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CHANGES IN THE URINARY METABOLITES OF PHYLLOQUINONE (VITAMIN K_1) IN MAN FOLLOWING THERAPEUTIC ANTICOAGULATION WITH WARFARIN

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Abstract—The metabolism of [1′,2′-$^3H_2$]phylloquinone was studied in five normal male volunteers both before and after treatment with therapeutic doses of warfarin for 8–10 days. Warfarin treatment caused an almost 2-fold increase in the urinary excretion of phylloquinone metabolites; a decrease in glucuronide conjugated metabolites and the excretion of an unidentified conjugate fraction resistant to hydrolysis by both β-glucuronidase and phenolsulphatase.

In addition, excretion of the normal aglycones of phylloquinone was markedly reduced and was replaced by the excretion of more polar, hydroxylated metabolites which appeared to be derived from phylloquinone-2,3-epoxide.

The results are consistent with the hypothesis that warfarin exerts its anticoagulant effect in man by blocking the regeneration of vitamin K from its 2,3-epoxide metabolite.

The K vitamins, of which phylloquinone (vitamin K_1) is an important member, are essential for the formation of blood clotting factors II, VII, IX and X and recent evidence suggests that the vitamin is involved in the carboxylation of certain specific glutamic acid residues in "precursor" clotting factors thus enabling them to bind Ca^{2+} [1, 2]. A recent hypothesis is that the synthesis of physiologically active clotting factors is linked to the epoxidation of phylloquinone to phylloquinone-2,3-epoxide [3, 4] and that drugs of the coumarin group, such as warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin), exert their anticoagulant effect by preventing the regeneration of phylloquinone from the epoxide metabolite [5, 11]. This hypothesis was originally proposed to explain the large amounts of phylloquinone epoxide which accumulated in the livers of warfarin-treated rats [6] and has received further support from recent experiments which have suggested that there is a close link between the inhibition of prothrombin synthesis and inhibition of the regeneration of phylloquinone from phylloquinone epoxide both *in vivo* and *in vitro* [12, 13].

The present study was prompted by our earlier demonstration that warfarin caused phylloquinone epoxide to accumulate in the plasma of human subjects [7]. This finding suggested that further insight into the mode of action of oral anticoagulant drugs might be gained from a more complete study of the metabolic fate of phylloquinone in subjects undergoing warfarin therapy.

MATERIALS AND METHODS

Radiochemicals and chemicals. [1′,2′-$^3H_2$]phylloquinone and 2-methyl-3-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-naphthoquinone lactone (phylloquinone γ-lactone) were obtained from Hoffmann–La Roche and Co., Basle, Switzerland. The [1′,2′-$^3H_2$]-phylloquinone used for oral administration had a specific activity of 95 Ci/mole. The preparations used for intravenous injection had specific activities of 5.4 and 98 Ci/mole and were received in sealed, sterile ampoules containing 1 mg and 45 μg [1′,2′-$^3H_2$]phylloquinone per 0.5 ml polyethylene glycol ester solvent, respectively.

Non-radioactive phylloquinone (Konakion) was obtained from Roche Products Ltd., Welwyn Garden City Herts. Bovine liver β-glucuronidase (E.C. No. 3.2.1.31) activity 600,000 Fishman units/g and phenolsulphatase (E.C. No. 3.1.6.1) from *Helix pomatia*, activity 20,000 units/g, plus β-glucuronidase, activity 300,000 units/g were obtained from Sigma (London) Chemical Co. Ltd., Kingston, Surrey.

Sodium warfarin {3-(α-acetonylbenzyl)-4-hydroxycoumarin} was obtained from B.D.H., Poole, Dorset, as tablets of 'Marevan'. Phylloquinone-2,3-epoxide was synthesised from phylloquinone [14]. Saccharo-1,4-lactone was obtained from Calbiochem Ltd., London, W1. 1,3-Bis-(chloromethyl)-1,1,3,3-tetramethyldisilazane (CMTMDS) and chloromethyldimethylchlorosilane (CMDMS) were obtained from Field Instruments Richmond, Surrey. Diazomethane in diethyl ether solution was prepared from *N*-methyl-*N*-nitroso-*p*-toluene sulphonamide, "Diazald" (Aldrich Chemical Co. Ltd., Gillingham, Dorset) [15].

All other chemicals were either Analar or the best available grade. All solvents were redistilled before use, cyclohexane and diethyl-ether having first been dried over sodium wire.

Chloroform was restabilised following distillation by the addition of 2% (v/v) methanol.

Spectroscopic grade cyclohexane, for use in ultraviolet absorption spectroscopy, was obtained from B.D.H.

Plan of study. Four normal male volunteers aged 22–30 yr were studied both before (control study) and after treatment with therapeutic doses of warfarin. In each study, [1′,2′-$^3H_2$]phylloquinone was injected into a forearm vein. Three subjects received 1 mg and one subject 45 μg phylloquinone. Total urine was collected up to 72 hr. In two subjects samples were collected separately from 0–8 hr, pooled from 8–24 hr

Table 1. Time courses of urinary excretion of radioactivity

Subject	Study	% Administered dose [1′,2′-$^3H_2$]phylloquinone Period of urine collection (hr) 0–2	2–5	5–8	8–24	24–48	48–72	0–72
1	Control	2.1	4.7	2.7	8.1	5.5	2.4	25.5
	Warfarin	1.3	3.3	10.9	13.8	6.8	2.6	38.7
2	Control	1.7	3.9	2.5	6.2	2.4	0.9	17.6
	Warfarin	3.7	8.6	6.5	14.0	5.7	1.5	40.0
3	Control	←	2.6	→	2.6	2.7	1.1	9.0
	Warfarin	←	3.9	→	6.2	4.8	1.9	16.8
4	Control	←	6.1	→	3.9	4.3	1.7	16.0
	Warfarin	←	14.0	→	12.8	7.0	2.7	36.5

Each subject was studied both before (control study) and during treatment with therapeutic doses of warfarin (warfarin study). In each study subjects 1, 3 and 4 were injected intravenously with 1 mg [1′,2′-$^3H_2$]phylloquinone and subject 2 with 45 μg [1′,2′-$^3H_2$]phylloquinone.

and separate 24 hr collections made for the remainder of the study. For the other two subjects 0–8 hr urine samples were pooled and the remaining samples collected as for the previous subjects. Urine collections were preserved with sodium azide (0.02%, w/v) and stored at −20°.

Two to six days after the end of the control study the subjects were started on therapeutic doses of warfarin for periods of 8–10 days. After an initial loading dose of 30–40 mg the subjects were maintained on daily doses of warfarin which ranged from 0.12–0.19 mg/kg body weight. At the start of the vitamin K study the prothrombin times ranged from 39–51 sec, corresponding to a concentration of the vitamin K-dependent clotting factors of 10–5 per cent of normal as obtained from a dilution curve of normal plasma using barium sulphate adsorbed oxalated plasma as diluent. After the injection of [1′,2′-$^3H_2$]-phylloquinone, the subjects continued warfarin therapy for the 3 day period of urine collections.

Thin layer chromatography (t.l.c.). (a) Adsorption t.l.c. was performed as described previously [16]. The solvent systems were:

(1) cyclohexane–ethyl acetate (7:3, v/v), (2) chloroform–diethyl ether (9:1, v/v).

(b) Reversed-phase partition t.l.c. was performed as described previously [17]. The solvent system was acetone–water (2:3, v/v).

Phenolsulphatase hydrolysis. Portions of ether extracts of urine containing conjugated metabolites were evaporated under N_2, redissolved in 0.1 M acetate buffer, pH 5.0 (4.5 ml), and incubated at 37° for 24 hr with varying concentrations (1–10 mg/ml) of phenolsulphatase. Controls were set up without enzyme or with enzyme plus 1% (w/v) bovine liver β-glucuronidase. Hydrolyses were terminated and treated as described previously [16].

Solvolysis. Solvolysis was performed in ethyl acetate by one of the procedures described by Burstein and Lieberman [18]. Ether extracts of urine conjugates were dissolved in water, adjusted to pH 1 with 1 M HCl and sodium chloride added to a final concentration of 20% (w/v). The mixture was extracted once with an equal volume of ethyl acetate. The upper ethyl acetate layer was incubated at 37° for 24 hr and the solvent was removed under a stream of N_2.

Other procedures. The following procedures were performed as reported earlier [16, 17]: radioactivity measurements, ether extraction of metabolites from urine, β-glucuronidase and acid hydrolyses, treatment of metabolites with diazomethane, CMTMDS and CMDMS, distribution studies, $NaHCO_3$ extraction and determination of organic acid fraction.

RESULTS

Excretion, extraction and solubility properties of urinary metabolites. The time course of urinary excretion of radioactivity is shown for the control and warfarin studies in Table 1. The total excretion of radioactivity was similar for both 45 μg and 1 mg doses of [1′,2′-$^3H_2$]phylloquinone and in all subjects warfarin caused an almost 2-fold increase in the excretion of urinary radioactivity over the 3 day period of study.

The solubility properties of the labelled metabolites were investigated by shaking aliquots of freshly voided urine with equal volumes of chloroform and water, and measuring the distribution of radioactivity between the two phases. In the control group 96–99 per cent of the radioactivity distributed in the water phase compared with 87–96 per cent in the warfarin group.

The acidic nature of the urinary metabolites was shown by the results of continuous ether extraction of urine at pH 2. By this procedure 70–80 per cent of the radioactivity could be extracted from the urine of both normal and warfarin-treated subjects.

Conjugate nature of urinary metabolites. Ether extracts of urine from control and warfarin studies were subjected to hydrolysis by bovine liver β-glucuronidase and distribution studies performed on the hydrolysate. Treatment with β-glucuronidase rendered lipid-soluble 85–90 per cent of the radioactivity present in ether extracts of urine from normal subjects compared with 50–55 per cent for warfarin-treated subjects (Table 2). In both control and warfarin studies the release of lipid-soluble radioactivity was greatly reduced in the absence of enzyme or by addition of 0.02 M saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase.

The possible presence of an inhibitor of β-glucuronidase in the urine of warfarin-treated subjects was

Table 2. Chloroform-extractable radioactivity after β-glucuronidase hydrolysis of ether extracts of urine

Enzyme	Incubation time (hr)	Radioactivity in chloroform phase (%) Control study Period of urine collection (hr) 0–2	2–5	5–8	8–24	Warfarin study 0–2	2–5	5–8	8–24
Active	2	86.9	88.8	83.8	87.3	55.0	55.2	51.8	54.2
Active	24	91.4	92.9	88.8	90.8	58.8	59.2	56.0	57.2
Inhibited	2	25.6	36.5	39.6	44.8	24.2	30.7	33.9	37.9
None	2	14.0	22.8	28.0	32.0	19.5	26.2	31.7	33.7

Portions of urine ether extracts were incubated at 37° with bovine liver β-glucuronidase (600 Fishman units/0.1 ml) in 0.1 M-acetate buffer pH 5. The inhibitor was 0.02 M saccharo-1, 4-lactone. The hydrolysates were shaken with an equal volume of chloroform, centrifuged and the radioactivity in equal volumes of each phase determined. The results are shown for ether extracts of urines from a subject injected intravenously with 45 μg [1′,2′-$^3H_2$]phylloquinone before (control study) and during treatment with therapeutic doses of warfarin (warfarin study), and are the means of duplicate hydrolyses.

investigated by adding unlabelled urine from a subject taking warfarin to incubation mixtures of labelled urine from normal subjects. No decrease in the fraction of radioactive conjugates hydrolysed by β-glucuronidase was observed.

Although for the warfarin group only 50–55 per cent of the radioactivity present in ether extracts of urine was rendered lipid-soluble by β-glucuronidase, treatment with dilute HCl increased this to 80–90 per cent. Since this suggested the presence of conjugated metabolites other than glucuronides, the possible presence of sulphate conjugates was investigated.

When ether extracts of urine from warfarin-treated subjects were incubated with phenolsulphatase about 50 per cent of the radioactivity was rendered lipid-soluble. However, inclusion of β-glucuronidase in the incubation mixture did not increase this value, suggesting that the hydrolysis observed was due to β-glucuronidase activity present in the phenolsulphatase preparation.

To further investigate the presence of sulphate conjugates, ether extracts of urine from warfarin studies were subjected to solvolysis, a mild hydrolytic procedure which has been used to hydrolyse steroid sulphates [18]. This procedure, which does not hydrolyse the glucuronide conjugates [17], rendered lipid-soluble almost 30 per cent of the radioactivity present in ether extracts of urine from warfarin-treated subjects.

Nature of aglycones. The number and nature of aglycone fragments released by β-glucuronidase, solvolysis and acid hydrolysis of ether extracts of urine were investigated by t.l.c. Figure 1 shows the distribution of radioactivity on chromatograms following enzyme hydrolysis and methylation of the aglycones released. For the control studies, a single peak of

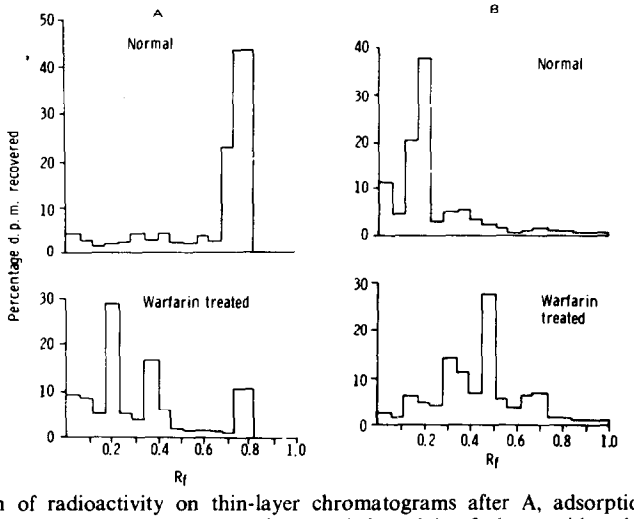

Fig. 1. Distribution of radioactivity on thin-layer chromatograms after A, adsorption t.l.c. and B, reversed-phase partition t.l.c. of methylated aglycones (released by β-glucuronidase hydrolysis) from control and warfarin studies in a subject injected with 45 μg [1′,2′-$^3H_2$]phylloquinone. The adsorption t.l.c. was performed in chloroform–diethyl ether (9:1, v/v). The reversed-phase partition t.l.c. was performed on paraffin impregnated layers of Kieselgel GF_{254} and developed with acetone–water (2:3, v/v). The radioactivity in each zone of adsorbent is expressed as a percentage of the total d.p.m. recovered from each chromatogram.

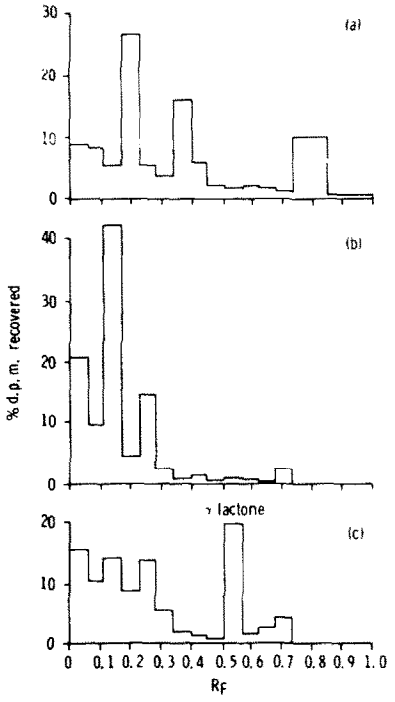

Fig. 2. Distribution of radioactivity on thin-layer chromatograms after adsorption t.l.c. of methylated aglycones released by (a) β-glucuronidase hydrolysis, (b) solvolysis, and (c) dilute acid hydrolysis of ether extracts of urine. The results are shown for a subject injected with 45 μg [1′,2′-$^3H_2$]phylloquinone during treatment with therapeutic doses of warfarin. The chromatograms were developed with chloroform–diethyl ether (9:1, v/v).

per cent of the total radioactivity recovered from the chromatogram. This peak contains the methyl esters of the two major metabolites of phylloquinone normally excreted in the urine [16, 17]. In all the warfarin studies this peak was reduced to about 10 per cent and three peaks of radioactivity which represented more polar metabolites were separated by both

Table 3. Organic acid fraction of aglycones released by β-glucuronidase hydrolysis

Period of urine collection (hr)	% Administered dose [1′,2′-$^3H_2$] phylloquinone	
	Control study	Warfarin study
0–2	0.78	1.43
2–5	1.79	4.35
5–8	1.21	2.34
8–24	2.49	4.82
0–24	6.27	12.04

The organic acid fraction refers to the lipid-soluble radioactivity (obtained by β-glucuronidase hydrolysis of ether extracts of urine) which was extracted by 5% $NaHCO_3$ (w/v). Results are shown for a subject injected with 45 μg [1′,2′-$^3H_2$]phylloquinone before (control study) and during treatment with therapeutic doses of warfarin (warfarin study) and are the means of duplicate estimations.

adsorption and reversed-phase partition systems of t.l.c. (Fig. 1).

Similar results were obtained after solvolysis and dilute acid hydrolysis of ether extracts of urine from warfarin-treated subjects (Fig. 2). The only difference was the presence after acid hydrolysis of a peak of radioactivity which co-chromatographed with carrier phylloquinone γ-lactone, and which appeared to be mainly derived from the central of the three polar peaks of radioactivity (Fig. 2).

The organic acid nature of the aglycones released by β-glucuronidase hydrolysis was demonstrated by their ready extraction from organic solvents with 5% (w/v) $NaHCO_3$ and by their methylation with diazomethane. The proportion of the aglycones released by β-glucuronidase which were extracted from chloroform by two extractions with an equal volume of $NaHCO_3$ was designated the organic acid fraction and is shown for control and warfarin studies in Table 3.

Reaction of aglycones with reagents which form silyl ethers. The effect of silyl ether forming reagents on aglycones released by β-glucuronidase is shown for a warfarin study in Fig. 3. In two different solvent

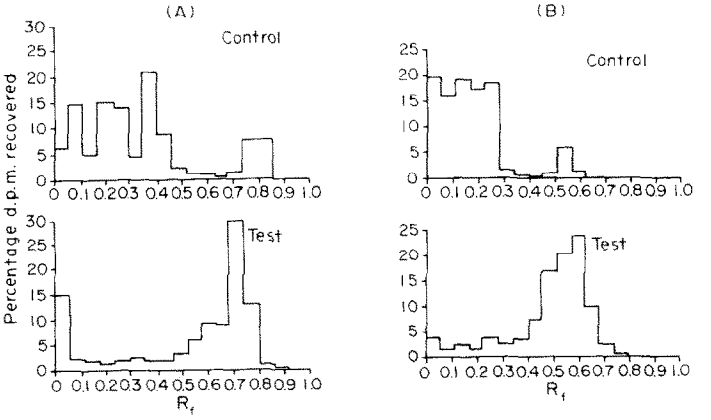

Fig. 3. Distribution of radioactivity on thin-layer chromatograms after adsorption t.l.c. of aglycones (released by β-glucuronidase hydrolysis) from a subject injected with 45 μg [1′,2′-$^3H_2$]phylloquinone during treatment with therapeutic doses of warfarin. Aglycones were treated with either diazomethane and pyridine only (control) or with diazomethane, pyridine, CMTMDS and CMDMS (test). The chromatograms were developed with A, chloroform–diethyl ether (9:1, v/v), or B, cyclohexane–ethyl acetate (7:3, v/v).

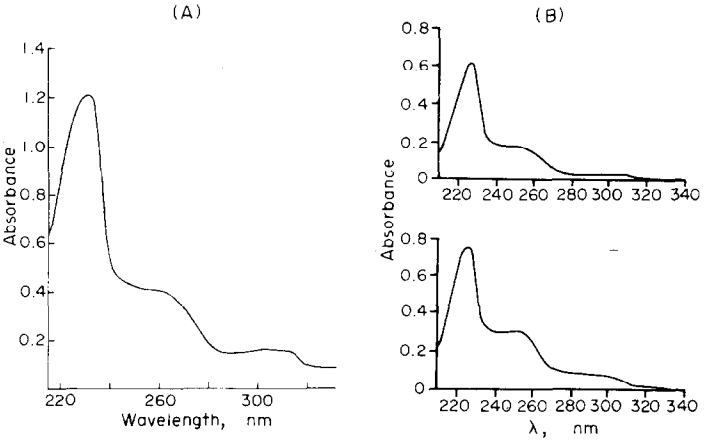

Fig. 4. Ultraviolet absorption spectra in cyclohexane of A, authentic phylloquinone-2,3-epoxide and B, two major "epoxide" metabolites isolated from the urine of a subject given 400 mg unlabelled phylloquinone plus 50 μCi [1',2'-$^3H_2$]phylloquinone during treatment with therapeutic doses of warfarin.

systems, treatment of aglycones with CMTMDS and CMDMS in the presence of pyridine resulted in their increased mobility on thin layer chromatograms.

In addition to the radioactive tracer studies, preliminary preparative experiments were performed in which two subjects maintained on warfarin ingested 400 mg unlabelled phylloquinone in addition to 50 μCi [1',2'-$^3H_2$]phylloquinone. Metabolites were isolated from the 0–24 hr urine collection, purified and examined by ultraviolet absorption spectroscopy and mass spectrometry.

In both subjects, in addition to the three metabolites of phylloquinone normally excreted, a series of metabolites was excreted which had u.v. absorption spectra similar to phylloquinone-2,3-epoxide (Fig. 4). Two of the series of "epoxide" metabolites, which accounted for 70 per cent of the total labelled "epoxides" recovered, were examined by mass spectrometry. Although strong signals in the low-mass regions (m/e 104, 141 and 186) were consistent with a phylloquinone origin [19], the characteristic phylloquinone fragment signal at m/e 225, which is indicative of a double bond at the 2' position of the phytyl side chain, was absent. Instead the "epoxide" metabolites had strong fragment signals at m/e 211, 213 and 239. The molecular weight of the two major "epoxide" metabolites was found to be 326.

DISCUSSION

The results reported here suggest that, in man, treatment with warfarin increases the urinary excretion of phylloquinone metabolites. This finding differs from results obtained in warfarin-treated rats which after injection of [6,7-$^3H_2$]phylloquinone showed no increase in the urinary excretion of labelled metabolites [20].

Warfarin was also found to alter the conjugation pattern of the metabolites such that the glucuronide fraction of conjugates was decreased. A second conjugate fraction comprising about 30 per cent of the total proved to be unstable to heat but resistant to both β-glucuronidase and phenolsulphatase hydrolysis. Hydrolysis by solvolysis suggests that this may be a sulphate conjugate. Siimilar changes in the conjugation pattern following drug administration have been reported for the urinary excretion of various steroids in man [21].

The most striking effect of warfarin in the present study was the decrease in the excretion of the normal aglycone fragments of phylloquinone. In man phylloquinone is normally degraded and excreted in the urine as two major aglycone fragments in which the side chain is reduced to 7 carbon atoms (2-methyl-3-(5'-carboxy-3'-methyl-2'-pentenyl)-1,4-naphthoquinone) and 5 carbon atoms (2-methyl-3-(3'-carboxybutyl)-1,4-naphthoquinone) respectively [16, 17]. The decrease in the proportion of these aglycones was similar for all the warfarin studies with doses of phylloquinone ranging from 45 μg to 400 mg and represented about 15 per cent of the values found in the control studies (Fig. 1). The reduced excretion of these normal metabolites was, however, replaced by the excretion of more polar radioactive aglycones which chromatographed as three peaks (Fig. 1) and which were also shown to be organic acids. Experiments with reagents which form silyl ethers suggested that after methylation of the carboxyl function these polar aglycones still contained one or more free hydroxyl groups. The normal metabolites with no free hydroxyl groups after methylation do not react with silyl ether forming reagents [16, 17]. The results of acid hydrolysis also suggest that one of these more polar aglycones may be a γ,δ-unsaturated acid. This would account for the peak of radioactivity which co-chromatographed with carrier phylloquinone γ-lactone (Fig. 2). Lactonisation has been shown to occur for the normal metabolite of phylloquinone with a seven carbon atom side chain under these acid hydrolysis conditions [16].

The results of preliminary preparative studies suggested that the "hydroxylated" metabolites found in warfarin-treated subjects were derived from phylloquinone-2,3-epoxide. Together with our previous identification of phylloquinone epoxide as a major plasma metabolite in warfarin-treated humans [7], the results reported here have clearly demonstrated that warfarin blocks the normal metabolism of phyl-

loquinone and are consistent with the hypothesis that oral anticoagulants interfere with the synthesis of the vitamin K-dependent clotting factors by inhibiting the regeneration of vitamin from its 2,3-epoxide metabolite.

At present the pathway for the metabolism of phylloquinone epoxide in the presence of warfarin is unknown. However, it has been shown in rats that warfarin increases the level of hepatic metabolites of phylloquinone which are more polar than phylloquinone or phylloquinone epoxide and that this increase is not affected by a drug which inhibits microsomal oxidases [20].

Other studies on the epoxides of various polycyclic aromatic hydrocarbons have shown two pathways for epoxide metabolism; conjugation with glutathione, or hydration by the enzyme epoxide hydratase to produce dihydrodiols [22]. It is possible that the unidentified conjugate excreted in the urine of warfarin-treated subjects is derived from a glutathione conjugate of phylloquinone epoxide and that the "hydroxylated" aglycone fragments are derived by enzymic hydration of the epoxide ring to produce a diol.

A non-specific epoxide hydratase which is unaffected by microsomal oxidase inhibitors has been detected in human liver [23]. It is possible that this enzyme is also involved in the metabolism of phylloquinone epoxide in man. Further work is required to elucidate the metabolic fate of phylloquinone epoxide in the presence of warfarin and it is hoped that this in turn will clarify the anticoagulant action of warfarin in man.

REFERENCES

1. J. Stenflo, P. Fernlund, W. Egan and P. Roepstorff, *Proc. natn. Acad. Sci. U.S.A.* **71**, 2730 (1974).
2. G. L. Nelsestuen, T. H. Zytkovicz and J. B. Howard, *J. biol. Chem.* **249**, 6347 (1974).
3. A. K. Willingham and J. T. Matschiner, *Biochem. J.* **140**, 435 (1974).
4. J. T. Matschiner and A. K. Willingham, *J. Nutr.* **104**, 660 (1974).
5. R. G. Bell and J. T. Matschiner, *Archs Biochem. Biophys.* **141**, 473 (1970).
6. J. T. Matschiner, R. G. Bell, J. M. Amelotti and T. E. Knauer, *Biochim. biophys. Acta* **201**, 309 (1970).
7. M. J. Shearer, A. McBurney and P. Barkhan, *Br. J. Haemat.* **24**, 471 (1973).
8. J. T. Matschiner, A Zimmerman and R. G. Bell, *Thromb. Diath. haemorrh. Suppl.* **57**, 45 (1974).
9. R. G. Bell and J. T. Matschiner, *Nature, Lond.* **237**, 32 (1972).
10. R. G. Bell, J. A. Sadowski and J. T. Matschiner, *Biochemistry* **11**, 1959 (1972).
11. P. Ren, R. E. Laliberte and R. G. Bell, *Molec. Pharmac.* **10**, 373 (1974).
12. M. J. Shearer, A. McBurney, A. Breckenridge and P. Barkhan, *Clin. Sci. and Molec. Med.* **52**, 621 (1977).
13. P. Ren, P. Y. Stark, R. L. Johnson and R. G. Bell, *J. Pharmac. exp. Ther.* **201**, 541 (1977).
14. M. Tishler, L. F. Fieser and N. L. Wendler, *J. Am. chem. Soc.* **62**, 2866 (1940).
15. T. J. De Boer and H. J. Backer, *Recl Trav. chim. Pays-Bas Belg.* **73**, 229 (1954).
16. M. J. Shearer and P. Barkhan, *Biochim. biophys. Acta* **297**, 300 (1973).
17. A. McBurney, M.Phil. Thesis, London University (1975).
18. S. Burstein and S. Lieberman, *J. biol. Chem.* **233**, 331 (1958).
19. S. J. Di Mari, J. H. Supple and H. Rapoport, *J. Am. chem. Soc.* **88**, 1226 (1966).
20. P. T. Caldwell, P. Ren and R. G. Bell, *Biochem. Pharmac.* **23**, 3353 (1974).
21. H. L. Bradlow, B. Zumoff, D. K. Fukushima, L. Hellman, D. R. Bickers, A. P. Alvares and A. Kappas, *Ann. N.Y. Acad. Sci.* **212**, 148 (1973).
22. D. M. Jerina and J. W. Daly, *Science, N.Y.* **185**, 573 (1974).
23. F. Oesch, *Biochem. J.* **139**, 77 (1974).