Studies on menadione reduction and reoxidation with *Azotobacter vinelandii*

The respiratory-chain preparations of *Azotobacter vinelandii*, called washed small particles (WSP) by TISSIÈRES[1-3], differ in many respects[4] from the respiratory particles obtained by disruption of mammalian mitochondria (KEILIN AND HARTREE heart-muscle preparation[5]).

One of these differences is that the *Azotobacter* particles contain a very active DPNH-menadione reductase, whereas the heart-muscle preparation is very poor in catalysing this reaction[6]. On the other hand, COLPA-BOONSTRA AND SLATER[6] demonstrated that the same heart-muscle particles were able to catalyse very actively the oxidation of reduced menadione (K_3H_2) and that in rat-heart sarcosomes this oxidation was coupled with phosphorylation.

In the present communication it is shown that also the WSP fraction of *Azotobacter vinelandii* catalyses the oxidation of K_3H_2 in a reaction coupled with the synthesis of ATP.

Methods of preparing substrates and enzyme fractions have been described in previous papers[1-7].

DPNH oxidation in the presence of WSP was measured by following the decrease in absorbancy at 340 mμ with a Zeiss spectrophotometer and expressed in terms of equivalent μl O_2/mg protein/h (Q_{O_2}). The reactions were started by adding the WSP fraction in a suitably diluted suspension to the spectrophotometer cuvette containing DPNH and the other components of the reaction mixture listed in Table I.

TABLE I

INFLUENCE OF MENADIONE ON DPNH OXIDASE OF WSP IN THE PRESENCE AND ABSENCE OF KCN

Final concentrations of reagents: DPNH, 10^{-4} *M* (only in test cuvette); phosphate buffer (pH 7.0), 0.04 *M*; ethanol, 1 % (v/v); WSP, diluted in distilled water, 3 mg protein/ml; K_3 and KCN as shown. Temperature of Expt. 1, 28.9°; cf Expt. 2, 22.1°.

Expt.	K_3 (10^{-4} *M*)	*KCN* (10^{-2} *M*)	Q_{O_2}
1	—	—	3000
	—	+	0
	+	—	700
	+	+	745
	+*	—	3290
	+*	+	3010
2	—	—	1400
	+	—	1355
	+	+	835
	+*	—	2440
	+*	+	1140

* K_3 added 10-50 sec after WSP.

When K_3 was added after the enzyme the rate of the reaction was either the same or higher than in the absence of K_3 (Table I); in no case was an inhibition by K_3 observed.

Abbreviations: WSP, washed small particles; DPNH, reduced diphosphopyridine nucleotide; AMP, ADP, ATP, adenosine mono-, di- and triphosphate; K_3, 2-methyl-1,4-naphthoquinone; K_3H_2, 2-methyl-1,4-naphthohydroquinone.

When, however, K_3 was present before the enzyme was added the DPNH oxidase was sometimes inhibited considerably. This probably means that K_3 is inhibitory if the enzyme (presumably the flavoprotein) is oxidized, but not when it is reduced as is the case when K_3 is added after the enzyme. The oxidation of DPNH in the presence of K_3 at pH 7.0 is largely cyanide-insensitive (Table I).

Manometric experiments with the same type of enzyme preparation and succinate as the substrate revealed that the oxidation of succinate by K_3 was only a few percent of the succinic oxidase activity. This result is similar to that obtained with heart-muscle preparation, and shows that the K_3 reductase, which is present in the bacterial preparation but not in the mammalian, does not work with succinate as the substrate. Other examples of a DPNH–K_3 reductase have been reported by WOSILAIT AND NASON[8] in *Escherichia coli*, by WEBER *et al.*[9] in *Mycobacterium phlei*, and by ERNSTER *et al.*[10] in liver.

K_3H_2 is rapidly oxidized by the WSP fraction of *Azotobacter vinelandii* at pH 6.24 where the autooxidation of K_3H_2 is negligible[6]. The reaction was completely inhibited by 10^{-2} *M* KCN. The course of the reaction follows first-order kinetics and the K_3H_2 oxidase activity, expressed in terms of the first-order velocity constant k ($min^{-1} \times [mg\ protein/ml]^{-1}$), was 20–25 at 28°, not very different from the value of 10–15 at 22° obtained with heart-muscle preparation[6]. This is in contrast to the difference in DPNH oxidase activities of both types of preparations, the bacterial preparation being 10–15 times more active than the mammalian. Antimycin A, in concentrations much higher than necessary completely to inhibit the oxidation of DPNH, K_3H_2 or succinate by heart-muscle preparation[6], had no effect on the oxidation of these substrates by the *Azotobacter* particles. The DPNH oxidase activity of these particles was also insensitive to Amytal (1.8 m*M*). The insensitivity of *Azotobacter* preparations to antimycin is in agreement with the work of REPASKE AND JOSTEN[11] and BRUEMMER *et al.*[12].

The results presented in Table II show that oxidation of K_3H_2 by WSP is accompanied by phosphorylation, the P:O ratios varying between the narrow limits, 0.19 to 0.22. The ratio obtained with DPNH as substrate in the same experiment was sometimes the same, sometimes much higher. Thus it seems that the phosphorylative activity accompanying the oxidation of DPNH is more labile than that accompanying K_3H_2 oxidation. Oxidative phosphorylation with DPNH as substrate was uncoupled by K_3 (*cf.* PINCHOT[13], who found a similar uncoupling with *Alcaligenes faecalis*). The degree of uncoupling was not affected by pre-incubation of the WSP with K_3 for 45 min. It has been suggested[9] for *Mycobacterium phlei* that there are two different pathways for the oxidation of DPNH in the presence of K_3. Though this possibility is not excluded for the *Azotobacter* particle, it is clear from the results presented in Table II, Expts. 2 and 3, that this cannot be an explanation for the uncoupling of phosphorylation by K_3, since such a sidepath would not cause P:O ratios lower than those obtained with K_3H_2 as substrate. In this respect it must be emphasized that these experiments were carried out at pH 6.24, where the autooxidation of K_3H_2 formed from DPNH and K_3 is very low, so that any reoxidation of K_3H_2 would need an enzymic pathway. A more likely explanation is that menadione reacts with an enzyme necessary for phosphorylation, thus acting as a real uncoupling substance. This appears to be in accordance with the finding of BRODIE AND BALLANTINE[14] that vitamin K_1 can restore phosphorylation in light-inactivated preparations of

TABLE II

OXIDATIVE PHOSPHORYLATION BY THE WSP FRACTION OF *Azotobacter vinelandii* WITH K_3H_2 OR DPNH AS SUBSTRATE

The procedure has been described in ref. 3, 6. Reaction mixture: phosphate, 0.03 *M*; glucose, 0.007 *M*; ethylenediaminetetraacetate, 0.001 *M*; $MgCl_2$, 0.005 *M*; AMP, 0.0002 *M*; ADP, 0.0002 *M*; hexokinase; DPNH, K_3 and K_3H_2 as indicated. The pH was 6.24, the reaction volume 2.6 ml. Trichloroacetic acid was added after the substrate was completely oxidized and Δ (esterified phosphate) measured as described in ref. 7.

Expt.	*Substrate*		*Addition K_3 (mM)*	*P:O*
	DPNH (mM)	*K_3H_2 (mM)*		
1	0.45	—	—	0.43
	—	0.42	—	0.19
2	0.76	—	—	0.22
	0.76	—	0.1	0.08
	—	0.65	—	0.22
3	0.74	—	—	0.24
	0.74	—	0.1	0.08
	—	0.65	—	0.21
	—	0.65	0.1	0.18
4	0.68	—	—	0.49
	0.68	—	0.1	0.33
	—	0.77	—	0.22
	—	0.77	0.1	0.19

Mycobacterium phlei, and that this phosphorylation is competitively inhibited by lapachol or dicoumarol. It seems possible that the phosphorylative step(s) of the respiratory chain measured with DPNH as substrate is not the same as that measured with K_3H_2 and that the former is more susceptible to uncoupling by K_3.

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Laboratory of Physiological Chemistry, University of Amsterdam (The Netherlands)

D. J. J. SCHILS
H. G. HOVENKAMP
J. P. COLPA-BOONSTRA

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