THE POSSIBLE ROLE OF VITAMIN K IN THE RESPIRATORY CHAIN

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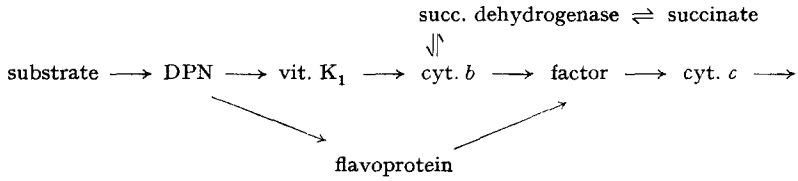
On the basis of experiments with mitochondria isolated from the livers of vitamin K_1-deficient chicks, MARTIUS AND NITZ-LITZOW[1] postulated that vitamin K_1 is concerned in oxidative phosphorylation. A little later, MARTIUS[2] proposed that the vitamin actually entered the phosphorylating respiratory chain, transferring hydrogen (or electrons) according to the following scheme



According to this scheme**, the reaction between DPN (diphosphopyridine nucleotide) and the factor through flavoprotein represents a non-phosphorylating side-path.

If this correct, it follows that, in the presence of preparations of the respiratory chain:

(1) Reduced vitamin K_1 should be rapidly oxidized.

(2) Reduced vitamin K_1, or DPNH + vitamin K_1 in the presence of phylloquinone reductase (which catalyses the reaction between these two substances[3]) will rapidly reduce cytochrome *b*.

(3) Fumarate will rapidly oxidize reduced vitamin K_1, or, in the presence of vitamin K_1 and phylloquinone reductase, it will rapidly oxidize DPNH.

We have attempted to test these predictions. Unfortunately, we have been unable to prepare phylloquinone reductase, following the directions of MARTIUS[3]. The enzyme preparation made in this way showed activity with menadione (vitamin K_3) as hydrogen acceptor, but none with vitamin K_1 dispersed in Tween 80. The activity towards vitamin K_3 was not inhibited by Tween 80. The lack of activity towards vitamin K_1 is in agreement with the experience of WOSILAIT AND NASON[4] with their enzyme called menadione reductase.

We were able to prepare reduced vitamin K_1 by chemical reduction[5], but it was found very difficult to handle owing to auto-oxidation. Reduced menadione or vitamin K_3 (K_3H_2), also prepared chemically, was much easier to use. It was found that K_3H_2 is rapidly oxidized by respiratory chain preparations. The precise point at which K_3H_2 enters the respiratory chain has, therefore, been investigated in the

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** This scheme is modified from that given by MARTIUS[2] by introducing succinic dehydrogenase between succinate and cytochrome *b* and by making these reactions reversible.

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expectation that if vitamin K_1 is indeed a member of the chain, it will enter at the same point.

A preliminary account of part of this work has already appeared[6].

METHODS

Heart-muscle preparation was made from horse heart by the method of KEILIN AND HARTREE[7]. Heart sarcosomes were prepared from rat heart as described by CLELAND AND SLATER[8]. Rat liver mitochondria were prepared according to the method of HOGEBOOM[9]. Protein was determined by the biuret method[8]. K_3H_2 was prepared according to FIESER[5] by reduction of menadione (K_3) with $Na_2S_2O_4$ in ethereal solution and precipitation with petroleum ether. A greyish white powder was obtained (m.p. 160°–170° C). Reduced diphosphopyridine nucleotide (DPNH), adenosine diphosphate (ADP) and hexokinase were prepared as described by SLATER[10].

The DPNH oxidase activity (espressed as $Q_{O_2} = \mu l\ O_2$/mg protein/h) was measured spectrophotometrically as described by SLATER[11]. The method of determining K_3H_2 oxidase activity is described below.

Oxidative phosphorylation with K_3H_2 as substrate was measured exactly as described by TISSIÈRES, HOVENKAMP AND SLATER[12] for DPNH as substrate.

RESULTS

K_3H_2 oxidase system

The absorption spectra of K_3 and K_3H_2 are given in Fig. 1. The bands at 250, 262–263 and 340 mμ disappear on reduction, while new bands at 241 and 330 mμ appear*. A wavelength of 262 mμ was chosen as the most suitable for following the oxidation of K_3H_2.

The pH used for studying the oxidation of K_3H_2 was 6.24. This was a compromise

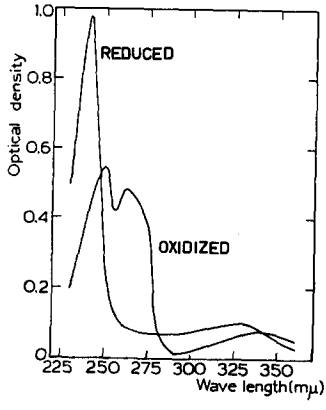

Fig. 1. Absorption spectra of oxidized and reduced vitamin K_3. K_3 and K_3H_2, $2.9 \cdot 10^{-5}$ *M*; phosphate buffer, pH 6.24, 0.04 *M*; ethylenediamine tetraacetate (EDTA), 10^{-3} *M*. Optical path, 1 cm.

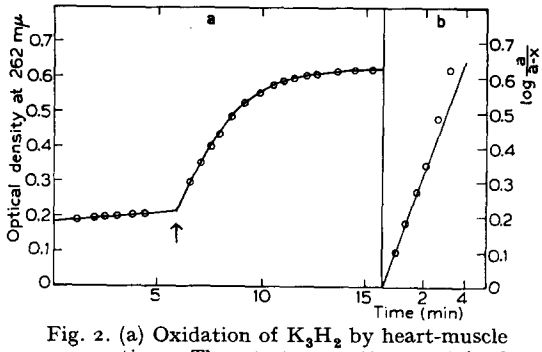

Fig. 2. (a) Oxidation of K_3H_2 by heart-muscle preparation. The test cuvette contained K_3H_2, $3.8 \cdot 10^{-5}$ *M*; phosphate buffer pH 6.24, 0.04 *M*; EDTA, 10^{-3} *M*. The reference cuvette contained the buffer and EDTA. Heart-muscle preparation (final concentration, 26 μg protein/ml) was added to both cuvettes at the arrow. Temp, 22.2° C. The optical densities have been corrected for the dilution caused by the addition of the heart-muscle preparation. (b) Data of *a* plotted according to first order equation $k't = 2.3 \log a/(a - x)$, where a = concentration of K_3H_2 originally present and x = concentration of K_3H_2 that has been oxidized in t min.

* The shape of the K_3H_2 spectrum is given only for the sake of comparison. Because of the auto-oxidizability of K_3H_2, the optical density was measured by the same extrapolation procedure as described on p. 128 for the succinate-K_3 reaction. The optical densities are more accurate at those wavelengths measured within a short time after preparing the solution (262 and 250 mμ) than at the other wavelengths.

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between higher pH's where the oxidase activity is higher, but where the auto-oxidation of K_3H_2 is too great for reliable studies of the enzymic reaction, and lower pH's where the auto-oxidation rate is very low, but the oxidase activity of the enzyme preparation is also suppressed.

The method of measuring the K_3H_2 oxidase activity is described in Fig. 2. After determining the rate of auto-oxidation, the enzymic reaction was started by adding a suitably diluted heart-muscle preparation to each cuvette. After the completion of the reaction, the spectrum was identical with that of K_3. The course of the oxidation followed first order kinetics, and the activity of the enzyme was expressed in terms of the first order velocity constant, k'. Doubling the initial concentration of K_3H_2 doubled the initial rate of oxidation, k' remaining the same (see Fig. 3).

Since first order kinetics were obtained with K_3H_2 and zero order kinetics are found with comparable concentrations of DPNH, it is not possible to compare the specific activities of the two oxidase systems. The oxidation of K_3H_2 is, however, a rapid reaction, the initial rate of oxidation in the experiment illustrated in Fig. 2 corresponding to a Q_{O_2} of 365 at pH 6.24 and 22.2° C, compared with 284 for the DPNH oxidase at pH 7.3 and 20° C. A good quantitative correlation was found between the K_3H_2 oxidase and DPNH oxidase activities of different heart-muscle preparations (Fig. 4).

The effect of added cytochrome *c* on the reaction rate could not be studied since, like other hydroquinones, K_3H_2 rapidly reduces ferricytochrome *c* in the absence of heart-muscle preparation. However, unlike other hydroquinones and reducing agents such as ascorbic acid, K_3H_2 does not need the addition of soluble cytochrome *c* for its oxidation in the presence of heart-muscle preparation. In this respect, K_3H_2 resembles the naturally occurring substrates succinate and DPNH and the artificial substrate *p*-phenylenediamine. However, the oxidation of K_3H_2 resembles more that of DPNH and succinate than that of *p*-phenylenediamine, since it is completely inhibited by antimycin A. The relative sensitivities of the different

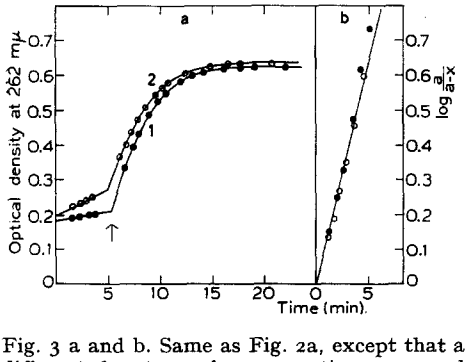

Fig. 3 a and b. Same as Fig. 2a, except that a different heart-muscle preparation was used (27 μg protein/ml). Curve 1, 1 cm optical path; K_3H_2, $3.8 \cdot 10^{-5}$ *M*. Curve 2, 0.5 cm optical path; K_3H_2, $7.6 \cdot 10^{-5}$ *M*. b. Data plotted in same way as Fig. 2b.

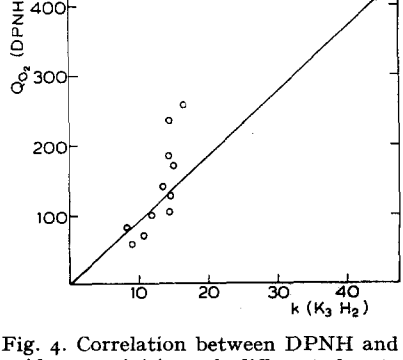

Fig. 4. Correlation between DPNH and K_3H_2 oxidase activities of different heart-muscle preparations. DPNH oxidase activity is expressed as Q_{O_2} (μl O_2/mg protein/h) at 20° C. K_3H_2 oxidase activity is expressed as the first order rate constant (22° C) k (min^{-1} × [mg protein/ml]$^{-1}$), where $k = k'/w$ and w = concentration of protein (mg/ml). k' can be calculated from the first order equation $k't = 2.3 \log a/(a - x)$ (see Fig. 2b and 3b).

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oxidase systems to antimycin are compared in Table I. The K_3H_2 oxidase system is the most sensitive; the DPNH oxidase system is considerably more sensitive than the succinic oxidase system, while as would be expected from the work of POTTER AND REIF[15], the oxidation of *p*-phenylenediamine was completely unaffected.

The K_3H_2 oxidase system was completely inhibited by cyanide (95% inhibition by 0.001 *M* cyanide and 99.5% inhibition by 0.01 *M* cyanide).

TABLE I

SENSITIVITY OF OXIDASE SYSTEMS TO ANTIMYCIN A

Concentrated heart-muscle preparation (35.5 mg protein/ml) was treated with different amounts of antimycin according to Method II of THORN[13] and after dilution, the rates of oxidation of succinate, DPNH and K_3H_2 were determined on the same suspension. The experiment with *p*-phenylenediamine was carried out with the same heart-muscle preparation treated separately with antimycin. The rates of oxidation of succinate, DPNH and *p*-phenylenediamine were measured as previously described[14,11].

Antimycin (μg/mg protein)	*Inhibition (%) of oxidation of*			
	succinate	*DPNH*	*K_3H_2*	*p-phenylenediamine*
0.07	22	24	46	0
0.105	41	80	85	—1
0.14	99	97	91	—1

Observations with micro-spectroscope

When K_3H_2 (final concentration, $2 \cdot 10^{-3}$ *M*) was added to a concentrated suspension of the heart-muscle preparation in the presence of air, the bands of cytochromes *a*, *b* and *c* appeared within a few seconds. (A few seconds are required to deplete the suspension of oxygen by reaction with K_3H_2). The speed of reduction, and the final spectrum were very similar to those obtained with succinate under comparable conditions.

K_3 as hydrogen acceptor

Since the same particles catalyse the oxidation of DPNH, succinate and K_3H_2, it might be expected that, in the absence of oxygen, they would also catalyse reactions between the various substrates. A slow oxidation of DPNH by fumarate under these conditions has already been reported[11]. Table II, which describes an experiment

TABLE II

EFFECT OF VITAMIN K_1 ON THE RATE OF OXIDATION OF DPNH BY FUMARATE

Phosphate buffer pH 7.3, 0.03 *M*; EDTA, 10^{-3} *M*; DPNH, 10^{-4} *M*; KCN, 0.01 *M*; fumarate 0.01 *M*. Vitamin K_1 (where indicated), 10^{-5} *M*, Tween 80 (where indicated), 0.0003 %; heart-muscle preparation. Temp, 20° C.

Addition	*Rate of oxidation of DPNH (ΔD_{340} mμ/min)*
none	0.0110
Tween	0.0068
Vitamin K_1 + Tween	0.0068

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carried out by Dr. P. E. GREENGARD in the Molteno Institute, University of Cambridge, shows that vitamin K_1 had no effect on the DPNH-fumarate reaction under these conditions.

DPNH-K_3 reductase

The oxidation of DPNH by K_3 was measured by following the decline of the absorption at 340 mμ of the DPNH in a Thunberg-Beckman cuvette, after removal of oxygen as described by SLATER[16]. During the evacuation procedure, the main tube of the Thunberg-Beckman cuvette containing the K_3 (or, in some experiments, vitamin K_1) was protected from light to avoid decomposition of these compounds. The reaction was begun by tipping in the heart-muscle preparation from the hollow stopper. After measuring the anaerobic reaction for about 10 min, the tube was opened and the rate of the aerobic reaction was then measured. The difference in absorption of K_3 and K_3H_2 at 340 mμ is so small that no correction is necessary (Fig. 1).

Table III includes measurements with vitamin K_1, dispersed in Tween 80, as well as with K_3. The effect of Tween 80 on the reaction with K_3 was also studied. The results clearly show that, even in the presence of Tween 80, DPNH reacts with K_3, but no reaction could be demonstrated with vitamin K_1, even in the presence of five times the protein concentration. Expts. 4 and 5, Table III, show that Tween 80 has a much more powerful inhibitory action on the DPNH oxidase system than on the DPNH-K_3 reductase.

TABLE III

DPNH-K_3 REDUCTASE ACTIVITY OF A KEILIN AND HARTREE HEART-MUSCLE PREPARATION

Final concentrations of reagents in main flask of Thunberg-Beckman cuvette: DPNH, 10^{-4} *M*; vitamin K_1, K_3 and Tween 80 as indicated; phosphate buffer pH 7.4, 0.04 *M*; EDTA, 10^{-3} *M*; at zero time the heart-muscle preparation (amount as indicated) was tipped in from the hollow stopper.

Expt. No.	*Vitamin K_1 (10^{-4} M)*	*K_3 (10^{-4} M)*	*Tween 80 (0.5 mg/ml)*	*Protein (mg/ml)*	*ΔD_{340} mμ/min*	
					absence of O_2	*presence of O_2*
1	—	—	—	0.026	0.000	0.040
2	+	—	+	0.026	0.000	0.0016
3	+	—	+	0.130	0.000	0.008
4	—	+	—	0.026	0.007	0.062
5	—	+	+	0.026	0.005	0.007

Since the DPNH oxidase activity of the heart-muscle preparation was damaged somewhat by the evacuation procedure, the relative activities of the various systems oxidizing DPNH were compared in another series of experiments, in which the oxidase activity was nearly completely inhibited by cyanide or antimycin (Table IV). After measuring the inhibited rate, in the absence of acceptor, for about 5 min, fumarate was added to both cuvettes and the rate again determined for about 5 min. K_3 was then added, and a further measurement of the rate made. In a second series of experiments, the order of addition of fumarate and K_3 was changed. No catalytic effect of K_3 on the DPNH-fumarate reaction was observed, the rate of the reaction in the presence of both the acceptors fumarate and K_3 being the sum of the rates with each alone.

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TABLE IV

REACTION VELOCITIES FOR DPNH-OXIDATION WITH DIFFERENT HYDROGEN-ACCEPTORS

The test cuvette contained: DPNH, 10^{-4} M; EDTA, 10^{-3} M; phosphate buffer pH 7.4, 0.04 M; KCN, 10^{-2} M or antimycin A, 0.4 μg/mg heart-muscle protein; fumarate, 10^{-2} M; K_3, $6 \cdot 10^{-5}$ M; heart-muscle preparation, 0.122 mg/ml. The rates have been corrected for dilution caused by the various additions of reagents and are given for a final volume of 3 ml. Brackets indicate determination in a single cuvette. In the reference cell only DPNH was omitted. Temp. 28° C.

Exp. no.	*Hydrogen-acceptor*	*Inhibitor*	ΔD_{340} *mμ/min*	*Difference*	*% of DPNH-oxidase activity*
1	O_2	—	0.570		
	O_2	KCN	0.004		0.7
	Fumarate	KCN	0.012	0.008	1.4
	Fumarate + K_3	KCN	0.039	0.027	4.7
2	O_2	—	0.570		
	O_2	KCN	0.004		0.7
	K_3	KCN	0.034	0.030	5.3
	K_3 + Fumarate	KCN	0.037	0.003	0.5
3	O_2	—	0.285		
	O_2	Antimycin A	0.005		1.7
	Fumarate	Antimycin A	0.009	0.004	1.4
	Fumarate + K_3	Antimycin A	0.027	0.018	6.3
4	O_2	—	0.285		
	O_2	Antimycin A	0.005		1.7
	K_3	Antimycin A	0.022	0.017	6.0
	K_3 + Fumarate	Antimycin A	0.025	0.003	1.1

In agreement with earlier measurements, the rate of the DPNH-fumarate reductase reaction was found to be about 1.4% of the DPNH oxidase activity. The DPNH–K_3 reductase was somewhat greater, amounting to about 5–6% of the DPNH oxidase activity. The fact that these percentages were about the same whether antimycin or cyanide was the inhibitor shows that antimycin does not inhibit these anaerobic reactions.

Succinate-K_3 reductase

On the basis of oxidation-reduction potentials, the reaction

$$\text{succinate} + K_3 \rightleftharpoons \text{fumarate} + K_3H_2$$

could be equally easily studied in both directions. However, the fact that fumarate but not succinate absorbs in the ultra-violet light made it much easier to use succinate as substrate in the reaction.

The reduction of K_3 by succinate was followed at 262 mμ. The relationship between the optical density change ΔD in a 1 cm cell at this wavelength and the changes in the concentrations (mole·cm^{-3}) of K_3, K_3H_2 and fumarate is given by the following equation.

$$\Delta D = \Delta[K_3] \cdot \varepsilon_{K_3} + \Delta[K_3H_2] \cdot \varepsilon_{K_3H_2} + \Delta[\text{fumarate}] \cdot \varepsilon_{\text{fum.}},$$

where ε_{K_3}, $\varepsilon_{K_3H_2}$ and $\varepsilon_{\text{fum.}}$ are the extinction coefficients at 262 mμ.

$$\text{Since } \Delta[K_3] = -\Delta[\text{fumarate}] = -\Delta[K_3H_2],$$

$$\Delta D = \Delta[K_3]\{\varepsilon_{K_3} - \varepsilon_{K_3H_2} - \varepsilon_{\text{fum.}}\},$$

$$i.e.\ \Delta[K_3] = \frac{\Delta D}{\varepsilon_{K_3} - \varepsilon_{K_3H_2} - \varepsilon_{\text{fum.}}}$$

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The value of $\varepsilon_{K_3H_2}$ was determined by following for 5 min the auto-oxidation of a freshly prepared solution and extrapolating the optical density to zero time. The respective values at 262 mμ for K_3, K_3H_2 and fumarate were found to be $1.66 \cdot 10^7$, $0.31 \cdot 10^7$ and $0.084 \cdot 10^7$ cm^2 $mole^{-1}$ respectively. Thus

$$\Delta[K_3] = \frac{\Delta D}{1.27 \cdot 10^7}$$

The reaction was carried out in a Thunberg-Beckman cuvette. The change of optical density at 262 mμ was first followed in the absence of air. After 15 min, the tube was opened and the rate determined in the presence of air (Fig. 5, curve 1). When air was admitted, there was a rapid increase of the optical density to the original value, owing to re-oxidation of K_3H_2 by oxygen (the fumarate produced during the anaerobic reaction makes only a very small contribution to the extinction at 262 mμ). The further slow increase in the optical density is due to oxidation of succinate to fumarate by the succinic oxidase sytem.

Fig. 5, curve 2 shows that malonate inhibits the anaerobic reaction by 80% indicating the participation of succinic dehydrogenase. Table V shows that succinate is oxidized by K_3 at about 3% of the rate of the succinic oxidase system.

TABLE V

COMPARISON OF THE RATES OF SUCCINATE OXIDATION BY HEART-MUSCLE PREPARATION WITH EITHER K_3 OR OXYGEN AS THE FINAL HYDROGEN-ACCEPTOR

Final concentrations of reagents in the main compartment of the Thunberg-Beckman cuvette: EDTA, 10^{-3} *M*; phosphate buffer pH 7.4, 0.04 *M*; succinate, 0.001 *M*; K_3, 10^{-4} *M*; heart-muscle preparation (tipped from hollow stopper at zero time), 0.083 mg protein/ml. Reference cell: same reagents without succinate and K_3. Temp. 26.6° C.

Malonate (M)	*Rate of oxidation of succinate (μmoles/l/min)*	
	by K_3	*by O_2*
0	0.76	26.2
0.01	0.14	0.24

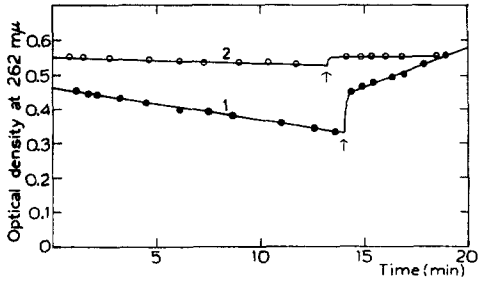

Fig. 5. Reduction of K_3 by succinate. Curve 1, no malonate; curve 2, in the presence of 0.01 *M* malonate. Heart-muscle preparation added at zero time, air admitted at the arrow. Other experimental conditions as in Table V.

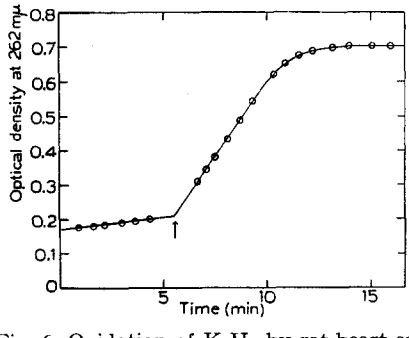

Fig. 6. Oxidation of K_3H_2 by rat-heart sarcosomes. Experimental conditions same as in Fig. 2, except for the addition of rat-heart sarcosomes instead of horse heart-muscle preparation at the arrow. Sarcosomes, 48.5 μg protein/ml; K_3H_2, $4.4 \cdot 10^{-5}$ *M*. Temp. 23.2° C.

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Oxidative phosphorylation with K_3H_2 as substrate

Fig. 6 shows that K_3H_2 can also be oxidized by intact mitochondria (sarcosomes) isolated from rat heart. A peculiar difference between this reaction and that obtained with the sarcosomal fragments (KEILIN AND HARTREE heart-muscle preparation) is that the former follows zero order kinetics until nearly all the K_3H_2 was oxidized, in contrast with the first order kinetics obtained with the latter preparation and the same concentration of K_3H_2. The Q_{O_2} with the rat sarcosomes (ranging from 40 to 150, with a mean of 76) was much lower than that of the KEILIN AND HARTREE horse heart-muscle preparation (about 400). With liver mitochondria, the oxidation was very slow (Q_{O_2} about 6). After ageing of the mitochondria, followed by repeated freezing and thawing, according to the procedure of MYERS AND SLATER[17], higher activities were obtained (40 with liver, 200 with heart). It appears that K_3H_2, like ferrocytochrome *c*, does not readily penetrate intact mitochondria. First order kinetics were again obtained with these mitochondria or sarcosomes which had been aged, frozen and thawed.

The oxidation of K_3H_2 by rat-heart sarcosomes was accompanied by phosphorylation when a suitable phosphate-acceptor system was added (Table VI). In these experiments, the oxidation of K_3H_2 could not be followed directly, because ADP in the reaction mixture absorbs too strongly at 262 mμ. The time required for complete oxidation of the K_3H_2 was, therefore, determined in a separate control experiment, in which ADP was omitted. The P:O ratios are calculated on the assumption that all the K_3H_2 initially added was oxidized by the enzyme preparation.

The phosphorylation observed with K_3H_2 was completely sensitive to 2:4 dinitrophenol.

TABLE VI

PHOSPHORYLATION OF ADP COUPLED WITH THE OXIDATION OF K_3H_2 BY RAT-HEART SARCOSOMES

The reaction vessel contained in 2.5 ml (final concentrations): phosphate buffer pH 6.24, 0.03 *M*; glucose, 0.0064 *M*; EDTA, 0.001 *M*; NaF, 0.04 *M*; adenosine monophosphate, $2 \cdot 10^{-4}$ *M*; ADP, $1.8 \cdot 10^{-4}$ *M*; $MgCl_2$, 0.0048 *M*; serum albumin, 0.04%; hexokinase 120 units[18]; rat-heart sarcosomes, 0.3–0.6 mg protein/ml. At zero time, the amount of K_3H_2 indicated was added. After a few minutes, when all the K_3H_2 had reacted*, 0.4 ml 40% trichloroacetic acid (TCA) was added to stop the reaction. To the zero time control, TCA was added before the sarcosomes and the K_3H_2. The precipitated proteins were removed by centrifugation, the solution brought to pH 7.0, and samples analysed for esterified P. Different experiment numbers refer to different preparations.

Expt. No.	*Dinitrophenol (M)*	*K_3H_2 added (μmole)*	*Δ esterified P*	*P:O*
1	0	0.28	0.16	0.56
2	0	0.25	0.21	0.85
3*a*	0	0.29	0.20	0.70
3*b*	10^{-4}	0.29	—0.01	0.00

* Determined in a control experiment (see text).

DISCUSSION

MARTIUS AND NITZ-LITZOW brought forward the following evidence in favour of the view that vitamin K_1 is concerned in oxidative phosphorylation. (1) Dicoumarol and other substances chemically related to vitamin K uncouple phosphorylation

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associated with the oxidation of β-hydroxybutryrate by liver mitochondria[19]. (2) Mitochondria isolated from the livers of vitamin K-deficient chicks give lower P:O ratios with β-hydroxybutyrate as substrate than obtained with normal mitochondria. Phosphorylation coupled with the oxidation of succinate was little affected[1]. (3) The addition of vitamin K_1 to the mitochondria from deficient chicks raised the P:O ratio to normal[1]. The activity of added vitamin K_3 in stimulating the analogous process of photosynthetic phosphorylation[20] could also be considered as support for MARTIUS' views.

On the other hand, COOPER AND LEHNINGER[21] have brought forward the following evidence against this hypothesis. Dicoumarol, which according to MARTIUS AND NITZ-LITZOW uncouples oxidative phosphorylation by inhibiting vitamin K_1, is just as effective against the phosphorylation which occurs in the region of cytochrome oxidase as against that in the region of pyridine nucleotide. Although it could be argued that dicoumarol has more than one action, this argument removes one of the three pieces of evidence in favour of the idea that vitamin K is concerned in oxidative phosphorylation.

In this paper, we do not wish further to discuss the general proposition that vitamin K_1 is concerned in oxidative phosphorylation. Rather, the discussion will be confined to a consideration of the specific role in the respiratory chain assigned to this vitamin by MARTIUS.

The evidence in favour of this specific role is: (1) The oxidation-reduction potential of vitamin K_1 lies between those of DPNH and cytochrome *b*. (2) The isolation of phylloquinone reductase[3]. (3) The ability of vitamin K_1, in the presence of phylloquinone reductase, to catalyse the reduction of cytochrome *b* by DPNH.

On the other hand, MARTIUS has brought forward no evidence that reduced vitamin K_1 can be oxidized by respiratory chain preparations. We have attempted to confirm MARTIUS' experiment with the microspectroscope on the catalytic effect of vitamin K_1, in the presence of phylloquinone reductase, on the reduction of cytochrome *b* by DPNH, but found this experiment very difficult to do. In the first place, we were unable to prepare the phylloquinone reductase. In the second place it was necessary in our experiments, as in those of MARTIUS, to disperse the vitamin K_1 in Tween 80. We sometimes found that Tween itself, like antimycin, was able to increase the intensity of the band of cytochrome *b*, presumably by increasing the proportion of the cytochrome *b* which is reduced in the steady state (*cf.* ref. 22).

Our investigations of the postulated function of vitamin K_1 as an intermediary carrier between DPNH and cytochrome *b* were, therefore, based on an investigation of the K_3H_2 oxidase system, on the assumption that K_3H_2 could be regarded as a "model" for the naturally occurring vitamin. Our results do not support the view that K_3 acts between DPNH and cytochrome *b*. On the contrary, they bring forward the following evidence against it.

(1) The anaerobic reaction between DPNH and K_3 is much slower than the aerobic oxidation of DPNH.

(2) The anaerobic reaction between DPNH and fumarate was not catalysed by the addition of K_3 (or of vitamin K_1).

(3) The anaerobic reaction between K_3 and succinate is much slower than the aerobic oxidation of K_3H_2, succinate or DPNH.

(4) The heart-muscle preparation which contains a very active DPNH oxidase

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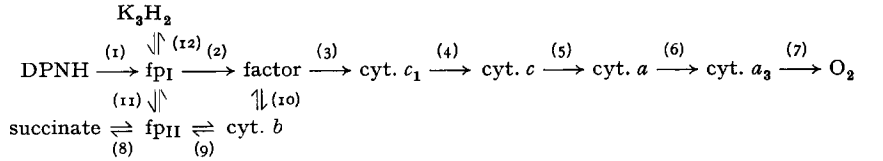
system contains no detectable vitamin K[23] (less than 0.01 μmole vitamin K/g protein compared with 0.8 μmole cytochrome *c*/g protein).

Although these results clearly show that K_3H_2 does not act directly between DPNH and cytochrome *b* in the non-phosphorylating sarcosomal fragments, the facts that the K_3H_2 oxidase system was inhibited by antimycin and cyanide and that the anaerobic reactions were not inhibited by antimycin suggest that K_3H_2 enters the respiratory chain in the region of the flavoprotein or cytochrome *b*. Its oxidation-reduction potential and the relatively low P:O ratio obtained with intact sarcosomes (of the same order as found with succinate) are consistent with this conclusion.

The various reactions discussed in this paper can be satisfactorily explained by the following extension of the previous reaction scheme[11] in the sarcosomal fragments



(fp_I and fp_{II} represent the flavoproteins, diaphorase and succinic dehydrogenase, respectively). This scheme differs from that previously proposed by the introduction of cytochrome c_1[24] and the addition of reactions (11) and (12). It is recognized that the role of cytochrome *b* is uncertain.

The oxidase systems comprise the following reactions:

DPNH oxidase. (1) to (7).

Succinic oxidase. (8), (9), (10), followed by (3) to (7).

K_3H_2 oxidase. (12), followed by (2) to (7). (The alternative or additional reaction of K_3H_2 with fp_{II} is not excluded)*.

The anaerobic reactions follow the following pathways:

DPNH ⟶ fumarate. (1), (2), (10), (9), (8) or (1), (11), (8). (This reaction is about 60% inactivated by BAL-treatment[11], but is not affected by antimycin).

DPNH ⟶ K_3. (1), (12).

Succinate ⟶ K_3. (8), (11), (12).

Antimycin (or Tween 80) by acting in the region of the factor could promote the reduction of cytochrome *b* by DPNH.

The position assigned to K_3H_2 is very different from that proposed by MAHLER *et al.*[25] These authors suggest that "quinonoid" compounds can act as a by-pass between the flavine prosthetic group of cytochrome reductase and cytochrome *c* or cytochrome oxidase. However, their scheme is clearly not consistent with the antimycin-sensitivity of the K_3H_2 oxidase system. The latter shows that K_3H_2, like other hydroquinones, cannot react directly with the endogenous cytochrome *c* of the particles, although it readily reduces soluble cytochrome *c*.

The very high activity of the K_3H_2 oxidase system in sarcosomes and sarcosomal fragments suggests that this may represent a model reaction of the naturally occurring vitamin. The failure to demonstrate reduction of vitamin K_1 by DPNH or succinate

* Recent experiments with amytal which inhibits the oxidation of DPNH, but not that of succinate[26] or of K_3H_2, suggest that K_3H_2 enters the respiratory chain by reaction with fp_{II} rather than with fp_I.

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under conditions in which K_3 was reduced is not conclusive evidence against this, since the water-insoluble vitamin K_1 might not penetrate to the active site. However, there is a strong possibility that the reactions described in this paper are entirely artificial. Flavoproteins are relatively unspecific with respect to their hydrogen-acceptors. With acceptors of the appropriate oxidation-reduction potential, the reaction will be easily reversible and one must expect that the reduced form of the acceptor can act as a hydrogen donor for respiratory chain preparations. Oxidative phosphorylation has often been demonstrated with artificial donors and acceptors.

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SUMMARY

1. At pH 6.24 where its auto-oxidation is very slow, reduced vitamin K_3 (K_3H_2) is rapidly oxidized in the presence of respiratory chain preparations. The oxidation was followed by measuring the increase of the optical density at 262 mμ.

2. With the KEILIN AND HARTREE heart-muscle preparation, the course of the oxidation followed first order reaction kinetics with respect to K_3H_2.

3. Unlike other hydroquinones and other reducing agents such as ascorbic acid, K_3H_2 does not require the addition of cytochrome *c* for its oxidation.

4. The enzymic oxidation of K_3H_2 is completely inhibited by cyanide and antimycin.

5. The heart-muscle preparation also catalyses anaerobic reactions between reduced diphosphopyridine nucleotide (DPNH) and K_3, and between succinate and K_3. However, the rates of these reactions are only a few percent of the DPNH, K_3H_2 or succinic oxidase activities. Addition of vitamins K_1 or K_3 has no effect on the rate of the anaerobic reaction between DPNH and fumarate, which is also catalysed by these preparations. None of the anaerobic reactions are inhibited by antimycin.

6. The oxidation of K_3H_2 by rat-heart sarcosomes (mitochondria) is accompanied by a dinitrophenol-sensitive oxidative phosphorylation.

7. The possible point of entry of K_3H_2 into the respiratory chain is discussed. The results do not support the view that K_3 acts as a hydrogen- or electron-carrier between DPNH and cytochrome *b*.

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THE POSSIBLE ROLE OF α-TOCOPHEROL IN THE RESPIRATORY CHAIN

II. REACTIVATION BY α-TOCOPHEROL

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In the previous paper of this series, it was shown that an active preparation of the respiratory chain, the KEILIN AND HARTREE heart-muscle preparation, as well as liver mitochondria, contained α-tocopherol in amounts of the same order of magnitude as of known components of the respiratory chain[1]. Thus, α-tocopherol fulfills the first of three criteria for a member of the respiratory chain which were listed. The present paper is concerned with the second criterion, namely that removal of a component of the respiratory chain from an enzyme preparation leads to inactivation of the chain, and the activity can be restored by the addition of the proposed component.

The studies of NASON and his co-workers[2-4] appeared to provide good evidence that α-tocopherol satisfied this second criterion. They found that extraction of a preparation of rat skeletal muscle with iso-octane inactivated the reduced diphosphopyridine nucleotide (DPNH) oxidase and DPNH–cytochrome *c* reductase, and that the activity could be specifically restored by the addition of either the lipid extracted from the enzyme preparation by iso-octane or of α-tocopherol.

In similar experiments with the KEILIN AND HARTREE heart-muscle preparation, we have found that the α-tocopherol, isolated from the heart muscle, and pure α-tocopherol were equally effective in restoring the activity of an iso-octane-extracted preparation. On the other hand, an examination of the effects of extraction with iso-octane and of the reactivation have led us to question whether these processes can be simply explained on the basis of removal and restoration of a specific component of the respiratory chain.

A preliminary account of some of these findings has been given[5].

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