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# Iodine, Hemin and Heminester as Oxidants in a Synthesis of ATP from ADP and $P_i$ Mediated by Thiols and Disulfides

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The conversion of oxidation energy into the energy-rich phosphoryl linkage of ATP has been demonstrated in a system consisting of the tertiary-butylammonium salts of ADP and $P_i$ in pyridine and utilising several mercapto carboxylic acids or their disulfides as mediators and iodine or hemin or hemindimethyl ester as oxidants.

Iodine with several thiols in 1:1 molar proportions produces relatively poor yields of ATP (1.5–5.4%) which can be considerably increased (10–15%) when the corresponding disulfides are used, whereas a thioether (*N*-acetylmethionine) had no effect. This led to the conclusion that disulfides are better substrates and accordingly much higher amounts of ATP were formed from all thiols on using a two-fold amount of oxidant (up to 25.6%). Taking this into account a mechanism has been formulated in which the formation of a phosphorylating species, $RS\text{-}OPO_3H_2$, is assumed to be formed from disulfides.

Hemin and (less efficiently) its dimethyl ester used as oxidants also give rise to the formation of ATP in our system, but only under aerobic conditions. Here optimal yields (up to 16.7%) were obtained with thioglycolate or its disulfide as mediators, a thioether (*N*-acetylmethionine) being almost ineffective, and no difference in efficiency between thiol acid and disulfide was observed at a hemin : sulfur ratio of 1:1. The yields depend in some way on the hemin : S ratio, lower values giving higher amounts of ATP on oxidation of the mercapto compound, but not of its disulfide. Therefore a reaction mechanism different from that of iodine must be presumed. The formation of the same phosphorylating species as above has been assumed to occur preferently with a thiol and to a lesser extent with a disulfide. The role of oxygen is still rather obscure, for it can not be determined whether it is the terminal electron acceptor or electron carrier. A catalytic participation of hemin could not be demonstrated, perhaps on account of the slow autoxidation rate of the ferrous heme iron.

The basic mechanism of mitochondrial oxidative phosphorylation, the process by which the energy of electron transport is converted into the high energy pyrophosphate bond of ATP, is still rather obscure. In order to produce a chemical hypothesis which includes the formation of high-energy intermediates during electron transfer, model reactions are being studied in several laboratories (for a recent review see [1]). It has been found that during oxidation of a variety of compounds in the presence of ADP and $P_i$ considerable amounts of ATP are formed. In our laboratory a number of model reactions have been investigated that involve sulfur compounds. Most surprisingly the oxidation by bromine (5 equiv.) of a simple thiol, like thioglycolic acid under anhydrous conditions (pyridine) led to the formation of a yield as high as 35% ATP from ADP and $P_i$ [2]. In this system, at every step of oxidation leading eventually to the highest oxidized state of sulfur (hexavalent), a coupled phosphorylation can occur. In order to simplify the system we have studied the effect of the milder oxidizing agent, iodine. Simultaneously the number of mediator compounds investigated as models for protein bound sulfur was extended to 2-mercaptopropionic acid, *N*-acetylcysteine, their corresponding disulfides, inorganic hydrogensulfide and *N*-acetylmethionine. Since hemin, as a model of the series of cytochromes, had turned out to be an ATP-producing oxidant in an analogous reaction of thioglycolic acid [3], this observation was followed by using the above mentioned substrates and various proportions of the reactants. Furthermore hemindimethylester was included, and the temperature dependence of some reactions has been studied.

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*Unusual Abbreviation.* Tertiary butylammonium, *t*BuAm.

*Enzymes.* 3-Phosphoglycerate kinase (EC 2.7.2.3); adenylate kinase or myokinase (EC 2.7.4.3).

This is part XV of a series *Model Experiments for Oxidative Phosphorylation*. For part XIV see E. Bäuerlein and Th. Wieland in *Proceedings of the Colloquium on Bioenergetics* (Bari, Sept. 1970). Part XIII: Bäuerlein and Wieland [3].

## MATERIALS AND METHODS

### *Materials*

Materials were obtained from the following sources. 0.1 N tertiary-butylammonium (*t*BuAm) hydroxide solution in isopropanol/methanol, pyridine, puriss., glacial acetic acid p.a. 100%, acetic acid anhydride p.a. and $Br_2$ (Suprapur) from Merck (Darmstadt); $I_2$ p.a. from Riedel-de-Haën (Seelze, near Hannover); ADP (free acid), 3-phosphoglyceric acid, glyderaldehyde-phosphate dehydrogenase and phosphoglycerate kinase from Boehringer Mannheim GmbH (Mannheim) Germany. Extremely purified myokinase was a generous gift of Dr. H. Schirmer. *N*-Acetyl-DL-methionine puriss. and hemin were from Fluka (Eschborn, near Frankfurt a. M.). Hemin was also isolated from fresh bovine blood according to Gattermann-Wieland [4] and, as well as the commercial product, recrystallized as described by Nencki and Zaleski [5]. L-Cysteine, *N*-acetylcysteine and thioglycolic acid, 2-mercaptopropionic acid, ethylthioglycolate which were distilled before use, were products of Th. Schuchard (München). Dihydrogensulfide (99.6%) was delivered in a special steel pressure flask from J. T. Baker Chemical (Phillipsburg, N.J., U.S.A.) $N_2$ puriss. from BASF (Ludwigshafen).

The following reagents have been prepared in our laboratory. Dithiodiglycolic acid (m.p. 103—105 °C) was prepared by oxidation of thioglycolate with $H_2O_2$ according to Schöberl [6], diethylsulfide-2,2'-dicarboxylic acid (m.p. 154—155 °C) according to Huffmann and Ellis [7]. Hemindimethylester was prepared according to Küster *et al.* [9], and purified by chromatography of 500-mg portions on a column (5.5×200 cm) of Sephadex LH-20 (Pharmacia, Uppsala) in a puriss. solvent mixture of chloroform and methanol (1:1, by vol.).

### *Preparation of tBuAm-Salts*

*(tBuAm)$_3$ADP + (tBuAm)$_2$HPO$_4$.* The procedure used was basically that outlined by Wieland and Bäuerlein [2,3,10]. In a typical experiment 1/9 mmol solid ADP (free acid), 1/9 mmol $H_3PO_4$ in dioxane were added to 5/9 mmol 0.1 N *t*BuAm-OH in 2-propanol-methanol solution to produce (*t*BuAm)$_3$ADP + (*t*BuAm)$_2$HPO$_4$. The solution was evaporated under vacuum and the residue was dried for 20 min at 0.1 torr.

*(tBuAm)HS and (tBuAm)HS$_8$.* A sufficient quantity of 0.1 N *t*BuAm-OH in 2-propanol-methanol was saturated with pure dihydrogen sulfide (99.6%) to form (*t*BuAm)SH. For preparation of (*t*BuAm)$_2$S an equivalent amount of *t*BuAm-OH was added to the (*t*BuAm)SH solution. (*t*BuAm)HS$_8$ was obtained by dissolving therein the calculated amount of elemental sulfur. The solutions were evaporated as described above, the residual oils were dissolved in anhydrous pyridine so that 1 ml of the solution contained 1/9 mmol of the hydrogensulfide used.

### *Reaction Conditions*

*Oxidation with Iodine.* In a typical experiment the residual gum of (*t*BuAm)$_3$ADP + (*t*BuAm)$_2$HPO$_4$ was dissolved in 3.98 ml pyridine which had been dried for at least four weeks over calcium hydride. The reaction was initiated by adding first 1/9 mmol thioglycolic acid by a micropipette directly into the solution and immediately after 1.11 ml of a 0.2 N solution of iodine (1/9 mmol) in anhydrous pyridine. The mixture was stirred magnetically at room temperature for 16 h. In all experiments the final volume was 5 ml.

*Oxidation with the Hemin-Oxygen System.* In a typical experiment the residual gum of (*t*BuAm)$_3$ADP + (*t*BuAm)$_2$HPO$_4$ was dissolved in 5 ml of dry pyridine. The reaction was initiated by adding first 1/9 mmol thioglycolic acid by a micropipette directly into the solution and immediately after that 2/9 mmol solid hemin. The mixture was stirred magnetically at 30 °C for 16 h under oxygen.

### *Analysis of the Reaction Mixture*

The reaction mixture was evaporated under vacuum at 35 °C and the residue was dissolved in a mixture of 4 volumes of pure pyridine and 1 volume of 0.05 M Tris buffer pH 7.55 to a final volume of 5 ml. As described elsewhere [2,3,10] it was separated by quantitative thin-layer chromatography. The eluates were analyzed for ATP by the phosphoglycerate-kinase method of Bücher [11], followed by a further assay by the myokinase method of Gruber [12].

## RESULTS

### *Oxidation by Iodine*

The oxidation of several thiols, disulfides, hydrogensulfide ($SH^-$) and of a polysulfide ($HS_8^-$) by iodine in the presence of the *t*BuAm salts of ADP and phosphoric acid yields ATP to various extents depending on the nature of the thiol and on the molar ratio of oxidizing agent to S-compound. The data in Table 1 show that in all cases the differences in yields of ATP between using 1 or 2 molar equivalents of $I_2$ are much greater than expected from stoichiometry.

The yields of ATP obtained with 1 mol equiv. of $I_2$ increase distinctly if disulfides (Nos. 2,6) are used as substrates thus pointing to disulfides as much better mediators than thiols of oxidative phosphorylation. All of the thiols investigated induce only small amounts of ATP during oxidation with 1 mol equiv. of $I_2$. That fact justifies the assumption that in the oxidation of thiols with 1 mol $I_2$ the corresponding disulfides are formed mainly, without a coupled acti-

Table 1. *Net yields of ATP from ADP + $P_i$ on oxidation of several thiols and their disulfides by various amounts of $I_2$*
The *t*BuAm salts of the phosphates were reacted with the substrates in dry pyridine for 16 h at 20 °C. ATP was analyzed as described by Bäuerlein and Wieland [3]

| No. | Substrate | ATP obtained with | |
|---|---|---|---|
| | | 1 mol equiv. $I_2$ | 2 mol equiv. $I_2$ |
| | | % | % |
| 1 | Thioglycolic acid | 5.4 | 18.8 |
| 2 | Dithiodiglycolic acid | 14.5 | 25.3 |
| 3 | 1:1 Mixture of 1 and 2 | 14.9 | 19.2 |
| 4 | Ethylthioglycolate | 2.8 | 23.2 |
| 5 | 2-Mercaptopropionic acid | 1.5 | 25.6 |
| 6 | Disulfide of 5 | 9.9 | 25.5 |
| 7 | 1:1 Mixture of 5 and 6 | 6.2 | 23.2 |
| 8 | *N*-Acetylcysteine | 1.8 | 12.8 |
| 9 | Hydrogensulfide ($SH^-$) | 1 | 8.1[a] (12.8 with 4 $I_2$) |
| 10 | $SH^- + 7\,S$ ($S_8H^-$) | | 12.5[a] (16.6 with 4 $I_2$) |
| 11 | *N*-Acetylmethionine | 0.7 | 1.4 |
| 12 | Without S-compound | 0.5 | 0.9 |

[a] The corresponding values with $Br_2$ (2, 3 and 5 mol) were for 9: 11.0, 17.7 and 19.4 respectively and for 10: 14.8, 14.2 and 14.8 respectively.

vation of $P_i$ (or ADP), and that ATP formation occurs essentially in the next step, *i.e.* the further oxidation of the disulfides.

The inorganic substrates $HS^-$ (No. 9) and $HS_8^-$ (No. 10) also induce on oxidation the formation of ATP although in smaller amounts than organic thiols. Similarly in these cases a very distinct increase in ATP formation is observed on going to greater relative amounts of oxidizing agent, *i.e.* oxidation of di- or polysulfide bonds. Higher yields of ATP were obtained in the inorganic system by using bromine instead of iodine. *N*-Acetylmethionine, a suitable substrate in oxidation with $Br_2$ [13] did not give considerable amounts of ATP on oxidation with $I_2$.

### *Oxidation with Hemin*

Hemin as an oxidant accepts one electron per mol, corresponding to one halogen atom. As we have found, very poor yields of ATP are obtained if the system is operated without oxygen. Thus the net yield of ATP from ADP and $P_i$, which was about 16% on oxidation with 2 mol hemin in air [3], fell to 1.5% on repeating the reaction in a nitrogen atmosphere. Therefore the system hemin · $O_2$ has to be considered as the true oxidant in all experiments treated in the following section.

On reacting 2 mol hemin with thioglycolic acid (Table 2, No. 1) or its ester (No. 3) in presence of ADP and $P_i$ a remarkable amount of ATP is formed as compared with the yield on oxidation with 1 mol iodine (Table 1, Nos. 1 and 4). Hemindimethylester is also able to produce considerable amounts of ATP when reduced by thioglycolate, which seems an outstanding mediator in this system. The 2-mercapto compounds 4 and 7 induce only poor ATP formation, hydrogensulfide (No. 8) is without any effect and *N*-acetylmethionine is only weakly potent. Hemin also stimulates strong ATP formation with dithiodiglycolic acid as reducing agent (No. 2). There is an increasing amount of ATP on decreasing the hemin: sulfur ratio for the thiol-induced reaction as shown in Table 3.

Table 2. *Net yields of ATP from ADP + $P_i$ on oxidation of several thiols and their disulfides by 2 equivalents of hemin or hemindimethylester in air*
The *t*BuAm salts of phosphates were reacted with the substrates in dry pyridine for 16 h at 30 °C. ATP was analyzed as described by Bäuerlein and Wieland [3]

| No. | Substrate | ATP obtained with | |
|---|---|---|---|
| | | Hemin | Hemin-dimethylester |
| | | % | % |
| 1 | Thioglycolic acid | 15.8 | 9.5 |
| 2 | Dithiodiglycolic acid | 16.7 | 10.8 |
| 3 | Ethylthioglyconate | 10.1 | 9.8 |
| 4 | 2-Mercaptopropionic acid | 4.6 | 2.8 |
| 5 | Disulfide of 4 | 3.5 | 3.6 |
| 6 | 1:1 Mixture of 4 + 5 | 8.1 | 5.6 |
| 7 | *N*-Acetylcysteine | 4.4 | 3.5 |
| 8 | Hydrogensulfide | 0 | 0 |
| 9 | *N*-Acetylmethionine | 3.2 | 2.9 |
| 10 | Without mercapto compound | 5.1 | 1.1 |

Table 3. *Oxidative ATP formation in a hemin · $O_2$-sulfur system under various hemin: sulfur ratios*
Substrates: thioglycolate or/and its disulfide

| Ratio hemin/S | State of sulfur | Yield of ATP |
|---|---|---|
| | | % |
| 2 | —SH | 15.8 |
| 1 | —SH | 17.8 |
| 1 | —S—S— | 16.7 |
| 0.67 | —SH | 24.3 |
| 0.67 | —S—S— | 12.5 |
| 0.67 | —SH + —S—S— | 17.5 |

The disulfide is a poorer substrate particularly at a lower hemin: S ratio. 2-mercapto acids and their disulfides generally give inferior yields (Nos. 4, 5 and 7 in Table 2).

Increasing the relative concentration of hemin did not lead to higher but to lower ATP values. In the presence of 4 mol, the adenylate-kinase-like activity of hemin [3] became so strong that the amount of ATP formed by disproportionation of ADP equalled or surpassed the yield of the oxidatively generated product. On lowering the hemin concentration below 0.2 mol per sulfur atom no marked ATP generation occurred.

Hemindimethylester has nearly no adenylate-kinase-like effect; apparently a catalyst needs two

carboxylic groups in a suitable spatial configuration, as in hemin. The ATP yields produced by the ester are in most cases lower than those obtained with hemin.

The temperature dependence of the oxidative ATP-formation was also studied in a few examples. In the reaction of thioglycolate and hemin the ATP yields at 20°, 30° and 40 °C were 6.0 and 15.8, 15.5, respectively, a result which was to be expected.

## DISCUSSION

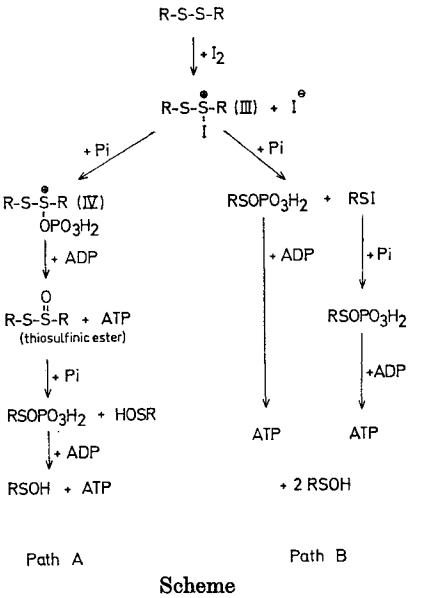
### *Oxidation with Halogens*

A mechanism has been previously suggested by us for the generation of ATP from ADP and $P_i$ during oxidation of mercapto compounds by bromine [2]. A rapidly formed sulfenylbromide was assumed to react with $P_i$ to form a mixed sulfenic phosphoric anhydride (I), a presumably very reactive phosphorylating agent.

$$RSH + Br_2 \rightarrow Br^- + RSBr$$
$$RSBr + H_2PO_4^- \rightarrow RSOPO_3H_2\ (I)$$
$$(I) + ADP^- \rightarrow ATP + RSO^-\ (\text{sulfenate})$$
$$RSO^- + Br_2 \rightarrow RS(=O)Br + Br^-$$
$$RS(=O)Br + H_2PO_4^- \rightarrow RS(=O)-O\,PO_3H_2\ (II)$$
$$(II) + ADP^- \rightarrow ATP + RSO_2^-\ (\text{sulfinate}).$$

On doubling the amount of $Br_2$ a two-fold yield of ATP was obtained (11.2% *cf.* 5.8%). This can be explained by assuming a further reaction of the sulfenate, formed in the first phosphorylating step, with bromine to give *via* sulfenylbromide a sulfinyl phosphate (II), also a phosphorylating agent. The use of iodine instead of bromine resulted in a comparable ATP formation by 1 mol $I_2$ using thioglycolate as mediator (No. 1) and less with other thiols (Nos. 4, 5, 8), but considerably higher amounts of ATP were generated by 2 mol $I_2$ (Nos. 1, 4, 5, 8) as compared with $Br_2$ (Table 1). This points to a different mechanism which may in addition be involved. This suggestion is confirmed by our observation that ATP is amply generated from ADP and $P_i$ on oxidation of disulfides by 1 mol $I_2$ (Nos. 2, 6). Thus it appears that here disulfides are the true substrates. In contrast with $Br_2$, whose reaction product with thiols, RSBr, will react rapidly with $P_i$ to form (I), $I_2$ *via* RSI will mainly produce a disulfide, since the sulfenyliodide most probably is less reactive with $P_i$ as a partner.

As a primary step in the energy-converting reaction an addition of $I^+$ to one of the sulfur atoms of a disulfide can be formulated. The electrophilic species (III) could react with $P_i$ (or ADP) by a nucleophilic substitution (path A) to give a sulfonium phosphate (IV) which represents, after its phosphorylating action upon ADP, a thiosulfinic ester (disulfide monoxide). These compounds, which easily transfer their sulfenyl moiety (*e.g.* to mercaptanes forming disulfides [14]) can react with $P_i$ thus forming the phosphorylating species (I). Alternatively, (III) could also react with $P_i$ at its adjacent electron depleted sulfur atom (path B). In this way also anhydride (I) would be formed, and additionally a second molecule of (I) by reaction of the sulfenyliodide with $P_i$.




Scheme

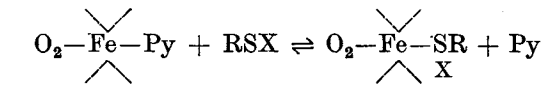
The even higher yields of ATP on oxidizing the disulfides No. 2 and No. 6 with 2 mol $I_2$ may be due to ADP—$P_i$ coupling during further oxidation of the sulfenate first formed in a way analogous to that formulated for $Br_2$ above.

Remarably, rather good yields of ATP are formed from ADP and $P_i$ on oxidation of polysulfides with $I_2$ or $Br_2$ (No. 10). It is conceivable that similar inorganic systems could have acted in converting the energy of electron transfer into pyrophosphate in primeval times.

### *Oxidation with Hemin and Oxygen*

In contrast to oxidative ATP-formation with halogens, hemin (+ $O_2$) produces rather good yields from thioglycolate with 1 mol per sulfur atom (Table 2). A little less ATP is produced on oxidation of the disulfide. The thiol-dependent ATP-formation is even increased by lowering the hemin: sulfur ratio, whereas that induced by oxidation of disulfide is distinctly lowered. (In earlier experiments [3] the essential role of oxygen had not yet been recognized,

therefore at low hemin: sulfur ratios the conditions were partially anaerobic.) A 1:1 mixture of thioglycolate and its disulfide yields, at the same hemin:S ratio of 0.67, an amount of ATP lying between the individual yields. These results point to a mechanism different from that suggested for halogens. A rapid oxidation by hemin of thiols to disulfides is not very probable. It can be assumed that the formation of a phosphorylating species (I) readily occurs following the binding of a sulfur atom, preferably of a thiol and to a lesser extent of a disulfide, the the hemin · $O_2$ complex.



(Py = pyridine, X = H or SR).

Since the concentration of the adduct becomes higher on increasing the amount of the thiol (hemin: sulfur ratio $< 1$) an increase in yields of ATP from the SH-compound can well be understood (Table 3). On increasing the concentration of hemin the adenylate kinase-like activity, *i.e.* the blank value of ATP is raised to such an extent that the net yield of ATP oxidatively formed is drastically diminished.

The result obtained with a mixture of thiol plus disulfide as a substrate is included in Table 3; this on oxidation gives rise to about the mean amount of ATP. This experiment was carried out with reference to the publication of Painter and Hunter [15], in which oxidative phosphorylation in an aqueous system of cytochrome *c*, gluthathione (GSH) and its disulfide (GSSG) was reported. The enhancing effect of GSSG on the autoxidation of GSH was ascribed to a complex which should be formed with $GS^-$ and which was considered more easily oxidizable than either of the components. The phenomenon is explained by the recent discovery of Massey *et al.* [16] of the catalytic effect of $S^-$-containing impurities in commercial samples of GSSG. Using our thin-layer technique for analysis of the reaction products, no ATP formation could be found in the aqueous system.

From Table 2 it appears that thioglycolate or its disulfide proved the best mediators with hemin · $O_2$ as oxidant It is difficult to find an explanation except the frequently claimed steric hindrance exerted by the bigger molecules (Nos. 4—7). Inorganic sulfides are without any effect in the hemin · $O_2$ system and *N*-acetylmethionine, which is considered as a model mediator for its ligand function in cytochrome *c* by Lambeth and Lardy [13], did not produce significant amounts of ATP.

The role of oxygen is still to be explored. Since the oxidative formation of ATP is far from being a stoichiometric reaction, it is difficult to decide if oxygen is the terminal electron acceptor and what kind of reduction product is formed. Hemin seems not to be a catalyst in the usual sense for it was not possible to generate considerable amounts of ATP in an aerated system with a hemin : sulfur ratio of $< 0.2$. This may, however, be due to the autoxidation rate of hemochromes, *e.g.* of dipyridineprotoporphyrin-$Fe^{II}$ [17]. which is too slow to permit a sufficiently fast redisposal of the $Fe^{III}$-containing oxidant. Supporting this idea is the relatively long time needed for the oxidative phosphorylating action of the hemin · $O_2$ system, which, in spite of the failure of a clear catalysis, could represent a model for a cytochrome-*c* oxidase coupled oxidative phosphorylation.

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