A REINVESTIGATION OF HUNTER'S MODEL SYSTEM FOR OXIDATIVE PHOSPHORYLATION.

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Summary: The formation of ATP in aqueous solution during oxidation of GSH by cytochrome c in presence of GSSG, ADP, P_i and Mg^{++} is due to a adenylate kinase like reaction rather than to an oxidatively linked phosphorylation. This has been concluded from experiments using ^{14}C-ADP in Painter and Hunter's system (1).

In 1970 Painter and Hunter (1) claimed that ATP is formed if reduced glutathione (GSH) is oxidized by cytochrome c in presence of GSSG, ADP, P_i and MG^{++}. Froede and Hunter (2) reported on the phenomenon of oxidized glutathione (GSSG) enhancing the rate of reduction of cytochrome c by reduced glutathione (GSH). This stimulatory effect of GSSG was shown by Massey, Williams and Palmer (3) to be an catalytic effect of S^{o}-containing impurities, probably gluthathione trisulfide (GSSSG). The active form of the redox reagent is probably the persulfide ion, GSS^{-}. In similar experiments, but using an anhydrous solvent (dry pyridine) and hemin-oxygen as oxidant, we have also observed an increase in ATP formation on adding the corresponding disulfides to the mixture of a thiol, ADP and P_i (4). Recently, however, we presented evidence that the increase of the amount of ATP is not a function of the supposed molecular complex of a thiol and its disulfide, but only one of the hemin-sulfur ratio (5, 6).

To test if, in a homogenous aqueous medium as used by Painter and Hunter, sulfur compounds may be involved at all in the oxidative synthesis of ATP, a direct thin-layer chromatographic analysis of the reaction with ^{14}C-ADP was carried out, instead of trapping the ATP and assaying it by a hexokinase system.

Materials and Methods

The sources of the compounds were as follows: Sigma: Cytochrome c, type VI; Boehringer: GSH, GSSG, AMP-Na_2, ADP-Na_3, ATP-Na_2H_2, NADP-Na_2; Merck: $K_2HPO_4 \cdot 3H_2O$, H_3PO_4, $MgCl_2 \cdot 6H_2O$, Tris, EDTA; Armour: BSA (Armour cristalline bovine serum albumin); NEN: Adenosine-8-^{14}C-5'-diphosphate, trisodium salt, in ethanol-water 1:1, specific activity: 17.7 mC/mM. Macherey & Nagel: Polygram CEL 300 PEI/UV_{254}.

All components except ADP were present in the same concentrations as described by Painter and Hunter (1) in Table III, cuvette 7: 27 µM Cytochrome c, 1 mM GSH, 10 mM GSSG, 1 mM HPO_4^{2-}, 0.1 mM ADP (90.8 mM ADP + 9.2 µM ^{14}C-ADP), 4.5 mM $MgCl_2$, 0.35 mM $NADP^+$, 1 mM EDTA, 1 mg/ml BSA, 45 mM Tris • HCl pH 7.6. The solution of GSSG in 45 mM Tris-buffer was adjusted to pH 7.6 by carefully adding 1 N NaOH. Both $NADP^+$ and GSH were dissolved in cold 45 mM Tris-buffer pH 6.0.

In three parallel experiments (Table 1) the ATP formation in the complete system (a) was compared with a control without GSH and P_i (b), and with a solution of ^{14}C-ADP alone (c) in 45 mM Tris-buffer pH 7.6. Each reaction volume of 3 ml contained 0.5 µC (0.0275 µmole) ^{14}C-ADP, which was dried by evaporation of its solution in ethanol-water and subsequent lyophilization. Before addition of GSH, the solutions were flushed thoroughly

with purified nitrogen. Samples of 0.030 ml were taken at 0, 5, 15 and 60 min after addition of GSH. ATP (R_F = 0.32), ADP (R_F = 0.53) and AMP (R_F = 0.68) were separated by thin-layer chromatography on PEI-cellulose with 0.85 KH_2PO_4, pH 3.4 as a solvent. The origins had been impregnated before with a solution of the cold nucleotides. The radioactivity was counted by a thin layer scanner, type LB 2722, Berthold (Wildbad, GFR). Values are given in percent of the sum of radioactivities of ATP, ADP and AMP.

Results and Discussion

Using ^{14}C-ADP the experiment of Painter and Hunter (cuvette 7, table III) (1) was repeated. In this experiment with a concentration of 0.4 mM ADP they found 5.5 % ATP formed after 4 min. We obtained 4.4 % ATP in 5 min as analyzed by radio thin-layer chromatography (5). Since in table II of the same paper (1) a concentration of 0.1 mM ADP appeared more favorable (23 % ATP), this experiment was repeated under these apparently optimal conditions.

Table 1

Formation of ATP from ADP in an aqueous (1) system.

	0 min	5 min	15 min	60 min
	% ATP			
(a) complete system	1.6	4.8	8.7	17.1
(b) = (a) without GSH and P_i	2.1	5.5	8.3	16.3
(c) solely ^{14}C-ADP in buffer	0.2	0.4	0.5	0

Radioactivity of ATP + ADP + AMP = 100 %

As shown in table 1 a distinct formation of ATP took place in the whole system (a), but about the same yields were obtained after omission of GSH and P_i (b).

Table 2

Concentrations of AMP and ADP during the experiment of table 1

	0 min	5 min	15 min	60 min
	% AMP and (in brackets) % ADP			
(a) complete system	3.4 (95.0)	5.5 (89.7)	8.7 (82.6)	20.4 (62.5)
(b) = (a) without GSH and P_i	3.3 (94.6)	6.3 (88.2)	9.3 (82.4)	20.9 (62.8)
(c) solely ^{14}C-ADP in buffer	0.9 (98.6)	1.9 (97.7)	0.9 (98.6)	1.2 (98.8)

Concomitantly a similar increase in the amount of AMP and decrease in ADP occured in absence or in presence of reductant and of P_i. This points strongly to a disproportionation reaction of ADP.

In a later paper (7) Painter and Hunter suggested for the supposed redox reaction a sulfenyl phosphate intermediate as a chemical link. A mixed anhydride of sulfenic and phosphoric acid, $RSOPO_3H_2$, was previously formulated in 1968 by Wieland and Bäuerlein (8), to explain the formation of ATP from ADP and P_i by oxidation of a thiol by bromine in anhydrous pyridine. It has not been possible to isolate this type of compound (9), likely because it is extremely reactive. It is not very probable that such compounds will be found in presence of water, which is a strong competitor with ADP for the phosphoryl moiety. High sensitivity for 0.4 % water was also found by Lambeth and Lardy (10) for a similar model reaction of ATP formation during oxidation of N-acetylmethionine. Chemical experience would suggest the localization of an oxidative phosphorylation reaction in a lipophilic non-aqueous microenvironment of a protein or lipid structure.

As to the adenylate kinase like effect reference can be made to the action of hemin on ADP in dry pyridine, which yielded up to 7 % ATP. This reaction of a heme center, which can be demonstrated only in the presence

of both the central iron atom and the two free carboxyl groups of the protoporphyrin IX (11), may explain the formation of ATP. An alternative explanation may be contamination of the cytochrome c preparation with adenylate kinase.

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