Mechanism of Action of Warfarin. Warfarin and Metabolism of Vitamin K_1†

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ABSTRACT: The hypothesis that warfarin exerts its anticoagulant effect by causing the accumulation of phylloquinone oxide, an inhibitor of vitamin K_1, was further tested by examining the effect of warfarin on the metabolism of tritiated vitamin K_1 and oxide. Warfarin caused a greatly increased ratio of labeled oxide to vitamin K_1 in the livers of rats injected intracardially with the tritiated compounds as compared to untreated animals. The oxide was extensively converted to vitamin K_1, but warfarin almost completely blocked this conversion. To determine whether there is a correlation between the ratio of phylloquinone oxide and vitamin K_1 in the liver and the inhibition of prothrombin synthesis by warfarin, rats given warfarin 24 hr previously were injected with effective and ineffective doses of tritiated vitamin K_1. Although the oxide:K_1 ratios in the livers of animals in which prothrombin synthesis was inhibited were not large (1.4 to 1.9), they were all greater than the ratios in livers of animals in which the rate of clotting factor synthesis was high (0.90 to 0.95). The relationship between vitamin K_1 and warfarin in their effects on prothrombin synthesis appeared to be noncompetitive. Tritiated vitamin K_1 (200 μg) counteracted 20 times the dose of warfarin and elicited a maximal rate of increase in plasma prothrombin for 2 hr, but in a similar experiment, the response to 25 μg of vitamin K_1 was almost completely blocked by whatever remained of 0.4 mg of warfarin given 24 hr previously. Analyses of livers removed at 2 hr revealed a lower oxide:K_1 ratio in the livers from animals in which prothrombin synthesis was not inhibited. These results were consistent with the idea that warfarin inhibits prothrombin synthesis by increasing the ratio of phylloquinone oxide to vitamin K_1 in the liver.

The mechanism of action of coumarin anticoagulants is of great interest because of their therapeutic value against thromboembolic disease and for insight into the mode of action of vitamin K. It has been generally accepted that the coumarin drugs and vitamin K compete for an unknown active site but the effect of warfarin on the metabolism of vitamin K_1 has suggested an entirely different mechanism of action.

Warfarin had little effect on the subcellular distribution of vitamin K_1 (Bell and Matschiner, 1969a) but caused the accumulation of phylloquinone 2,3-oxide, a metabolite of vitamin K_1 (Matschiner *et al.*, 1970). The oxide stimulated prothrombin synthesis in vitamin K deficient rats but was ineffective in warfarin-treated animals (Bell and Matschiner, 1970). Evidence that the oxide is a competitive inhibitor of vitamin K_1 in warfarin-treated rats (Bell and Matschiner, 1972) led us to propose that warfarin exerts its effect by increasing the ratio of oxide to vitamin K_1 and the oxide inhibits the activity of the vitamin by competing with it for an active site. To test this hypothesis further, the effect of warfarin on the metabolism of tritiated vitamin K_1 and phylloquinone oxide and the correlation between the relative amounts of vitamin K_1 and oxide in the liver and prothrombin synthesis have been studied.

Materials and Methods

Male Carworth rats (10–12 weeks old) were used. Prothrombin was assayed by the method of Hjort *et al.* (1955).

6,7-Tritiated vitamin K_1 and tritiated phylloquinone oxide were prepared and purified as previously described (Matschiner, 1970). They were dissolved in Tween 80 and diluted with 0.9% NaCl to make solutions containing 5% Tween; 0.1 or 0.2 ml was injected intracardially.

Hexane extracts of liver were chromatographed on silicic acid (Matschiner *et al.*, 1970) and fractions containing vitamin K_1 and phylloquinone oxide were combined. Authentic K_1 and oxide were added and the mixtures were chromatographed on thin-layer plates (Matschiner *et al.*, 1970). The separated vitamin and oxide were assayed for tritium by liquid scintillation counting. To test the procedure for recovery, tritiated vitamin and oxide were added to portions of liver. Hexane extracted 94% of the added radioactivity and after silicic acid and thin-layer chromatography, the oxide:K_1 ratio was the same as the added ratio.

Results

Warfarin and Metabolism of K_1 and Oxide. Warfarin had little effect on the amount of radioactivity in the liver at 2 hr or on the conversion of vitamin K_1 to polar metabolites not extracted by hexane, but caused a greatly increased ratio of oxide to vitamin K_1 (Table I). The oxide was extensively converted to vitamin K_1 but warfarin almost completely blocked this conversion. Phylloquinone oxide was apparently metabolized more slowly by the liver than vitamin K_1 since considerably more ^3H was eluted in the K_1–oxide zone after silicic acid chromatography of extracts from livers of animals injected with oxide relative to those given vitamin K_1.

Prothrombin Synthesis and Oxide:K_1 Ratios in the Liver. Rats given warfarin 24 hr previously were injected with 100 μg of tritiated vitamin K_1 which produces a maximal rate of increase in plasma prothrombin, 25 μg of vitamin K_1 which produces a small response and 5 μg which elicits virtually no

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TABLE I: Warfarin and the Metabolism of Vitamin K_1 and Phylloquinone Oxide.[a]

Injected	Warfarin	% of Injected ^3H at 2 hr: Liver	Extract	K_1–Oxide Chromatographic zone	Oxide: K_1
100 μg of [^{3}H]K_1	−	38	22	12	0.11 ± 0.02 (3)
100 μg of [^{3}H]K_1	+	32	17	8.5	0.90 ± 0.18 (3)
25 μg of [^{3}H]K_1	+	29	9.9	6.3	1.4 ± 0.12 (4)
100 μg of [^{3}H]oxide	−	34	20	18	0.57 ± 0.16 (3)
100 μg of [^{3}H]oxide	+	51	23	19	19 ± 2.9 (3)

[a] The values are the average plus and minus the standard error of the mean. The number of livers analyzed is shown in parentheses. Sodium warfarin (0.4 mg) was injected intraperitoneally where indicated 24 hr before administration of the radioactivity. Animals were killed and livers removed 2 hr after injection of radioactivity. Duplicate thin-layer plates were run for determination of the oxide: K_1 ratio for each liver.

response (Figure 1). Although the differences in the oxide: K_1 ratio in the livers of animals in which prothrombin synthesis was high or was inhibited are not great, the oxide: K_1 ratio is lower in animals in which clotting factor synthesis is normal. Surprisingly, there is little difference in the proportion of the injected tritium in the liver at 5 hr over the range of doses injected (Table II).

Reversal of Warfarin Inhibition by Large Doses of Vitamin K_1. Lowenthal *et al.* (Lowenthal and MacFarlane, 1964; Lowenthal and Birnbaum, 1969) found that warfarin inhibited the synthesis of factor VII at low doses of vitamin K_1 and anticoagulant, but as the doses were increased while maintaining the same ratio, the inhibition disappeared. We found a similar noncompetitive relationship between vitamin K_1 and warfarin in their antagonistic effects on prothrombin synthesis (Table III). Vitamin K_1 (200 μg) counteracted 4 mg of warfarin and elicited a maximal rate of increase in plasma prothrombin for 2 hr after which inhibition occurred. In a similar experiment 25 μg of vitamin K_1 had little effect on whatever remained of 0.4 mg of warfarin given 24 hr previously. Analyses of livers removed at 2 hr revealed that the oxide: K_1 ratio was higher in the livers from animals in which prothrombin synthesis was inhibited.

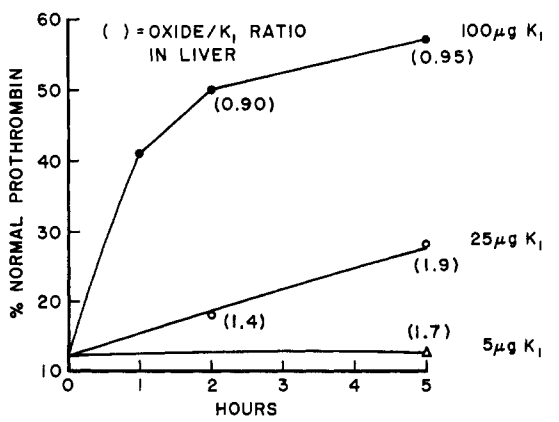

FIGURE 1: Response of warfarin-treated rats to [^{3}H]K_1. Sodium warfarin (0.4 mg) was injected intraperitoneally 24 hr before administration of the indicated amounts of [^{3}H]K_1. The prothrombin concentrations are the averages for three or more animals. For details of metabolism of [^{3}H]K_1, see Tables I and II.

Discussion

The principal effect of warfarin on the metabolism of vitamin K_1 was to change the relative proportions of vitamin K_1 and phylloquinone oxide in the liver and is consistent with the hypothesis that warfarin exerts its effect by increasing the ratio of oxide to vitamin K_1 in the liver. Although the oxide: K_1 ratios in the livers of animals in which prothrombin synthesis was inhibited were not large (1.4 to 1.9) they were all greater than the ratios in livers of animals in which the rate of clotting factor synthesis was high (0.90–0.95) (Tables I and II). It is possible that the oxide: K_1 ratio in the whole liver may not reflect the ratio at the active site for vitamin K.

A regulatory protein with binding sites for vitamin K and warfarin has been proposed by Olson *et al.* (1969), Hermodson *et al.* (1969), and O'Reilly (1970). We suggest that if such a protein exists, vitamin K_1 and the oxide compete for binding sites rather than the vitamin and coumarin drugs.

Suttie and his colleagues (Hermodson *et al.*, 1969; Thierry *et al.*, 1970) found little difference in the metabolism of vitamin K_1 between normal and warfarin-resistant rats, but studies in

TABLE II: Warfarin and the Metabolism of Vitamin K_1.[a]

Injected	% of Injected ^3H at 5 hr: Liver	Extract	K_1–Oxide Chromatographic Zone	Oxide: K_1
100 μg of [^{3}H]K_1	24	7.7	3.8	0.95 ± 0.15 (3)
25 μg of [^{3}H]K_1	23	6.4	3.2	1.9 ± 0.40 (5)
5 μg of [^{3}H]K_1	18	5.6	3.5	1.7 ± 0.34 (3)

[a] The values are the averages plus and minus the standard error of the mean. The number of livers analyzed is shown in parentheses. Sodium warfarin (0.4 mg) was injected intraperitoneally 24 hr before administration of tritiated vitamin K_1. Animals were killed and livers removed 5 hr after administration of [^{3}H]K_1. Duplicate thin-layer plates were run for determination of the oxide: K_1 ratio for each liver.

TABLE III: Metabolism of Vitamin K_1 and Reversal of Warfarin Inhibition.[a]

Injected	Prothrombin % of Normal: Zero	2 hr	5 hr	Oxide:K_1 in Liver at 2 hr
200 μg of [^{3}H]K_1 + 4 mg of warfarin	12	53	34	0.92 ± 0.19 (3)
25 μg of [^{3}H]K_1	12	18	28	1.4 ± 0.12 (4)

[a] Rats injected intraperitoneally with 0.4 mg of sodium warfarin 24 hr previously were injected with the indicated amounts of [^{3}H]K_1. One group was also given an additional 4 mg of warfarin as shown. The prothrombin concentrations are the average for five animals. Duplicate thin layers were run for determination of the oxide:K_1 ratio for each liver. The number of livers analyzed is shown in parentheses.

which the K_1–oxide interconversion is considered may reveal differences. If our hypothesis concerning the mechanism of action of warfarin is correct, resistance to the anticoagulant might occur by a mutation which renders the conversion of phylloquinone oxide to vitamin K_1 no longer sensitive to warfarin inhibition.

Bell and Matschiner (1972) found that vitamin K_1 and phylloquinone oxide were competitive antagonists while the relationship between the vitamin and coumarin anticoagulants is not a simple competitive one (Lowenthal and MacFarlane, 1964; Lowenthal and Birnbaum, 1969). We proposed that warfarin is unable to counteract large doses of vitamin K_1 because the oxide:K_1 ratio does not reach an inhibitory level. The experimental results are consistent with this idea (Table III).

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Rearrangement of [1-^{2}H]- and [2-^{2}H]Naphthalene 1,2-Oxides to 1-Naphthol. Mechanism of the NIH Shift†

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ABSTRACT: Synthetic [1-^{2}H]- and [2-^{2}H]naphthalene 1,2-oxides rearrange predominately to 1-naphthol, which retains 60–85% of the original deuterium. The magnitude of deuterium retention is dependent on the pH of rearrangement. Under neutral or basic conditions, both deuterated oxides give 1-naphthol with a deuterium retention of approximately 80%. Thus, a common intermediate, on the pathway from the deuterated naphthalene oxides to 1-naphthol, is indicated. This intermediate is probably the keto tautomer of 1-naphthol, enolization of which to 1-naphthol in neutral and basic pH regions must then be accompanied by an isotope effect (k_H/k_D) of 4.0. Under acidic conditions, [1-^{2}H]naphthalene 1,2-oxide forms 1-naphthol with a lower retention of deuterium than is the case with [2-^{2}H]naphthalene 1,2-oxide. Thus, acid-catalyzed isomerization of naphthalene 1,2-oxide is mechanistically distinct from the spontaneous isomerization observed in the neutral and basic range. Microsomal hydroxylation of *either* [1-^{2}H]- or [2-^{2}H]naphthalene at pH 9 produces 1-naphthol with approximately 65% retention of deuterium providing additional evidence for naphthalene 1,2-oxide as an intermediate in the metabolism of naphthalene. Hydroxylation of the deuterated naphthalenes with a peracid or a photolytic model system affords deuterium retentions similar to those observed with microsomes.

The NIH shift, an intramolecular migration of aryl ring substituents, has been established as a characteristic of monoxygenase-catalyzed oxidation of aromatic compounds to form phenols (Daly *et al.*, 1968a; Guroff *et al.*, 1967). Despite numerous investigations, the mechanism of this intramolecular migration is still not known with certainty. However,

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