THE USE OF PHENAZINE METHOSULFATE IN THE STUDY OF OXIDATIVE PHOSPHORYLATION.

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Phenazine methosulfate (PMS) was introduced by Kearney and Singer (1956) as an electron acceptor in the succinic dehydrogenase assay, where PMS accepts electrons from the flavin and is reoxidized by molecular oxygen. In these assays cyanide is routinely added to secure a non-enzymic oxidation of the dye. PMS has also been used in the study of photosynthetic phosphorylation (Geller, 1958). Finally the use of PMS as an artificial electron acceptor in the study of oxidative phosphorylation in submitochondrial particles has been proposed (Smith and Hansen, 1962).

We have introduced (Löw et al 1963) reduced PMS as a source of electrons in the study of the phosphorylation coupled to the passage of electrons through the cytochrome c oxidase region of the respiratory chain, in particles derived from beef-heart mitochondria by sonic treatment.

In table I the effect of PMS on oxidative phosphorylation in submitochondrial beef-heart particles is shown. The concentration is within the range used for the assay of succinic dehydrogenase. At this concentration the dye causes a partial uncoupling of the phosphorylation, whereas oxygen uptake is largely unaffected by the dye, both with generated DPNH or succinate as the substrate. The oxygen uptake under the conditions used in table I is slowed down only slightly by the addition of cyanide, whereas the phos-

phorylation on the other hand is completely eliminated by this addition. On the other hand the phosphorylation in the presence of PMS was unimpaired by antimycin A and by Amytal, inhibitors acting in the cytochrome b region and flavinregion respectively, which can be taken as an indication that the phosphate uptake is coupled to the cytochrome c oxidase of the respiratory chain.

Table I. The influence of phenazine methosulfate on oxidative phosphorylation.

Source of electrons	Additions			Cyanide, 10^{-3}M	
		Oxygen consumption μatoms	P_i-uptake μmoles	Oxygen consumption μatoms	P_i-uptake μmoles
DPNH (generated)	none	4.8	6.4	0.0	-
	PMS	6.3	2.5	2.5	0.2
	PMS + antimycin A	6.0	3.1	3.1	0.2
	PMS + Amytal	6.1	1.8	4.3	0.3
Succinate	none	5.2	6.1	0.0	-
	PMS	5.6	1.5	5.5	0.2
	PMS + antimycin A	4.1	1.5	5.3	0.3

The oxygen consumption was measured manometrically. Each reaction vessel contained 10 mM P-32 labelled phosphate buffer pH 7.5, 2 mM $MgCl_2$, 1 mM ATP, 0.25 M sucrose, 50 mM glycyl-glycine pH 7.5, 60 mM glucose, hexokinase in excess and 0.4 mg submitochondrial particle protein. Submitochondrial particles were prepared according to Löw and Vallin (1963). Further additions, either alcohol dehydrogenase corresponding to 100 μg of solid material, DPN to 0.33 mM and semicarbazide 10 mM or succinate 10 mM. Where indicated 0.4 mM PMS, 1 mM cyanide, 2 mM Amytal or 1.25 μg of antimycin A /mg particle protein were present. As a compensation for the antimycin A addition each vessel further contained 0.01 ml ethanol. Incubation time, 20 minutes at 30°C with a final volume of 1.0 ml. The oxygen consumptions correspond to the actual figures measured without subtraction of any blanks.

We are aware that with the concentration of DPN used, the reduction of PMS might to a smaller or greater extent be non-

enzymatic. In the presence of Amytal, the reduction of PMS is very likely completely due to a non-enzymatic reduction of PMS by DPNH.

With realization of the possibility that the observed phosphorylation was coupled to the cytochrome oxidase the use of flavosubstrate for the reduction of PMS became less desirable. In figure 1, ascorbate was used to reduce varying concentrations of PMS and the enzymic reduction was compared with the non-enzymic.

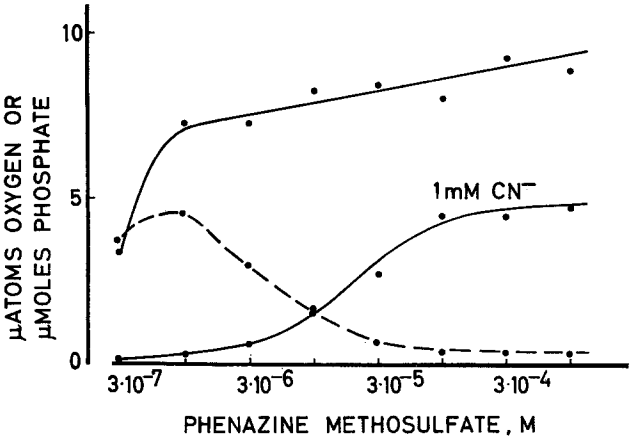

Figure 1. Oxidative phosphorylation mediated by varying concentrations of PMS. Oxygen consumption measured by Warburg technique. The incubation medium contained 1 mM ATP, 2 mM $MgCl_2$, 0.25 M sucrose, 10 mM P-32 labelled phosphate buffer pH 7.5, 50 mM glycyl-glycine pH 7.5, PMS as indicated, 15 mM ascorbate, 30 mM glucose, hexokinase corresponding to 0.01 mg of crystalline material and 1 mg particle protein. Where indicated KCN was added to 1 mM concentration. The final volume was 1.0 ml and the vessels were incubated for 20 minutes at 30°C. Solid lines refer to oxygen consumption. The dashed line refers to the phosphate uptake in the absence of cyanide.

As seen in the figure, at low concentrations the dye is only oxidized enzymatically, not non-enzymatically. The phosphorylation is uncoupled with increasing concentrations and this seems to be due to a toxic effect of the dye, rather than due to the increasing autooxidation. What clearly emerges from the

figure is that if the PMS-concentration is kept low, the dye can be used without interference of autooxidation.

The phosphorylation, which at low concentrations of the dye gives a P/O-ratio which approaches 1, is sensitive to 2,4-dinitrophenol and to oligomycin (table II) in concentrations which are typical for the inhibition of phosphorylations associated with the respiratory chain. The oxidation as well as the phosphorylation is unaffected by Amytal and antimycin A (table II) which classifies the phosphorylation as coupled to the span between cytochrome c and oxygen in the respiratory chain.

Table II. Effect of various inhibitors on oxidative phosphorylation mediated by phenazine methosulfate (PMS).

Addition	μatoms O	μmoles P
- - -	7.0	3.7
Antimycin A 0.3 μg/mg prot.	6.2	3.6
Amytal, 2 mM	5.3	2.8
Oligomycin 0.5 μg/mg prot.	6.9	0.1
- - -	5.2	2.8
DNP 0.1 mM	4.9	0.0

Conditions as in fig. 1. Antimycin A or oligomycin were added as 10 μl of alcoholic solutions. $3 \cdot 10^{-6}$ M PMS was added.

The point of entrance of electrons into the electron transport chain seems to be either cytochrome c or cytochrome c_1, since the respiration is antimycin insensitive (table II) but cyanide sensitive (figure 1).

This is further underlined by the fact that the reduced dye can be used as a source of electrons for the energy dependent reduction of DPN (table III). This reduction is sensitive to antimycin A, indicating that in this system the point of entry

Table III. The action of inhibitors on PMS-linked DPN reduction.

Inhibitor	Concentration	μmoles DPN reduced/min./ mg protein	% inhibition
Amytal	-	0.156	-
	0.125 mM	0.113	27.8
	0.25 mM	0.070	55.2
	0.50 mM	0.025	83.6
Rotenone	-	0.155	-
	0.024 μg/mg prot.	0.042	72.9
	0.048 μg/mg prot.	0.020	87
Antimycin A	-	0.180	-
	0.15 μg/mg prot.	0.156	13.6
	0.18 μg/mg prot.	0.107	40.9
	0.22 μg/mg prot.	0.025	86.1

The reduction of DPN was followed as an increase in absorption at 340 mμ in a Beckman DK-2 spectrophotometer with the cuvette chamber maintained at 30°C. Incubation medium: 50 mM Tris-HCl pH 8.0, 6 mM $MgCl_2$, 0.25 M sucrose, 1 mM KCN, 1 mM ATP and particles corresponding to 0.15 mg of protein/ml. This mixture was preincubated for 6 minutes whereupon the reduction was started by addition of PMS to $1.66 \cdot 10^{-6}$M, ascorbate 5 mM and DPN 1.5 mM concentrations. Rotenone and antimycin A were added as 1 μl of alcoholic solutions. Final volume 3.0 ml.

of electrons should be either on $\underline{c}_1$ or $\underline{c}$ as antimycin A blocks the path of electrons between cytochrome $\underline{b}$ and cytochrome $\underline{c}$.

In view of the present findings the use of PMS as an acceptor of electrons from the dehydrogenase end of the respiratory chain is not a tool for the study of the phosphorylations in this region (Smith and Hansen, 1962; Racker, 1963), since in the presence of cyanide in concentrations high enough to secure a terminal block of electrons no phosphorylation seem to occur.

On the other hand the PMS-ascorbate system opens new possibilities for the study of the phosphorylation coupled to the cytochrome c oxidase in submitochondrial particles.

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