DTE = dithioerythritol, DNP = 2,4-dinitrophenol, CCCP = m-chlorocarbonylcyanidephenylhydrazone, NS = not signifikant. Lit: [19] and [1)] in preparation

Labelling of well-coupled mitochondria (RCR > 4, succinate) with 30 µm ^{3}H-picrylacetate results in 0.30 to 0.34 nmol ^{3}H-acetyl/mg mitochondria into 30 kDa polypeptides and the proteolipid, respectively (Table III b). The capacity of both bands is about 2.10 to 2.25 nmol ^{3}H-acetyl/mg mitochondria and 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 (Table III b). We conclude, therefore, that in decoupled mitochondria the proposed relay mechanism does not work. There is presumably no activation of the involved disulfide bridges, and thus consequently no intermediate covalent binding to the 30 kDa polypeptides (incl. ca. 0,5 nmol/mg phosphate carrier, Table II) is possible (Table III b, 4 a, 4 b and 5). PA should then acetylate only mitochondria with activated bound glutathione (Fig. 2) and thereby release picrate to the inside (Table III b, 4).

Glutathione molecules may or may not be reduced by the NADPH specific glutathione reductase (depending on available NADPH). A rough calculation for well- coupled mitochondria (succinate as substrate, NADH and NADPH = 0) 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 eliminating RCR. Involved is the 30 kDa P_i/H^+-symport system. PA acetylates RCR dependently and sensitively to DTE and DNP 30 kDa membrane proteins, including the P_i/H^+- symport system, thereby eliminating RCR. Conclusion: RCR is dependent on glutathione bound to 30 kDa membrane proteins. DNP, its analogs, lipophilic NTU, and its nucleophilic analog compounds, as well as lipophilic NSPM, and most likely PA, share the same reaction site on these proteins (e.g. phosphate carrier). This could be shown for most compounds by performing competition experiments (Table IV):

Table IV. **Effects of uncouplers on 2-azido-4-nitrophenol binding to mitochondria**

Uncoupler (sites II to III)	Dissociation constants Ki/µm	Inhibition of "high affinity binding"/%	Inhibition of "low affinity binding"/%
NPA[a,b,c]	3	-	-
NPA			
+ 58 nmoles NSPM/mg[b)]	8,4	0	ca. 40
+ 69 nmoles NPTU/mg[c)]	15,4	0	0
+ 33 µM DNP[a)]	[ca. 3,8][g)]	28	ca. 70
+ 33 µM PCP[a)]	[ca. 4,1][g)]	36	n.d.
+ 33 µM CI-CCP[a)]	[ca. 4,8][g)]	59	n.d.
+ 1 µM S-13[a)]	[ca. 3,9][g)]	30	n.d.
+ 3 mM NaN_3	[ca. 4][g)]	33	n.d.
NPA[a,b,c)]	3	-	-
+ 1mM picrate[d)]	3	0	-
NPA, ETP_H[d)]	33	-	-
+ 1 mM picrate[d)]	-	100	-

	Inhibition of covalent binding to the Pi/H+-symport system/%	nucleotide carrier/%
20 nmoles NSPM/mg + 230 nmoles Diamide/mg[e)]	75	0

NPA	Inhibition of covalent binding to ~30 kDa peptides/%	the α-subunit of F/%
+ 33 µM DNP[f)]	66	93
+ 1 mM picrate[f)]	66	78

a) W.G. Hanstein and J. Hatefi (1974) J.Biol.Chem. 249, 1356-1362
b) R. Kiehl (1980) 1. EBEC, Urbino, 113-114
c) R. Kiehl (1980) 1. EBEC. Urbino, 113-114 and R. Kiehl (1996) manuscript in preparation
d) W.G. Hanstein and J. Hatefi (1974) PNAS 71, 288-292
e) R. Kiehl (1980) FEBS Letters 109, 280-282
f) H. Drosdat, R. Kiehl, E. Hoffinann-Posorske, S. Kordt and W.G. Hanstein (1982) 2. EBEC, Lyon, 19-20.
g) calculated from the high affinity binding data
n.d. = not determined

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. If so, this mechanism would then consequently imply new pharmacological treatments of diseases. Most of the results summarized by Ziegler [20] should be viewed with regard to our proposed mechanism.

Under the influence of glutathione, the other carriers presumably work similarly to the proposed P_i/H^+ symport system. Coupling between the carriers and the H^+- pumping ATPase could be shown [13]. Factor B and similar preparations (47, 42, 30, 11-12 kDa) [21-23] 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 particles supported energy-linked functions [24] (probably via contaminating oxidized thiols). Such results support our proposed model. The cation (proton)/anion pressure accompanied by membrane transitions (swelling and contraction) [13] regulate, therefore, the activity of respective carriers, of transhydrogenase and ATPase.

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 clear [see 8].

These results have been connected to cellular events and their importance for medicine emphasized (pharmacology and toxicology) [2-5, 25] . The functions of the human body are dependent on a certain "sulfer status" (e.g. thiol/disulfide balance) , and any disturbance in this balance leads to serious disfunctions of the body. For instance, heavy metal intoxication (Cd^{2+}, Hg^{2+}) may be the triggering factor for inflammations of the skin, immunological disfunctions, autoimmune diseases, collagen disorders, neurological disturbances, general metabolic disorders, cancer and AIDS. Our mechanism implies the same conclusion for other environmental chemicals [3, 5, 14, 26-31].

Impairment of the functions in one mitochondrion by a few heavy metal ions or chemicals [14, 15, 26] can result in the disfunction of the whole cell, which may lead to certain diseases. 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 [31] and described [30] .

ATP synthesis on the P_i/H^+-symport system with oxidized glutathione as a catalyst

In 1970, Painter and Hunter showed [32] high amounts of ATP formation in a model system containing cytochrome c, NADP+, GSH, GSSG, Pi-, ADP and $MgCl_2$. The disulfide GSSG was described as the essential catalyst. Wieland and Bäuerlein formulated in 1968 [33] a mixed anhydride of sulfenic and phosphoric acid, $RSOPO_3H_2$, to explain the formation of ATP from ADP and Pi by oxidation of a thiol with bromine in anhydrous pyridine.

During our attempt to elucidate the mechanism of oxidative phosphorylation in mitochondria, we came to the conclusion that an activated disulfide in the membrane had to be involved [8, 9]. We proposed a phosphate reaction with ADP on this activated disulfide in a synchronous mechanism without the real build-up of a sulfenylphosphate intermediate [8].

A mechanism for mitochondrial ATP synthesis on the 30 kDa P_i/H^+ symport system, with oxidized glutathione as a catalyst, has been presented [2-5, 25, 34] (Fig. 3). Fig. 3 describes our mechanism of phosphate/proton symport (Fig. 2) completed for ATP synthesis. The entire mechanism would then be correctly described as "glutathione-dependent proton/phosphate symport driven ATP synthesis". P_i- and H^+ activate the disulfides. A further H^+ from the outside, as well as ADP- from the inside, lead to the transfer of ~ $P_i^{\delta+}$ (synchronous build-up of a sulfenyl phosphate) onto the nucleophilic oxygen of ADP^-. ATP^{2-} and 2 H^+ are released to the inside. P_i and H^+ , again from the outside, free H_20 from the activated disulfides (sulfenic acid) for release (presumably) to the inside, allowing a new reaction sequence to start. This cycle is

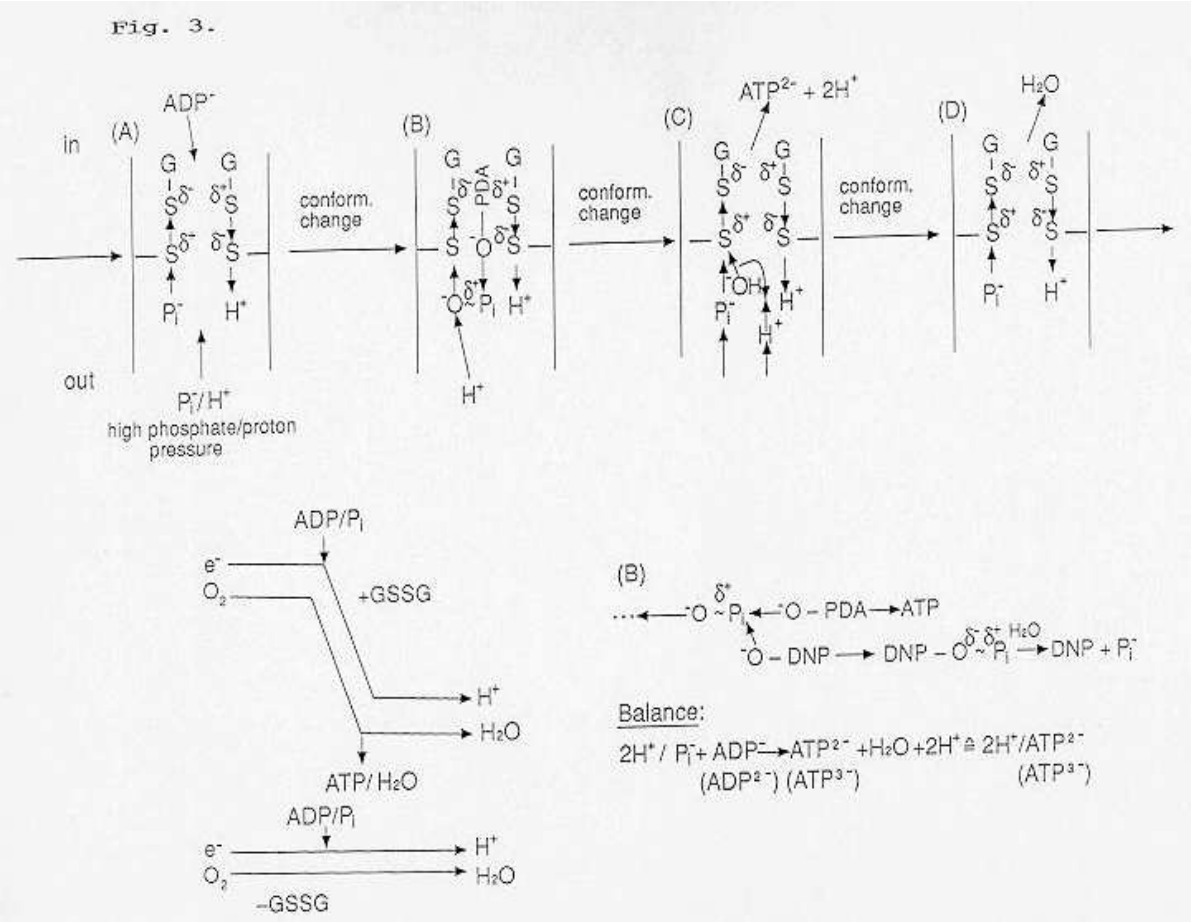


Fig. 3. Coupling between ATP synthase, membrane bound ATPase (F_0F_1) and ATP/ADP-translocator.

repeated as long as ADP is present. Otherwise, the system works as a proton/ phosphate symporter. The result: 2 protons are pumped from the outside to the inside during synthesis of one ATP.

Mitochondria are decoupled without GSSG and no ATP is synthesized: RCR = 1. Maximal coupling is achieved with sufficient GSSG, and high amounts of ATP may be obtained: RCR = ∞ (Fig. 3).

DNP and its analogs are bound and transported by our system. These compounds compete with phosphate at the specific binding site (Figs. 1,2 and 3a, Table IV), and they may lead to inhibition of ATP synthesis by blocking ADP reaction (Fig. 3b). The build-up of phosphorylated compounds by competition with ADP on the sulfenyl phosphate (Fig. 3b) is not very likely since the affinity of ADP should be much higher. On the other hand, one should consider that the compounds are rapidly taken up (Table 1, Figs. 1 and 2) and not available for this kind of reaction; a reaction which at sufficient extern steady state concentrations of the compounds, and thus also phosphorylated compounds, as well as energy dissipation by their hydrolysis inside mitochondria, would lead to uncoupling. A sufficient extern steady state concentration may be obtained by release of the accumulated anions (Fig. 1) [35]. Reaction of the compounds to the sulfenic acid (Fig. 3c) should result in their transport.

Low concentrations of the phosphate analog compound thiophosphate (0,3 mM or 280 nmoles/mg mitochondria) [8] block phosphate transport and ATP synthesis, most likely by functioning as a suicide inhibitor: This sulfur compound substitutes phosphate on the activated disulfide (Fig. 3a) leading to a stable sulfer analog of the "masked" sulfenyl phosphate (Fig. 3b). ATP synthesis is then blocked at this step (Fig. 3b) because P_i/H^+ and, of course, H_20 are not able to release the inhibition.

Anyone discussing oxidative phosphorylation should realize by now why no one has been able to detect a covalent intermediate in ATP formation - regardless of which method was used. Based on the results and mechanism described, we will attempt to establish more direct proof of sulfenylphosphate formation, although sulfenylphosphate participation should already be proven.

The described mechanism of ATP synthesis poses the question: What about the proton pumping ATPase (F_0F_1)? As discussed [8],

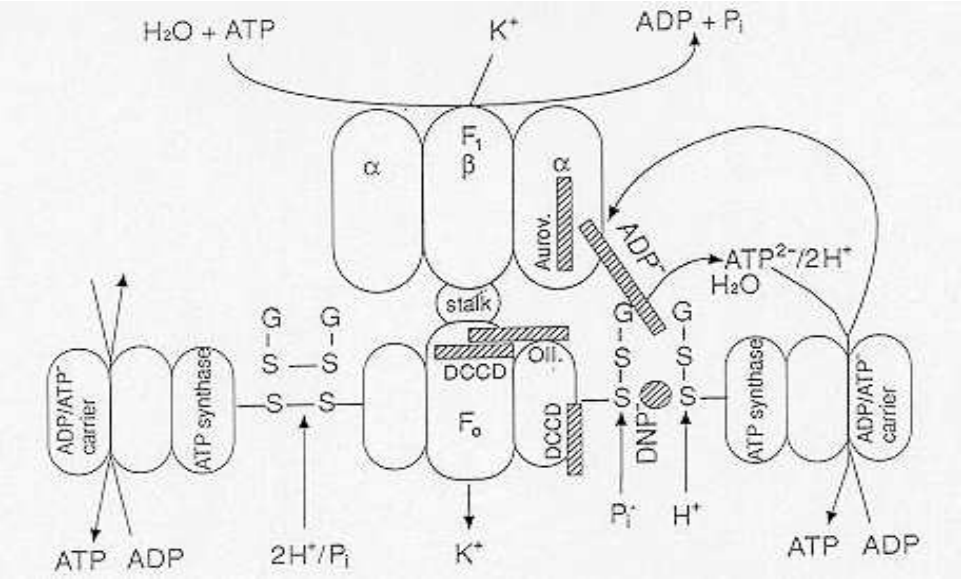


Fig. 4. ATP-synthesis in the glutathione dependent P_i/H^+ symport system.

the real function of this complex may not be the synthesis of ATP, but rather that mitochondrial F_0F_1 is under normal physiological conditions nothing more than a K-pump (Fig. 4).

Aurovertin and oligomycin, two inhibitors of ATP synthesis, bind at F_1 and F_0, respectively, demonstrating therefore a direct coupling between ATPase and ATPsynthase (Fig. 4) . Aurovertin may block a passing of ADP by F1α to the ATPsynthase and thereby also enhance DNP effects [36] . Coupling between F_0 and the phosphate/arsenate(Ca)-transport using oligomycin has been shown [14] and may also explain the oligomycin- and aurovertin- sensitive uncoupling by arsenate [37]. The synthesis of ADP-arsenate [38], a compound hydrolyzed inside the mitochondria, should be blocked. The cyclic movement of arsenate should normally complete the uncoupling mode in our proposed mechanism (Fig. 3) . The different sensitivities to inhibitors (NSPM/oligomycin-aurovertin) during the uncoupling action of DNP or arsenate are then best explained by DNP or arsenate substituting ADP or phosphate, respectively. The primary target of
another ATPase inhibitor, namely dicyclohexylcarbodiimide, for elimination of RCR in mitochondria are most probably the phospholipids **(** ~3 to 3,5 nmol/mg mitochondria) [8].

Our hypothesis represents the first description of a proton- driven build-up of high energy intermediates (activated disulfides, sulfenyl phosphate) , and the resulting phosphoryl transfer or transport activities. The continuing debate about Mitchel's chemiosmotic theory [39] is fully resolved by our formulated hypothesis [8, 9] (Figs. 5-7).
It should be added that our presented hypothesis in no way contradicts the isotope exchange data of Boyer [40], or the results of others. One has to be aware of the coupling between ATPsynthase and ATPase, and needs to compare the data with the SR-Ca^{2+}-pump-ATPase (ATP synthase) [41]. The nucleotide binding is dedendent not only on Mg^{2+}, but also more importantly on K^+ in an ATP regenerating system (PEP/PK) [42], K^+ modulates bound ATP concentrations [36] , which resembles the Ca^{2+}-pump of SR. The presented methods [36, 41, 42] are ideal for investigation of the involved K^+- pump activity. One should keep in mind, however, that Boyer's experiments were done at high concentrations of cations (incl. K^+) [40].

As mentioned, the ATPsynthase and ATPase are coupled under normal physiological conditions. ADP from cytosol is able to disrupt this coupled system. Masked sulfenylphosphate is not stable and under high phosphate and proton pressure results in immediate formation of ATP in the presence of ADP (α-sub. or cytosol). Sulfenylphosphate involvement is thus not seen under normal conditions, and therefore is not comparable to other high energy intermediates. Some modifications are required in order to trap this highly reactive "intermediate" (as described above by, for instance, thiophosphate). There is of course such a relationship between P_i/H^+ /ADP and ATP/H_20, but no stoichiometric relationship between former compounds and the sulfenyl groups or the ATPase molecules involved. Consequently, there is no contradiction of Jagendorf's experiments on chloroplasts [43]. The hypothesis and corresponding results have already been presented [8, 14, 16, 18, 19, 25, 34 - 36, 42, etc.] .

K +- Transport

Our experiments also prove that, under normal physiological conditions, the oligomycin-sensitive F_0F_1-ATPase actually represents a valinomycin- and oligomycin-sensitive ATP-driven K^+- pump [2-5, 25] (Fig.1). The synthesis of ATP on the glutathione-dependent P_i/H^+- symporter is thus indirectly proven (Fig. 3) . Further experiments demonstrate that mitochondria contain an energy-driven K^+/H^+-antiporter. This antiporter is sensitive to NSPM, Cd^{2+} , Mg^{2+} , and Ca^{2+} in addition to other various important compounds [25] , and relates to the K^+/H^+ antiport activities of cytochrome c oxidase [44] . New results show that crystallized beef heart cytochrome c oxidase contains one Mg^{2+}- and one Zn^{2+}-binding site, respectively [45]. a fact which has already been demonstrated in the eucaryotic enzyme [46,47] . The Zn^{2+}-ion is located at subunit Vb [48] in the cysteine-rich surroundings of a peripheral peptide area near the matrix side. Since Zn^{2+} binding may easily be disrupted by the presence of $Cd^{2+}(Hg^{2+})$-ions, and since Mg^{2+} is involved in the regulation of Ca^{2+}-binding and vice versa, the interplay of the different cations regulates the K^+ / H^+-antiport activities (discussed below). Modulations in K^+/H^+- antiporter activity lead to a corresponding swelling or contraction of the mitochondrial matrix space by the osmotically active K^+-ions, and indirectly to a modified oligomycin-sensitive (valinomycin, NSPM, Cd^{2+}, Ca^{2+}) or ruthenium red (Cd^{2+} , Ca^{2+}) -sensitive ATPase activity.

Valinomycin is able to eliminate K^+- gradients in mitochondria, which leads to K^+- cycling and a high proton gradient (by stimulation of the K^+/H^+-exchange and the ATPase), together with stimulated respiration (uncoupling) . This is not the case for submitochondria. If one considers all the results available for the explanation of this phenomenon (including binding of valinomycin to the F_0-part of the ATPase), one reaches the only possible conclusion: Valinomycin stimulates the ATPase activity (K^+-pump) of mitochondria sensitive to oligomycin from the outside (Figs. 5 and 6) . For these measurements, all the ions present (incl. Tris+) are important. It should be added

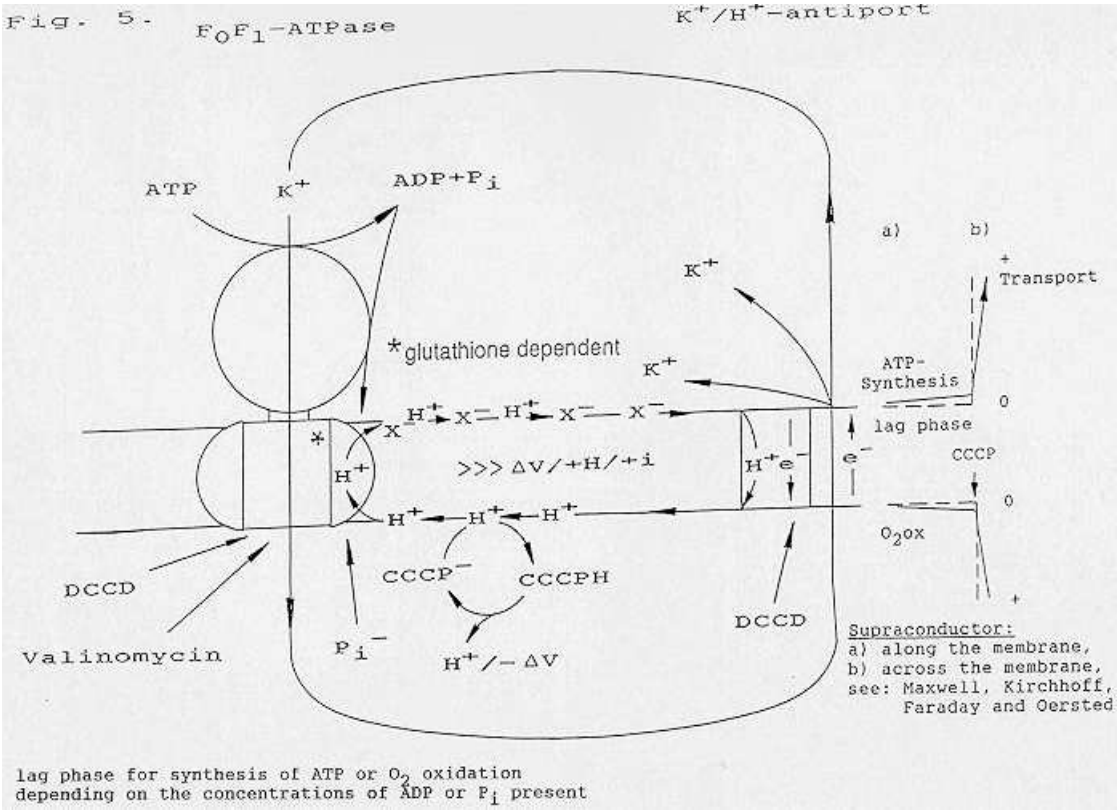


Fig. 5.
Electrophysiology of mitochondria: For simplicity take a disc capacitor as the comparing unit and the thickness of a mitochondriotic membrane as the disc gap transmitter. Δ V = voltage between the discs, H = magnetic field, i = current.

that the movement of valinomycin through pure phospholipid membrane is already very difficult, and could be considered almost impossible through mitochondrial membranes. Pressman's final conclusions [6] are mainly derived from phospholipid bilayer measurements and different from his original ones [49, 50].

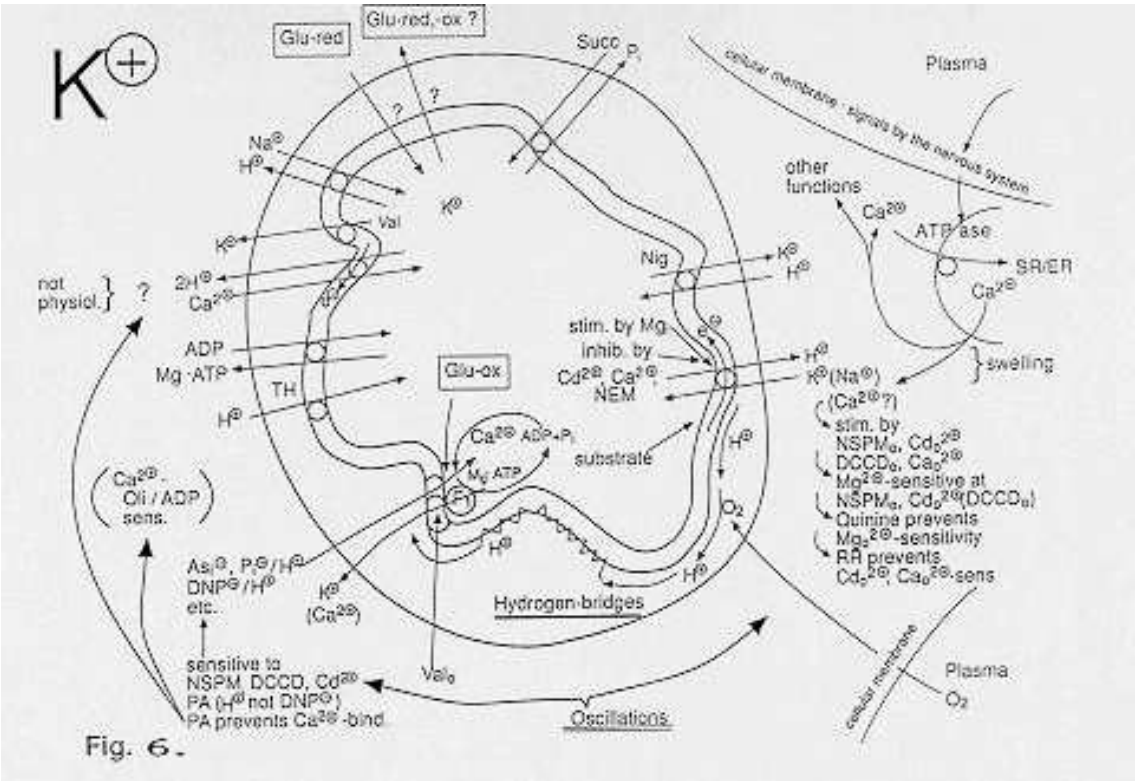


Fig. 6. Summation of various mitochondrial energy-linked functions. Location and effects of energy transter inhibitors and uncouplers on K^+/H^+-antiporter, K^+- pump and P_i/H^+-symporter. Mitochondrial coupling to the nervous system. Glu = glutathione, TH = transhydrogenase, Oli = oligomycin, PA = picrylacetate, Val = valinomycin, RR = ruthenium red, Nig = nigericin, SR/ER = sarcoplasmic/ endoplasmic reticulum.

Why should all the various microorganisms produce their highly complex "ionophores" solely for phospholipid bilayer membranes if their antibiotic activity is directed against systems containing only few or no phospholipid bilayers? A synthesis directly directed against the ion transporting units is much easier to imagine.

The cyclic decapeptide antamanide, for instance, has been described by Pressman as a neutral "ionophore" because he found stimulation of K^+- uptake by mitochondria and complexation of $Na^+ > K^+$ by this compound [6] . However, studies by Ovchinnikov [51] demonstrate that the biological action of antamanide is not connected to ionophoric action, and in support of this finding, photoaffinity labelling studies [52] demonstrate specific binding of this compound to membrane polypeptides. Our results on "protonophoric" uncoupling agents match and correspond perfectly to the experimental outcomes of "ionophoric" compounds.

Normally a model system has to be proved in every aspect and disregarded if not valid in all aspects. In the case just described, it is obviously easier to keep a wrong model than to perform difficult new experiments. We feel that everyone dealing with the current problems in the fight

against bacteria, fungi and viruses should go back about 30 years to find the real reasons for the problems we are facing today.

K^+ /H^+-antiporter (respiratory activity), K^+- pump (ATPase activity) and P_i/H^+- symporter/ATP synthase are linked together in versatile, energy-driven K^+/H^+-cycling, swelling/contraction phenomena and oscillations. This system is controlled by 0_2 and the free Mg^{2+} - and Ca^{2+} - concentrations in the cytosol of cells, and is most likely also able to handle "abnormal" Ca^{2+} concentrations (Fig. 6). A high physiological K^+- qradient between cytosol (K^+ = high, ~ 175 mM) and matrix (K^+ = low, ~ 1 mM) is established and maintained by the system (Table V):

Table V. **Distribution of various ions between plasma, cytosol and mitochondria**

Ion	Plasma/mM[a)]	Cytosol/mM[b)]	Mitochondria/mM[b)]
Na	150	10	< 0.1 – 10[d)]
K	5	175	< 2 - 175
Mg	2	l0[c)]	> 10[e)]
Ca	1 - 2	1 - 10 μM	< 2 - >30
Pi	1 - 2	5 - 10	< 2 - > 30
Cl	110	3	nd
$S0_4$	0.5	10	nd
ADP	« 0.1	0.7	6 - 8
ATP	« 0.1	4.0	5 - 7
Glutathione red.	< 0.1	ca. 1 - ca. 10[f)]	< 0.1 - ca. 10
Glutathione ox. nd	0.5	< 0.4 - > 3,2	

a) Labor und Diagnose (1988), 3. Auflage, Thomas, Ed., Med. Verlagsges., Marburg.
b) a) plus own values; Elbers et al (1974) Hoppe-Sevler's Z. Physiol. Chemie 355: 378-393; Klingenberg and Heldt (1982) in Metabolic compartmentation, Sies, Ed., Acad. Press, London, 101-122; Wahlländer et al (1979) FEBS Letters 97: 138-140.
c) free plus bound. d) in the presence of K+ as calculated out of the K+ values, the natural Na-reservoir in the cell is the nucleus.
e) above ATP conc. f) Meister and Anderson (1983) Am Rev Biochem 52: 711-760.

Under normal physiological conditions, no protons are detectable in the bulk phase, but are moving along the membranes via hydrogen bridges [see also 53] (Figs. 5-7): <u>The entire system is dependent on oxidized and reduced glutathione</u> (see Figs. 3 and 4) - To gain more insight into this system, one should read an introduction to the theory and equations of Maxwell and Kirchhoff. Further studies based on Faraday's and Oerstedt's equations should lead to the complexity of mitochondrial electrophysiology and its influence on memory and thinking.

A high noradrenaline/adrenaline-ratio in plasma leads to low glycolysis rates and, as a consequence, lowered ATP concentrations in the cell. ADP, P_i and free Mg concentrations in the cytosol, as well as ATP synthesis [54, 55] and oxidized glutathione concentrations in mitochondria, are concomitantly raised, NADH and NADPH concentrations decreased, but the reduced glutathione concentrations in the cells are lowered. The changed ATP/ADP - or Ca/Mg - ratio respectively, determines the respiratory rates in this case (Table V). Our results and

conclusions are based on extrapolating summarized results for mitochondrial oscillations [56] and are one way to explain the molecular mechanism leading to these phenomena.

Longer exposure of cells to noradrenaline leads to constantly lowered cAMP-concentrations and weakens, thereby, the immune response [30] by changing arachidonic acid, prostaglandin, leukotriene. and cytokine, etc. concentrations.

A rise in cytosolic Ca by extracellular signals leads to muscle contractions, myofibril mobility, and stimulation of respiration, etc., with liberation of ADP, P_i and Mg, resynthesis of ATP by creatine phosphate [55] , and rebinding of Mg. As a consequence, we have a constantly changing Ca/Mg ratio with oscillating mitochondria. In this system the changing Ca_{cyt} determines the Ca/Mg - or ATP/ADP-ratio respectively (Table V).

At concentrations of Cd^{2+} above 10 μM**,** both ions were taken up. But Cd^{2+} concentrations less than 10 μM, prevent Ca^{2+} (P_i-) accumulation by binding to the P_i/H^+- and Ca^{2+}-binding sites. Taken together, Ca^{2+}-ions behave similarly in many respects to K^+- ions (uptake, swelling/ contraction, ATPase, etc.). This is not surprising since the Ca^{2+}-concentration in the cytosol of our cells under normal physiological conditions is only about 1 to 10 μM (Table V), and the normal Ca^{2+}-reservoir and regulator is ER (SR) . It was not necessary for mitochondria to develop a similar system, and their K^+- cycling system may therefore also be able to handle high "abnormal" Ca^{2+}-concentrations, as well as Ca_i^{2+}/P_i (Fig. 6).

The results presented imply a direct connection and regulatory function between the nervous system (brain) and mitochondria (body and brain). Our body temperature may then be regulated by mitochondria via nerve signals from the brain. The normal substrate oxidation and 0_2 - uptake/-reduction of our body (anorexia/dystrophia) is directly linked to the nerve signals of our body, essentially to the brain. Substrate overload leads to a rise in cholesterol, hormone and vitamin (D3) concentrations. As a result, arteriosclerosis [57] , heart attack, and cancer are the most commonly developed diseases.

Oxygen deficiency or even depletion in our cells leads not only to an imbalance in the Na^+ /K^+- concentrations across the cellular and mitochondrial membranes, but also to osmotic imbalances resulting in H_20 decreases which damage mitochondria and cells, and as a consequence, result in the build-up of edema and probably epileptic manifestations [58] during anoxia/hypoxia. Shock and dilusion with coma, or even death, represent additional possible effects [2, l, 25]. These examples are just some of the possible interactions between nervous system and mitochondria. Mitochondria are in the true sense of the word the "hearts" of our cells.

The technical application of bioenergy production (via molecular biotechnology) is possible based on our results. Mitochondria are the ideal oscillators and predestined to mutual coupling with the plasma membranes thus influencing functions in the brain such as memory and thinking. New materials should then lead to the construction of thinking Computers.

Thermoregulation in man

The system described is also responsible for the thermoregulation of our body [25, 58]. The mechanism may best be described in Fig. 7. This steady state flow system releases heat (**q**) and the temperature is permanently raised (**ΔT**) by the cyclic hydrolysis/ synthesis of ATP and the concomitantly cyclic release/binding of Mg . The released heat is constantly distributed throughout the entire body by the oscillating mitochondria, as well as the pumping heart, and is used up by the normal body functions.

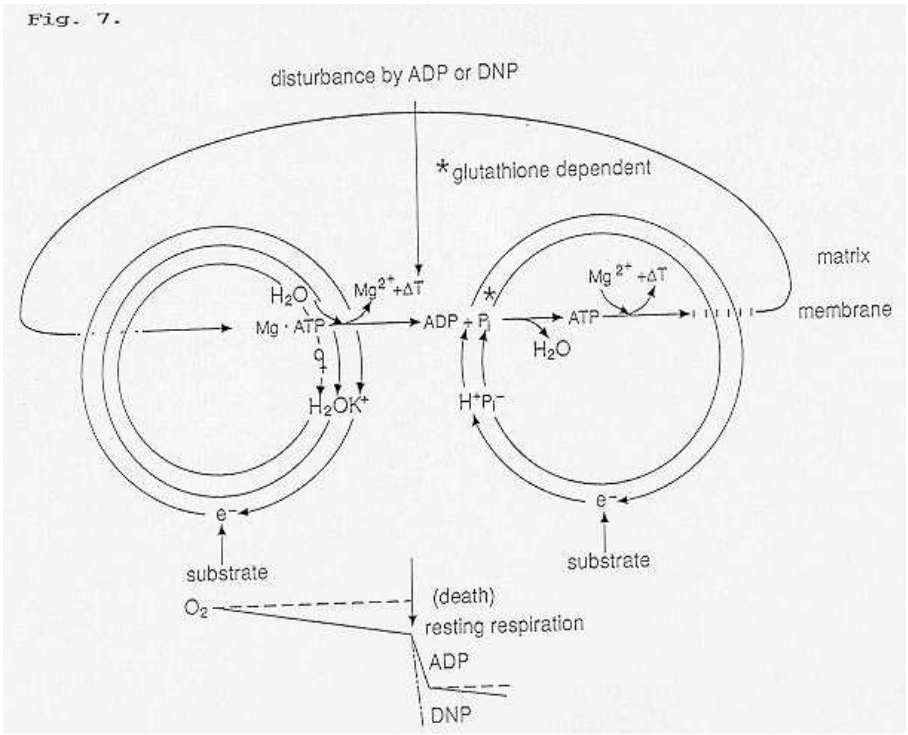


Fig. 7. Thermoregulation in mitochondria: Δ T = temperature change, q = heat exchange, DNP = 2.4-dinitrophenol.

The mechanical and thermal movement of the bulky water phase may also be responsible for muscle contraction and relaxation. The volume and enthalpy changes at the myosin - actin interaction sites [59] were not sufficient to explain the sliding events alone. Disturbances of this system are normally compensated for by lower/higher respiration rates. For clarification, one should study the theory of Carnot.

Acetylsalicylic acid used for pain relief (e.g. headaches) and for lowering of high temperatures, presumably acts via the nervous system (brain) on the center responsible for initiation of pain and thermoregulation. Its involvement in the arachidonic acid metabolism by acetylation of the prostaglandinendoperoxid-synthase with inhibition of associated cyclooxygenase activity and concomitant stimulation of the lipoxygenase pathway with build-up of LTC_4 by glutathione-S-transferase, makes it valuable as an antiinflammatory drug. Antipyretic malaria therapeutics such as chinine may change the Ca^{2+}/Mg^{2+}-binding to mitochondria [2, 4, 25] and in this way regulate the body temperature.

Other systems

Eukaryotes: Other eukaryotic systems may be similarly regulated as the one described above, although the parasitic amoeba Entamoeba histolytica lacks mitochondria altogether [60]. In this system, other enhanced energy-producing pathways should take over any necessary thermoregulation. Mitochondria are most closely related to purple photosynthetic and related bacteria [61]; a group that includes the gram-negative facultative anearobes and aerobes which produce glutathione. This suggests that eukaryotes may have acquired glutathione metabolism at the same time they acquired mitochondria, and that glutathione is thus essential to mitochondrial function in eukaryotes [60].

Bacteria: Glutathione and soluble thiol content have been investigated in a broad spectrum of bacteria [62] . Significant amounts of soluble thiol was present in all cases. The thiol compound was glutathione in most of the gram-negative bacteria, but not in most of the gram-positive bacteria studied. Glutathione was absent in four anaerobes and one microaerophile, but was present in blue-green bacterium. The synthesis of ATP in all these systems may be performed in solution (as described) by using glutathione oxidized [32] or other disulfides [62].

Glutathione functions in cells

In the cytosol: The functions of glutathione in cells have been extensively discussed and described [63-69]. These functions are mainly performed and made possible by the glutathione synthesizing system, γ-glutamyl transpeptidase (amino acid transport), glutathione-S-transferase (detoxification), glutathione peroxidase and glutathione reductase (peroxide metabolism). Important is the radioprotective action of glutathione [70]. On the other hand, glutathione seems not to be directly involved in the redox potentials responsible for development of atopic eczema, cancer and AIDS [4, 30] in contrast to reports by Meister [71].

In mitochondria: The glutathione functions in mitochondria are not as clear as in the cytosol of cells. In addition to the GSH/GSSG functions already described (P_i/H^+- symport/ATPsynthase), glutathione peroxidase and glutathione reductase were present. Glutathione-S-transferase activity has been detected in hepatic mitochondria [72] and consequently purified and characterized [73], but the presence of γ-glutamyl transpeptidase has not yet been shown and glutathione synthase is completely absent. Since eukaryotes presumably acquired glutathione metabolism by invading mitochondria [60], presumably via gene exchange, their should be one glutathione pool in stem cells. During growth and differentation (synergism between growth factor, erythropoetin, γ-interferon and cytokines [28- 31]) , one glutathione pool remains in erythrocytes, but at least two remain in the developed cells [74]. An active transport system for glutathione should not exist - but may be acquired by evolutionary pressure.

The uptake of glutathione by renal cortical mitochondria [75] was performed on almost decoupled mitochondria. It is not dependent on energy derived from oxidative phosphorylation, is inhibited to some degree by other anions/cations (ATP, KCN, $CaCl_2$, etc.), can be divided into a rapid equilibrium uptake to surrounding concentrations followed by a slower uptake phase (most likely due to mitochondrial swelling); points not relating to a physiological uptake of glutathione. Interestingly enough, however, an RCR (site II-III) of 3 decreased to 1.8 after exposure to 0,1 mM t-butylperoxide (t-BH) which initiates glutathione release (discussed below).

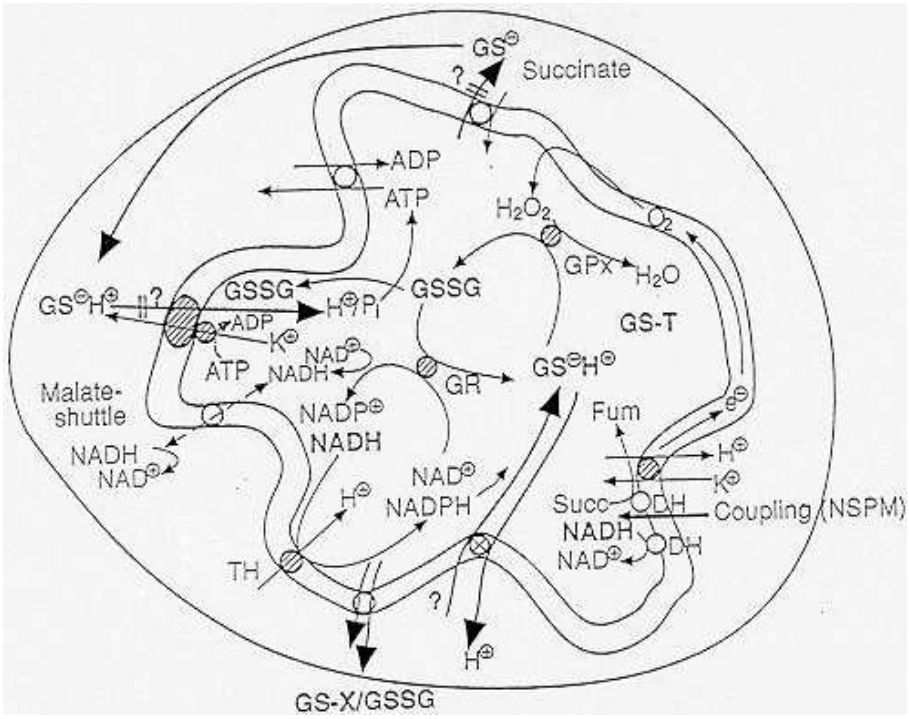


Fig. 8. Glutathione metabolism in mitochondria. GS = glutathione, GS-T = glutathione-S-transferase, GPx = glutathione-peroxidase, GR = glutathione-reductase, TH = transhydrogenase**,** DH = dehydrogenase, Succ = succinate, Fum = fumarate.

In Fig. 8 the known glutathione functions are summarized and connected to some energy-linked functions of mitochondria. The most probable way for cytosolic glutathione to reach and leave the mitochondrial matrix space is via a GS^-/H^+-symport system. Although other ways are also possible, they are not very likely. For instance, the ionophoresis of negatively charged

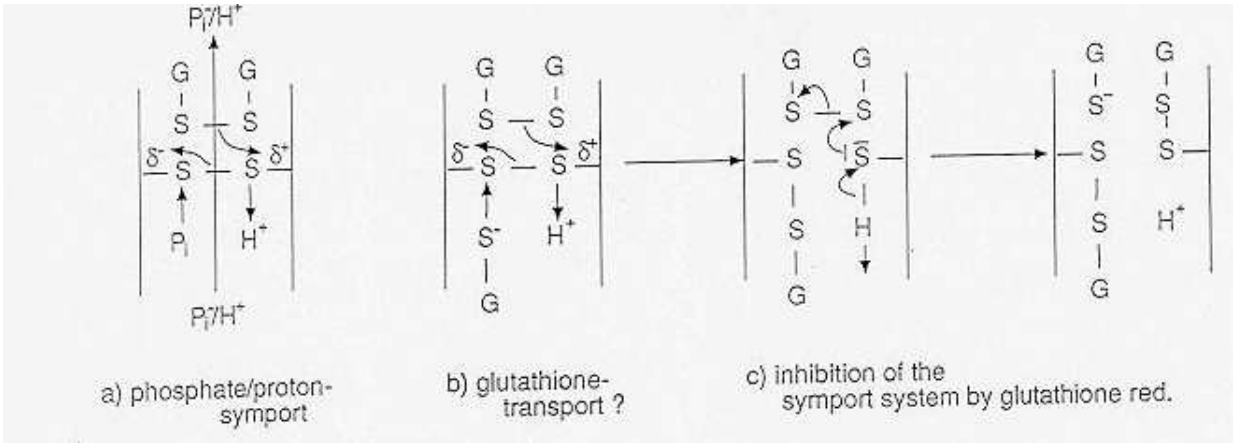


Fig. 9. Inhibition of P_i/H^+-symport by reduced glutathione.

compounds needs a positively charged mitochondrial membrane. Cycling across the ATP synthase and dicarboxylate carrier (Fig. 8) is not possible because both systems were inhibited by nucleophilic GS^- ions (Fig. 9), and the quite different molecular structures of GS^- and succinate. It should be stressed that the ATPsynthase should have a higher affinity to GSSG than to glutathione reductase, because otherwise there would be no ATP synthesis possible.

Jocelyn [76] 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 passive oxidation of GSH by t-BH in the presence of phosphate, he found most of the formed GSSG was lost as mixed disulfides with protein-SH groups and was released into the suspension medium. However, in the presence of oxaloacetate (without phosphate) he found most GSSG was retained by mitochondria and was lost as mixed disulfides with protein-SH groups. In both cases, he found small amounts of mixed disulfide before oxidation, but afterwards a 3-4 fold increase. This increase was prevented by succinate, which also prevented the formation of free GSSG. EDTA prevented the oxidation of internal GSH by external t-BH in the presence of phosphate and endogenous substrate.

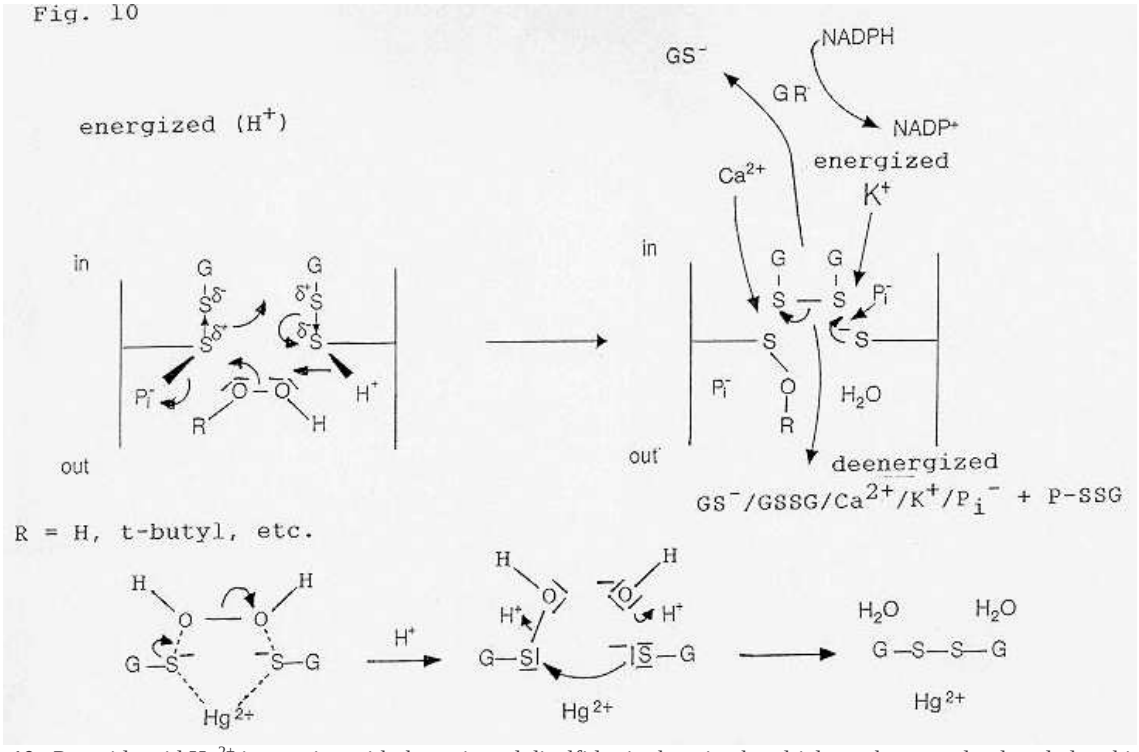


Fig. 10. Peroxide arid Hg^{2+} interaction with the activated disulfides in the mitochondrial membrane and reduced glutathione, respectively. P-SSG = mixed protein glutathione disulfide.

External t-BH may react directly with the activated disulfide bridges in the membrane (Fig. 10) and thereby lead to membrane alterations. In the absence of energy the transhydrogenase does not function. Therefore, NADPH will not be available for glutathione reduction and in the presence of H_20_2 the concentrations of oxidized glutathione will rise. Without energy there will be no Ca^{2+}/K^+-accumulation and in the presence of rotenone release of already present endogenous anions. Oxaloacetate further depletes mitochondria of reduced pyridinnucleotides

and enhances endogenous GSSG formation in the presence of mitochondrial H_20_2. t-BH reacts directly with the activated disulfide bridges in the membrane (Fig. 10), initiates release of anions (glutathione incl.), oxidation of released GSH, and build-up of mixed disulfides with protein-SH groups followed by mitochondrial damage. The glutathione reductase inhibitor BCNU, although potentiating t-BH induced oxidation of GSH, delays Ca^{2+} release in correlation to the more slowly occurring pyridine nucleotide oxidation since the energized state of mitochondria lasts longer [77-80]. Our studies on mitochondria or submitochondria (± reduced or oxidized glutathione) demonstrate that there are two extreme concentrations of reduced or oxidized glutathione where

Table VI. **Effects of thiol- and disulfide compounds on oxidative phosphorylation and respiration in the presence and absence of NSPM**

sites II-III 1 mg mitochondria, succinate, rotenone, RCR > 5	Stimuldtion of respiration/ % of DNP uncoupling	Inhibitation of State 4→ State 3 transition/%	Inhibitation of respiration/%
NSPM/25 nmoles/mg	75	--	--
+ 23 mM DTE	--	100	100
23mM DTE	--	--	ca. 5
+ 82 mM ß-ME[a)]	--	100	0
82 mM ß-ME	--	100	0
+ 11 mM GSH	--	100	100
11 mM GSH	--	100	100 (ADP stimulated, DNP inhibited)
+ 5 mM GSSG	--	100	100
5 mM GSSG	--	100	100 (ADP, DNP withou effect)

a) ß-ME = ß-mercaptoethanol

these particles do not perform ATP synthesis: 1) at very low concentrations (reaching almost zero values) and 2) at high concentrations (around 10 mM GSH and 5 mM GSSG respectively, Table VI) - as already mentioned - and in agreement to [81]. The question remains how mitochondria survive low depressed or high raised concentrations of surrounding oxidized or reduced glutathione?

Detoxification mechanism

The cytosol of the cells is detoxified not only into the plasma [69], but also actively into the mitochondria. Since mitochondrial ATP synthesis becomes uncoupled under these conditions, the respiratory chain greatly facilitates the uptake of chemicals across the P_i/H^+- symporter (i.e. ATP synthase) [25, 34] .

Glutathione-S-transferase [73] is then produced by mitochondria in order to survive "detoxification of cytosol". There is then a cyclic movement of chemicals from the plasma (P_i-

carrier?) into the cyctosol and mitochondria, back from the mitochondria into cytosol and plasma. The respective activity of the carrier involved determines the state of toxification. The highest amount of activities should be performed by the mitochondrial enzymes because they are driven by rising chemical concentrations which result in a gradual increase in respiratory chain activity. High enzyme activities were presumably reached by enhanced glutathione-S-transferase synthesis: This means that high chemical pressure causes an "evolutionary development of mitochondria" with lowered mitochondrial glutathione concentrations. In addition to these implications, the following important question ineveitably arisis: How are the glutathionethioether-carriers involved energetically be driven?

Other carriers, including phosphate, anion and glutathionethioether-carriers most likely gradually evolved from the ATPsynthase and probably belong to a gene superfamily comprised of the G-protein coupled receptors [82] responsible for signal transduction across membranes [8, 25]. High turnover of chemicals leads to a gradually increasing rate of glycolysis, build-up of lactate, and finally to the death of mitochondria. Mitochondria attempt to survive by stimulation of the cell and thus its own reproduction, which may lead to enhanced proliferation of cells.

Two different coupled respiratory chains

As shown in Fig. 8, two coupled respiratory chains exist in mitochondria: the well-known substrate oxidation, NADH or succinate to oxygen, and the oxidation of NADPH to H_20_2/ oxygen. By the inhibition of NADH respiration between coupling site I and II by certain chemicals (e.g. NSPM) [8] , NADPH respiration becomes dominant. This respiration may be supported by site II substrates**,** by transhydrogenase, or even the malate shuttle**,** which means that mitochondria have several ways of maintaining thermoregulation.

Glutathione metabolism in different cell types

Cell types from various tissues can be divided into at least 3 categories depending on the substrate used to maintain appropriate energy metabolism: brain cells use glucose, lymphocytes glutamate, and liver and kidney cells use mixtures of various substrates. This regulation is also a function of the variation that exists between mitochondria from different organs, and the need for caution when extrapolating data from one organ to another [27, 75, 80, 83, 84]. For instance, rat liver and kidney are similar in porphyrinogen oxidation rates and are of considerable interest with respect to the etiology of Hg - induced porphyria, inasmuch as the kidney is the principal target organ of mercury compounds. Renal mitochondrial GSH levels have been found to be at least 10 times lower than those of the soluble cellular fraction [27, 75]. Mercury concentrations can reach 100 μM or greater in rat renal proximal tubule cells during prolonged exposure to 5 or 10 ppm methyl mercury hydroxide in drinking water. 500 to 600 uM Hg^{2+} plus mitochondrial endogenously produced H_20_2 or Fe^{3+}- EDTA, in the presence of exogenously added H_20_2, leads to the oxidation of GSH (Fig. 10) and depletion of endogenous glutathione with excretion of porphyrins in the urine. These toxic mercury concentrations also lead to oxidative damage of mitochondria [75].

Brain mitochondria have a greater susceptibility to oxidative stress than liver mitochondria. For example, 50 μM t-BH resulted in irreversible loss of GSSG from brain mitochondria. In contrast, liver mitochondria can reverse t-BH induced formation of GSSG [80]. Glucose oxidation in active brain cells results in high site I oxidation rates with NADH oxidation (pyruvate DH) consumption of large amounts of 0_2 , production of CO_2 , concomitantly low NADPH levels (inactive TH) , but high GSSG (inactive GR) and high ATP concentrations (maximal coupled and active oxidative phosphorylation rates). On exposure to low concentrations of t-BH (50 μM), glutathione was recovered as the irreversible protein-SSG. These mitochondria, therefore, also have an increased sensitivity to toxic agents [27]. The same is essentially true for lymphocytes, although they utilize glutamate (glutamine) instead of glucose as fuel. In contrast, in mitochondria from liver cells, TH and GR are working at normal rates and these mitochondria are therefore less sensitive to oxidative stress [80] (Fig. 10) . The results described for Moris hepatoma [83] should be analyzed based on the above findings for mitochondrial glutathione concentrations.

Tumor mitochondria

The relationship between Mories hepatoma and glutathione metabolism is discussed above. The excellent article by Villalobo and Lehninger [84] describes inhibition of oxidative phosphorylation by calcium in ascites tumor mitochondria. Mg^{2+} restoration of the Ca^{2+}-inhibited oxidative phosphorylation in these mitochondria can be explained by Mg^{2+}-inhibition of Ca^{2+} accumulation as previously described [25] . The much greater sensitivity of NAD (NADP) -linked phosphorylating respiration to Ca^{2+} is most likely due to complexation of glutathione and inhibition of dependent enzymes, in addition to the possible causes mentioned by Villalobo and Lehninger [84]. Based on these explanations it becomes clear that all mitochondria are able to
be inhibited by Ca , and that this inhibition may lead to certain types of tumors. In this case, the highly increased glycolytic formation of ATP may play the most important role (see above). Mg^{2+} appears to be the most important factor in preventing Ca^{2+} and phosphate from building hydroxyapatite crystals in mitochondria. Since the Ca^{2+}/Mg^{2+}-ratio is most likely controlled by our brain, psychogenic factors like noradrenaline [above, 30, 85, 86] play an important role in the development of certain diseases including cancer [25], as already has been described for psoriasis vulgaris [86-89], (see also Babcock et al [90]), and in aging since aged mitochondria contain high amounts of Ca^{2+}/P_i-deposits (Fig. 6).

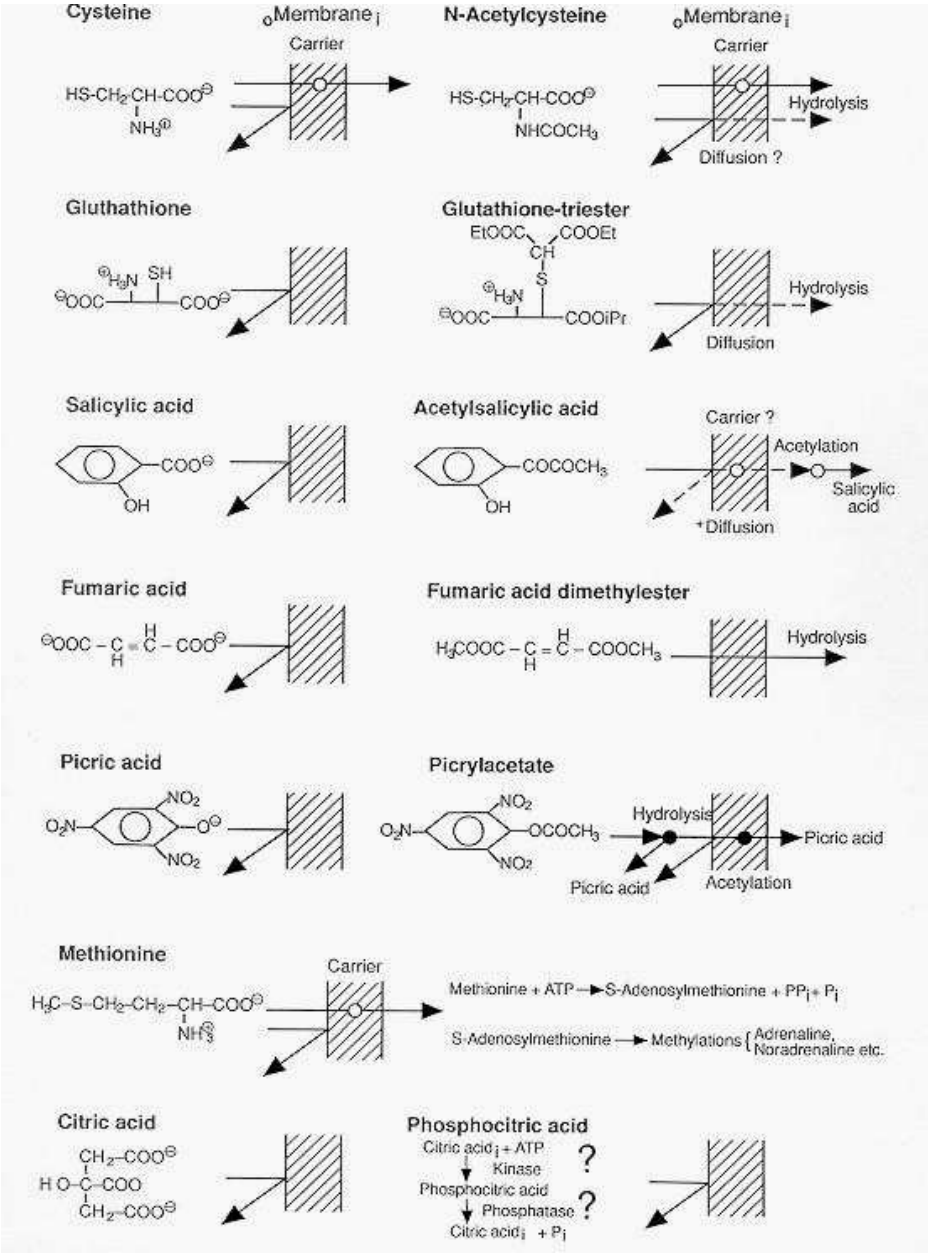
Therapeutics

Phosphocitric acid is present in cells, as well as in body fluids, and inhibits hydroxyapatite crystal growth [91]. It may therefore function to prevent Ca^{2+}/P_i -deposits in cells. It could be that cells then contain kinases and phosphatases for citric acid. Phosphocitric $acid_i$/Mg should be a good combination and one protective factor against cancer and aging.

The problem with potentially active therapeutics is membrane permeability. For instance, cysteine permeability is normally mediated via the amino acid transporter (e.g. γ-glutamyltranspeptidase). This amino acid can also cross membranes by diffusion after being N-acetylated. Its therapeutical activity is mainly expressed by antioxidative effects, either directly as a radical scavenger, or indirectly via the endogenous antioxidant system with glutathione and the glutathione system [92].

Glutathione may be used exogenously for detoxification of heavy metal ions [93] and is presumably sold for this purpose by some firms. For endogenous supplementation, glutathione has to be converted into a membrane permeable compound **.** This may be done by the reversible formation of triester derivates [94].

The essential amino acid methionine is transported into the cells and also synthesized inside the cells in a highly complex way. Methionine serves as the methyl-group donor to some 40 different methyl-group acceptors, including the psychogenic factor noradrenalin [30, 85, 86]. How phosphocitric acid reaches its target inside cells is totally unknown, and thus requires continued **r**esearch in this area (for membrane permeability of some therapeutically active compounds see Fig.11):



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March 21, 1995
5G11.doc

Manuscript No.: 5G 11
Author(s): R. Kiehl
Title: Glutathione: The essential factor for mitochondrial energy linked functions

Dear Dr. Kiehl,

Your manuscript entitled "Glutathione: The essential factor for mitochondrial energy linked functions" has been evaluated by two referees. Both reviewers felt that the manuscript addresses an important area of research, but that it does not provide sufficient in depth information on the role of glutathione in the mitochondria. We would like to suggest that you provide a more detailed discussion, including a thorough and up-to-date citation of references specifically focusing on glutathione energy linked functions. Ideally, the role and metabolism of glutathione in the mitochondria should be well described. In order to complete an extensive search and summary of the present literature, please feel free to work with one of your colleagues.

The reviewers also state that the organization, language and style of the manuscript could be improved. In particular, they comment that the text deviates into several different topics and requires a more structured format.

You may submit a new version of your work if you feel you are able to address the concerns of the reviewers. However, this version will be subjected to a completely new review process and there is no guarantee of eventual acceptance.

In the case that you would like to submit a new version of your manuscript, please include the above manuscript number with your paper.

Thank you for the submission of your work and I hope you find the reviewers' comments helpful.

Sincerely,

Detlev Ganten

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January 15, 1996

Manuscript No.: 5G 11 REVISED
Author(s): R. Kiehl
Title: Glutathione: The essential factor for mitochondrial energy linked functions

Dear Dr. Kiehl,

Your revised manuscript entitled "Glutathione: The essential factor for mitochondrial energy linked functions" has been evaluated. The reviewers comment that this version is improved, however, they did not give the manuscript sufficient priority for publication.

Thank you for the submission of your work.

Sincerely,

Detlev Ganten

1995: Prof.R.Huber, MPI Martinsried, meinte zu den Artikeln Nr.1-4 und 5 (ATPsynthesis and transport in mitochondria, Review on Glutathione), daß er die Artikel nicht in seinem Journal publizieren wolle.
1997: Prof.G.Lubec, Universitätsklinik Wien, erklärte, daß er erst etwas aus den Arbeiten machen müsse.

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Herrn
Dr. R. Kiehl
Saliterweg 1
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Jena, 29.11.2000

Sehr geehrter Herr Kollege Kiehl,

ich habe mir Ihr Manuskript aus dem Jahre 1995 näher angeschaut. Grundsätzlich halte ich die von Ihnen vorgelegte Substanz für außerordentlich interessant und auch im wesentlichen für publikationsfähig, jedoch unter Berücksichtigung folgender Aspekte:

1. In einem Review müssen die rezentesten Resultate auf dem jeweiligen Gebiet, das überstrichen wird, sorgsam berücksichtigt und ausdiskutiert werden. Sie kündigen selbst an, daß zwischenzeitlich auch von Ihnen neue Ergebnisse gewonnen worden sind. Diese müssen natürlich hinzugefügt werden.
2. 4 ausführliche Arbeiten avisieren Sie bei Herrn Michel und Herrn Huber und betonen, daß die Versuchsbedingungen zu Ihren Kapiteln 1-4 sich dort bei diesen Kollegen befinden sollen. Mit diesem Hinweis kann ich leider wenig anfangen, da Sie die Kapitel ohnehin nicht nummeriert haben und ich infolgedessen keine Zuordnung nachempfinden kann. Sie sollten daher diese Arbeiten aufnehmen und sich mit denen in ausreichender Form auseinandersetzen. Im übrigen scheint es mir nicht unwesentlich, obwohl dies für Reviews nicht zwingend ist, die Versuchsbedingungen, d. h. die experimentellen Voraussetzungen, die Sie gewählt haben, herauszukristallisieren und an Methodologien anderer Gruppen zu messen.
3. Rein formal entsprichen der Manuskriptaufbau sowie die äußere Form nicht den Instructions of Authors der Zeitschrift CMB. Das Manuskript müßte also inhaltlich erweitert und zum zweiten formal umgeändert werden.

Wenn dies soweit gediehen ist, bin ich gern bereit, das abgeänderte Manuskript zum peer reviewing an kompetente Kollegen weiterzuleiten. Ich darf Sie daher bitten, mir geeignete Reviewer zu nennen, die sinnvollerweise infrage kommen.

Mit freundlichen Grüßen

Prof. Dr. K.-J. Halbhuber
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Instructions