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SHORT COMMUNICATIONS

Effects of vitamin K and naphthoquinones on lipid peroxide formation and oxidative demethylation by liver microsomes

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VITAMIN K_3 (menadione), but not vitamin K_1 (phylloquinone) stimulates the rate of oxidation of NADPH by suspensions of liver microsomes (Gillette *et al.*,[1] Sato *et al.*).[2] NADPH is an essential component of the electron transport system for drug oxidation in the endoplasmic reticulum (Ernster and Orrenius)[3] but it may also initiate formation of lipid peroxide in microsomal suspensions (Wills).[4] This process of peroxidation is believed to be a consequence of a switching of the electron transport from drug oxidation to membrane lipids followed by loss of structure. As a consequence, the microsomal suspensions lose their capacity to carry out drug oxidation (Wills).[5] By regulation of the rate of NADPH oxidation, menadione and other related quinones may have an important biological role in controlling drug oxidation and lipid peroxidation in the endoplasmic reticulum, and to investigate this possibility, the effects of several forms of vitamin K and related quinones have been studied on these systems.

The microsomal fraction of rat liver was prepared as decribed (Wills).[4] Lipid peroxide formation was measured in suspensions of microsomes (1·5 mg protein/ml) incubated at 37° in pH 6·0 phosphate buffer with ascorbate (0·1 mM) or in pH 7·0 phosphate buffer in presence of NADPH (40 μM). Rate of malonaldehyde formation produced during peroxidation was measured by the thiobarbituric acid method (Wills).[4]

Oxidative demethylation of aminopyrine and of *p*-chloro-N-methyl aniline were measured by methods previously described (Wills,[5] Wills and Wilkinson).[6]

For measurement of the rate of oxidation of NADPH, microsomal suspensions containing 1·5 mg protein were incubated at 25° in a medium containing pH 6·0–7·0 phosphate or tris buffer (50 mM) NADPH (40–100 μM) in a total volume of 2·0 ml. Rate of decrease of absorbance at 340 nm was measured in a Hilger–Gilford reaction kinetics spectrophotometer. All values are means of four experiments.

The rate of oxidation of NADPH by microsomal suspensions was markedly stimulated by adding menadione to give a final concentration of 1–50 μM. Powerful stimulation of the rate of NADPH oxidation by microsomal suspensions was also produced by addition of vitamin K_5, (4 amino-2-methyl naphthol) but quinones such as benzoquinone, 1-4 Naphthoquinone or coumarin were not effective (Fig. 1). Phylloquinone containing a phytyl side chain was inactive, and furthermore, abolished the stimulatory effect of menadione.

Suspensions of liver microsomes incubated in presence of ascorbate or of NADPH rapidly form lipid peroxide (Wills).[4] Addition of menadione strongly inhibited peroxidation in incubated microsomal suspensions, whether ascorbate or NADPH was added (Fig. 2). The very low rate of peroxide formation of microsomal suspensions incubated with NADPH and menadione (50 μM) could not be increased by addition of further NADPH (40 μM) and furthermore, addition of menadione (5 μM) to incubated suspensions rapidly forming peroxide caused immediate inhibition of peroxide formation. Both these experiments indicated that menadione acts by a direct inhibitions of peroxide formation and not by reducing the concentration of NADPH. Vitamin K_5(4 amino-2-methyl naphthol) was also an effective inhibitor of peroxidation, but other quinones, such as benzoquinone, 1-2 naphthoquinone and 1-4 naphthoquinone were less effective inhibitors. Vitamin K_1 (phylloquinone), either natural or synthetic, did not cause inhibition of peroxidation when microsomal suspensions were incubated in presence of NADPH and even enhanced the rate (Table 1). The effects of menadione, vitamin K_5, and quinones on peroxidation in microsomal suspensions incubated in presence of ascorbate were similar to those observed using NADPH but phylloquinone had no stimulatory effect on this system.

Sato *et al.*[2] reported that the increased rate of peroxidation of NADPH caused by addition of menadione was accompanied by an increase in the rate of oxygen uptake. However, if the oxygen uptake observed when microsomal suspensions are incubated with NADPH or ascorbate is associated mainly with lipid peroxidation (Wills,[4] May and McCay)[7] and menadione strongly inhibits peroxidation in both systems, then inhibition of oxygen uptake might be expected on addition of menadione. Addition of menadione (10 μM) caused 70 per cent inhibition of oxygen uptake and 100 μM caused

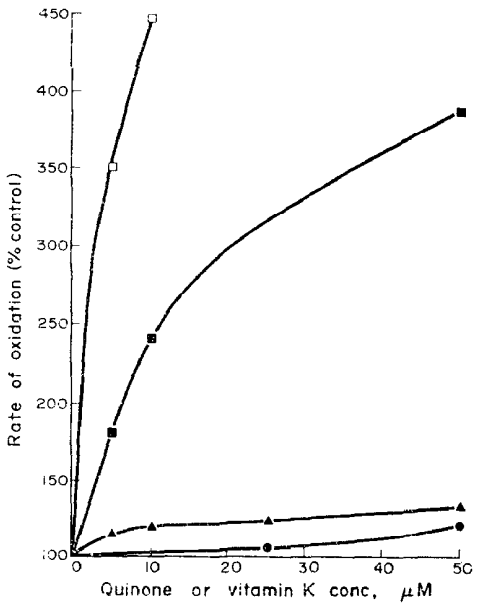

FIG. 1. Comparison of the effects of menadione, vitamin K_5, benzoquinone and 1·4 naphthoquinone on the rate of NADPH oxidation by liver microsomes.

Suspensions of liver microsomes (1·5 mg protein/ml) were incubated with NADPH (40 μM) in pH 7·0 phosphate buffer at 25°. The rate of decrease of absorbance at 340 nm was measured and the rate of oxidation of NADPH in absence of menadione was 12·8 nm/min. □——□ Menadione, ■——■ Vitamin K_5, ▲——▲Benzoquinone, ●——● 1·4 Naphthoquinone.

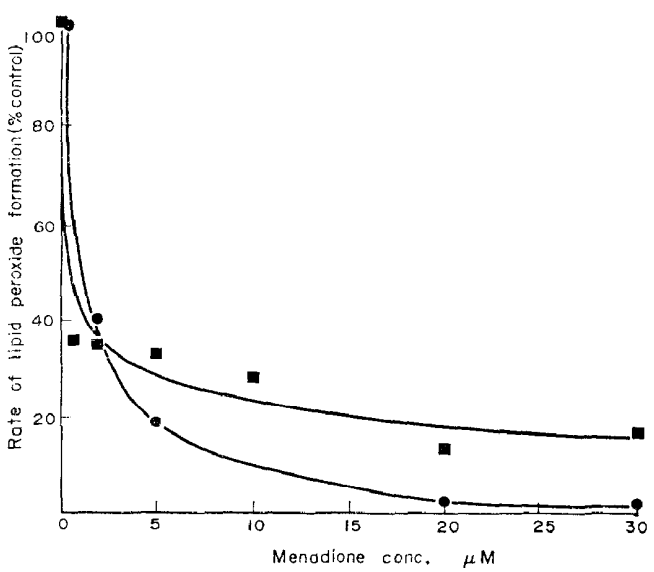

FIG. 2. Effect of menadione on the rate of lipid peroxidation by suspensions of liver microsomes.

Suspensions of liver microsomes (1·5 mg protein/ml) were incubated in pH 7·0 phosphate buffer with NADPH (40 μM) or in pH 6·0 phosphate buffer with ascorbate (0·2 mM). The rate of lipid peroxidation was determined as described (Wills[4]) and control rates were 14·4 nm malonaldehyde/mg protein/min for the NADPH system and 12·4 for the ascorbate system. ■——■ Ascorbate system. ●——● NADPH system.

TABLE 1. COMPARISON OF THE EFFECTS OF PHYLLOQUINONE, MENADIONE, QUINONES AND VITAMIN K_5 ON LIPID PEROXIDE FORMATION IN SUSPENSIONS OF MICROSOMES INCUBATED WITH NADPH

Addition	Concentration (μM)	Rate of lipid peroxide formation
None		100
Menadione	5	16
	10	8
	50	0
Phylloquinone	5	138
	50	161
1·4 Naphthoquinone	5	62
	50	30
1·2 Naphthoquinone	5	36
	50	19
Benzoquinone	5	40
	50	30
Vitamin K_5	5	0
	50	0

The rate of malonaldehyde formation in the control experiment was 14·4 nm/mg protein/min.

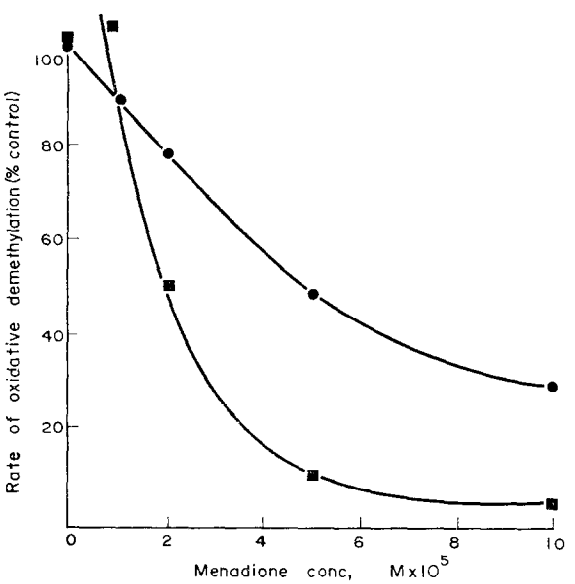

FIG. 3. Effect of menadione on the rate of oxidative demethylation of aminopyrine and *p*-chloro-N-methyl aniline.

Rates are expressed as a percentage of the control rates which were: 17·5 nm formaldehyde/min for aminopyrine and 8·55 nm *p*-chloroaniline/min for *p*-chloro-N-methyl aniline. ●——● *p*-chloro-N-methyl aniline. ■——■ Aminopyrine.

90 per cent inhibition but phylloquinone which, in contrast stimulated peroxidation, also stimulated oxygen uptake by 1·40 times when added in a concentration of 100 μM. Oxygen uptake in the presence of ascorbate was also inhibited by menadione but less effectively than in the NADPH system.

Menadione, added to suspensions of microsomes in a final concentration of 10–100 μM strongly inhibited the oxidative demethylation of aminopyrine and *p*-chloro-N-methyl aniline. (Fig. 3). Experiments were normally started by addition of microsomal suspensions to the incubation mixture. If

TABLE 2. COMPARISON OF THE EFFECTS OF VITAMIN K, QUINONES AND ANTIOXIDANTS ON OXIDATIVE DEMETHYLATION OF AMINOPYRINE AND OF *p*-CHLORO-N-METHYL ANILINE

Additives	Concn. (μM)	Rate of oxidative demethylation % Control	
		Aminopyrine	*p*-chloro-N-methyl aniline
None (control)		100	100
Menadione	100	8	29
	50	12	45
	10	66	89
	5	86	110
Phylloquinone	100	63	18
	50	84	94
	10	106	96
Vitamin K_5	100	15	
	10	35	77
1·4 Naphthoquinone	100	47	16
	50	100	100
Benzoquinone	100	110	
α-tocopherol	500	99	89
Butylated hydroxytoluene (BHT)	500	88	91
	200	103	96

Rates are expressed as a percentage of the control rates which were; 17·5 nm formaldehyde/min for aminopyrine and 8·55 nm *p*-chloroaniline/min. for *p*-chloro-N-methyl aniline.

however, the microsomal suspension was mixed with the substrate, aminopyrine and menadion, added after an incubation period of 5 min the inhibition produced was much reduced. Phylloquinone benzoquinone and naphthoquinones were much less effective than menadione (Table 2). Studies of the inhibition of lipid peroxidation by quinones indicated that this might be a consequence of an antioxidant action and a comparison was therefore made of effects of K vitamins and of antioxidants such as vitamin E and butylated hydroxy toluene, (BHT) on oxidative demethylation. These antioxidants, well established as powerful inhibitors of lipid peroxidation (Wills)[5] did not affect oxidative demethylation, in sharp contrast to vitamin K_5 and menadione, which inhibited strongly (Table 2).

The observation that oxidation of NADPH by liver microsomes was stimulated by menadione but that naphthoquinones or benzoquinone were much less effective stimulators suggests that the methyl group might be important in regulating the specificity of the activation and this view is confirmed by the fact that stimulation was observed after addition of vitamin K_5 (4-amino-2-methyl naphthol). Phylloquinone however does not stimulate NADPH oxidation, although this compound also possesses a methyl group in the 2-position. It is possible that this is due to the long lipid soluble phytyl chain on the 3-position of the ring which may play a role in locating the molecule in an area of the lipid membrane in which it cannot stimulate NADPH oxidation.

Menadione is a powerful inhibitor of lipid peroxidation, inhibiting both NADPH and ascorbate systems (Fig. 2). Naphthoquinone, or benzoquinone also inhibited to a smaller extent and the effect of the K vitamins and quinones on lipid peroxidation is therefore almost the exact opposite of their effect on NADPH oxidation.

Quinones and phenolic compounds have, for many years, been known to possess powerful antioxidant capacity against autoxidation of unsaturated fats[8] and recently, Mezick *et al.*[9] observed inhibition of lipid peroxidation in membranes of red blood cells by menadione. The inhibitory effect of K vitamins on peroxidation of microsomes in presence of NADPH may therefore be a result of two effects. Firstly, the stimulation of the rate of oxidation of NADPH so that less is available to initiate and sustain peroxidation and secondly, the result of antioxidant action blocking peroxidation. The fact that the antioxidant effect is important is shown by experiments utilizing addition of extra NADPH and by the inhibition of ascorbate peroxidation where, clearly, effects on NADPH oxidation are not relevant.

Stimulation of NADPH oxidation by menadione results in a marked decrease of the rate of oxidative demethylation of aminopyrine or of *p*-chloro-N-methyl aniline (Fig. 3), and when even a high concentration (10 mM) of aminopyrine is present, oxidative demethylation is strongly inhibited by a very low concentration of menadione (20 μM). Antioxidants such as vitamin E or BHT which like menadione, also strongly inhibit lipid peroxidation (Wills)[4] have little or no effect on oxidative demethylation. The antioxidant effect of menadione is therefore unlikely to be involved in reducing the rate of oxidative demethylation and this must be a result of the rapid oxidation of NADPH rendering it unavailable for oxidative demethylation. The affinity of menadione for the system oxidizing NADPH must be very high because it is effective in a concentration which is very much less than the aminopyrine although it is likely to associate with an earlier stage in the electron transport chain diverting the electron flow.

In summary, it may be suggested that a biological function of menadione and related quinones may be connected with stimulation of a rapid oxidation of NADPH rendering it unavailable for the initiation of the microsomal electron transport system on which oxidative demethylation of drugs and steroid hydroxylation depends. At the same time lipid peroxidation is inhibited, as a result of the reduction of the NADPH concentration but also as a result of an antioxidant effect. By this means, the integrity of the membranes could be preserved and stabilized for subsequent metabolism. It is therefore possible that menadione and related quinones may thus play an important role in regulating the metabolism and turnover of the membranes of the endoplasmic reticulum.

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REFERENCES

1. J. R. GILLETTE, B. B. BRODIE and B. N. LADU, *J. Pharmac, exp. Ther.* **119,** 532 (1957).
2. R. SATO, H. NISHIBAYASHI and T. OMURA, *Biochim. biophys. Acta* **63,** 550 (1962).
3. L. ERNSTER and S. ORRENIUS, *Fedn Proc.* **24,** 1190 (1966).
4. E. D. WILLS, *Biochem. J.* **113,** 315 (1969).
5. E. D. WILLS, *Biochem. J.* **113,** 333 (1969).
6. E. D. WILLS and A. E. WILKINSON, *Int. J. Radiat. Biol.* **17,** 229 (1970).
7. H. E. MAY and P. B. MCCAY, *J. biol Chem.* **243,** 2296 (1968).
8. G. SCOTT, *Atmospheric Oxidation and Antioxidants* Elsevier, Amsterdam, p. 361 (1965).
9. J. A. MEZICK, C. T. SETTLEMIRE, G. P. BRIERLEY, K. P. BAREFIELD, W. N. JENSEN and D. G. CORNWELL, *Biochim. biophys. Acta* **219,** 361 (1970).