In vivo inhibition of phosphorylation by menadione

Several recent publications by MARTIUS *et al.*[1,2,3] suggest that vitamin K_1 is involved in oxidative phosphorylation and electron transport. It has been demonstrated that vitamin K_1 *in vitro*[3] or menadione (2-methyl-1,4-naphthohydrochinone) given orally in small amounts[2] (100–200 γ), increases the P/O ratio for the oxidation of β-hydroxybutyrate by liver mitochondria isolated from chicks fed vitamin K-free diets. When added *in vitro* to liver mitochondria, however, menadione inhibits phosphorylation without disrupting electron transport[1].

As early as 1940 MOLITOR AND ROBINSON[4] reported the toxic effects of excessive dosages of menadione and phthiocol administered *per os* or by intraperitoneal injection. Vitamin K_1 given in one hundred times this concentration was without deleterious effect. It has been shown by BALL *et al.*[5] that 2-alkyl-3-hydroxynaphthoquinones inhibit the reduction of cytochrome *c*. Although numerous investigators have demonstrated the stimulatory effect of menadione on respiration[6,7], to our knowledge, the toxic effect of menadione in animal tissue has remained unexplained. In this communication we wish to report the inhibitory action of excessive amounts of menadione on phosphorylation in the intact rat.

Table I shows the effect of intraperitoneal injection of menadione on the incorporation of radioactive orthophosphate ($^{32}P_i$) into labile nucleotide phosphate ($\sim$P) in liver and heart tissue*. Menadione was dissolved in olive oil and administered in amounts of 110 to 120 mg/kg body weight.

TABLE I

Treatment	*Time**	*mg Glycogen/g liver (wet wt)*	*Specific activity*** *liver* $\sim$ *P*	*Specific activity*** *heart* $\sim$ *P*
Control	20 min	16.0	1235	238
Menadione	20 min	0.9	346	128
Control	40 min	14.2	1890	370
Menadione	40 min	1.8	660	320
Control	50 min	15.8	1940	334
Menadione	50 min	0.0	581	207

* Time interval between injection of $^{32}P_i$ and sacrifice.

** Counts/min/$\mu M \sim$P calculated on the basis of $5 \cdot 10^6$ counts/min P_i injected/kg body weight. Excised organs were immediately immersed in an ether-solid CO_2 solution. Tissue was extracted at 0° with 10% trichloroacetic acid. Glycogen and the barium salts of phosphates were prepared by the same method of SACKS[10]. Nucleotide phosphates were separated from other phosphates by absorption on norite[11] and the labile phosphate released by hydrolysis with *N* HCl at 100° for ten minutes.

Sixty minutes later the rats were given intraperitoneal injections of $5 \cdot 10^6$ counts/minute $^{32}P_i$ in 0.15 *M* KCl/kg body weight to which 25 γ carrier phosphate had been added. Menadione resulted in approximately 70% decrease in the incorporation of $^{32}P_i$ into $\sim$P in the liver and a 30% decrease in the heart. The greater inhibition in the liver is possibly due to the greater accumulation of menadione in this organ. A decrease in glycogen storage in the liver was noted concomitant with the decline in phosphorylative capacity.

These data suggest that the toxicity of menadione is due, at least in part, to a loss of the phosphorylative efficiency of the animal. The depletion of the carbohydrate reserve apparently represents an attempt on the part of the animal to compensate for the resultant energy deficit. These data are in agreement with results obtained in *in vitro* experiments on the effect of menadione in oxidative phosphorylation[1], and also with the conclusions of ISLER *et al.*[8] and MARTIUS AND NITZ-LITZOW[9] that menadione, *per se*, exhibits no vitamin K activity. MARTIUS AND NITZ-LITZOW postulate that menadione is a precursor of vitamin K and that the phytyl chain is required for attachment to the enzyme molecule. It seems reasonable to suppose that, when optimal amounts of 2-methyl-1,4-naphthoquinone are fed, the animal conjugates this structure with a phytyl chain. Excessive amounts of menadione, then, might surpass the ability of the animal to effect this conjugation with the result that unconjugated menadione is free to manifest its uncoupling effect on oxidative phosphorylation.

* The radioactive orthophosphate was supplied from an Atomic Energy Commission Project, No. AT(11-1)-34 (Kleiber).

We have been unable to demonstrate the uncoupling of phosphorylation and respiration in liver mitochondria isolated from menadione-toxic rats. Further experiments are in progress to investigate the possibility that menadione is lost during the isolation and preparation of the mitochondrial suspension.

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Received February 3rd, 1956

Complex nature of the proteolytic system of the thyroid gland

Crude preparations of thyroid protease were previously shown to hydrolyse N-acetyl-L-phenylalanyl-L-di-iodotyrosine[1]. Further studies have demonstrated that acetone fractionation of hog thyroid extracts, while providing a substantial purification of the protease[2], can also lead to a separation of the peptidase activity mentioned. The fractionation procedure, which will be described in detail elsewhere, utilised Extract II[2] as starting material. The main protease fraction (PRO/B16) used in the experiments cited below separated at higher acetone concentration and assayed at 620 units/mg§, whereas the fraction (PEP/B16) showing strong peptidase activity (substrate: N-acetyl-L-phenylalanyl-L-tyrosine) was only weakly active towards haemoglobin (1.5 protease units/mg) and separated at lower acetone concentration. The difference in behaviour of the two fractions towards various peptides is illustrated in Table I.

TABLE I

COMPARISON OF THE ACTION OF THYROID PEPTIDASE (PEP/B16) AND PROTEASE (PRO/B16) FRACTIONS ON PEPTIDE SUBSTRATES

Incubation: 1 h at 37° C; pH 3.5 (0.1 *M* ammonium acetate).
Estimation: chromatographically (*n*-butanol-acetic acid-water (80:20:20), ninhydrin sprayed).

Peptide (0.05 ml) (0.01 M)	*Peptidase (0.05 ml)*		*Protease (0.05 ml)*	
	Wt (mg)	*Result*	*Units*	*Result*
(I) N-Ac.-L-Phe.-L-Tyr.*	0.086	strong tyrosine spot (approx. 50% hydrolysis)	265	no hydrolysis
(II) L-Phe.-L-Tyr. Amide	0.17	no hydrolysis	100	no hydrolysis
(III) Cbz.-Gly.-L-Phe.	0.20	very weak phenylalanine spot	104	no hydrolysis
(IV) Phth.-Gly.-Gly.	0.17	no hydrolysis	104	no hydrolysis
(V) Gly.-L-Tyr.	0.17	no hydrolysis	104	no hydrolysis
(VI) L-Lys.-L-Tyr.-L-Leu.	0.20	weak leucine spot	100	no hydrolysis
(VII) L-CySH.-L-Tyr.**	0.27	negligible hydrolysis	108	approx. 75% hydrolysis

* This peptide and its di-iodo derivative behaved similarly.
** 0.04 *M*. N-Ethylmaleimide (0.05 *M*; 0.05 ml) added before chromatography[4].

§ One unit is the amount of enzyme required to liberate 10^{-4} milliequiv. tyrosine in 30 minutes at 37° C (haemoglobin substrate; pH 3.5); compare ANSON[3].