J. Pharm. Pharmacol. 1983, 35: 421–426
Received January 7, 1983

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# The effects of salicylate on enzymes of vitamin K metabolism

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The mechanism of salicylate-induced hypoprothrombinaemia has been investigated in the rat. Salicylate administration produced an increase in the percentage of the total liver vitamin that was present as vitamin K 2,3-epoxide, but the addition of salicylate did not influence vitamin K epoxide reductase activity in-vitro. Neither did it influence vitamin K-dependent carboxylase or vitamin K epoxidase activity. Both cytosolic and microsomal DT-diaphorase activities were, however, inhibited about 50% by 75 μM sodium salicylate. Salicylate inhibition was also observed when vitamin K quinone and NADH or dithiothreitol were used to support carboxylation. To achieve 50% inhibition required 0·5 mM salicylate with NADH as a reductant and 4 mM salicylate when dithiothreitol was the reductant. These results suggest that the main effect of salicylate on vitamin K metabolism is to inhibit quinone reductases and may be useful in explaining the inhibition of the biosynthesis of vitamin K-dependent clotting factors that occurs in salicylate-induced hypothrombinaemia. These data also demonstrate that the percentage of total liver vitamin present as vitamin K epoxide can be increased by agents that do not have a direct effect on the vitamin K epoxide reductase in-vitro.

Vitamin K is a required cofactor in the post-translational modification of Glu residues to γ-carboxyglutamyl (Gla) residues in microsomal protein precursors of plasma clotting Factors II, VII, IX, and X, and other vitamin K-dependent proteins (Suttie & Jackson 1977; Suttie 1980a). This microsomal carboxylase requires $O_2$, $CO_2$, the reduced form of vitamin K (vitamin $KH_2$), and appears to be the same enzyme that converts vitamin $KH_2$ to vitamin K 2,3-epoxide (Suttie 1980b). Liver microsomes also contain a vitamin K epoxide reductase capable of reducing the 2,3-epoxide to the quinone form and vitamin K reductases which convert vitamin K to vitamin $KH_2$. The presence of these microsomal activities suggests that there is cycling of vitamin K among its hydroquinone, epoxide, and quinone forms during the normal course of utilization of the vitamin in liver. The enzyme DT-diaphorase [EG 1.6.99.2] (Wallin et al 1978; Wallin & Suttie 1981; Fasco & Principe 1982a) and other NAD(P)H-linked reductases (Wallin & Hutson 1982), as well as uncharacterized dithiothreitol-linked reductases in microsomes (Fasco & Principe 1980, 1982b; Sherman & Sander 1981), have been suggested as physiologically-important vitamin K reductases in microsomes. The quantitative importance of these different reductases is not yet clear.

Large doses of salicylate have been reported (Meyer & Howard 1943; Shapiro et al 1943; Clausen & Jager 1946; Barrow et al 1967) to cause decreases in the plasma concentration of the vitamin K dependent clotting factors which can be of clinical significance where liver disfunction or clotting factor abnormalities are pre-existent. A salicylate-induced hypoprothrombinaemia has been demonstrated in rats (Link et al 1943) and rabbits (Rapaport 1943; Park & Leck 1981), and it has been shown (Owens & Cimino 1980) that the vitamin K-dependent production of Factor VII in a perfused rat liver could be blocked by salicylate. Although it is generally agreed that salicylates interfere with clotting factor synthesis by interfering with the normal function of vitamin K, the circumstances under which the hypoprothrombinaemic condition can be reversed by vitamin K administration and the mechanism of the inhibition have not yet been clarified. The present investigation was directed toward examining the manner in which salicylate interferes with vitamin K metabolism and its relation to the interruption of the biosynthesis of the clotting factors. A preliminary report of these studies has appeared (Uotila et al 1981).

## MATERIALS AND METHODS

### *Demonstration of the hypoprothrombinaemic effect of sodium salicylate*

Male Holtzman rats (200 g) were housed in wire-bottom cages and fed a standard rat diet (Purina Lab

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Chow No. 5012) for 2 days, then fed a vitamin K-deficient diet (Mameesh & Johnson 1959) for 3 days. Known amounts of vitamin K were administered each evening in 0·1 ml of corn oil. On the fourth day of controlled vitamin K intake, 100 mg $kg^{-1}$ sodium salicylate or water was injected intraperitoneally. At 0 and 10 h, 0·5 ml citrated blood samples were taken by cardiac puncture. One-stage clotting assays (fibrometer) were conducted on 4- or 5-fold diluted plasma samples using commercial thromboplastin.

*Effect of sodium salicylate treatment on the vitamin K epoxide to vitamin K ratio in rat liver*

Rats fed the vitamin K-deficient diet for 3 days were given an intraperitoneal injection of 100 mg $kg^{-1}$ sodium salicylate or 500 μg $kg^{-1}$ sodium warfarin. Control animals were given 2 ml $kg^{-1}$ water. After 30 min, all animals were given 4 μCi[3H]vitamin $K_1$ (5·3 Ci $mmol^{-1}$) in 0·2 ml 50% ethanol intracardially and killed at 210 min by cervical dislocation. The livers were removed, rinsed with cold SI buffer (0·25 M sucrose/0·025 M imidazole-HCl, pH 7·2), and frozen in plastic containers placed on dry ice.

Thawed livers were minced and homogenized with a Potter-Elvehjem homogenizer in 2 ml $g^{-1}$ of cold SI buffer and extracted in duplicate by a modified Bligh & Dyer (1959) procedure. To each 2·4 ml of homogenate, 2·7 ml each of 1:2 chloroform–methanol and water were added and mixed. After low-speed centrifugation, the chloroform phase was removed, 0·4 mg each of vitamin $K_1$ and vitamin $K_1$ epoxide were added, and the extracts were dried under a stream of nitrogen gas. The residues were redissolved in 1 ml methanol, filtered through glass wool, and 0·5 ml was applied to a semipreparative μBondapak C18 reverse-phase h.p.l.c. column. Separation of vitamin K and vitamin K epoxide was accomplished with methanol as solvent, and the carrier peaks detected at 254 nm were collected into scintillation vials. Radioactivity was determined in an LKB Ultrobeta 1210 liquid scintillation spectrometer with external standardization. Both [$^3$H]vitamin K and [$^3$H]vitamin K epoxide added to the homogenate were extracted into the organic phase with an efficiency of 69%. Total liver tritium was measured by digesting 0·5 ml of homogenate in 4 ml of NCS solubilizer followed by the addition of 0·068 ml of glacial acetic acid and 10 ml of Econofluor.

*Enzyme preparations*

Warfarin-resistant rats (Hermodson et al 1969) were obtained from a colony maintained in the laboratory. For enzyme preparations, animals were treated and subcellular fractions prepared from liver as previously described by Hildebrandt & Suttie (1982).

For vitamin K epoxide reductase assays in intact microsomes, the microsomal pellets were drained and resuspended in half the supernatant volume of cold SI buffer using a Dounce glass homogenizer and used immediately. When the solubilized epoxide reductase was studied, microsomal pellets were taken up in half the supernatant volume of a detergent buffer containing 0·25 M sucrose/0·025 M Na-cyclohexylaminopropanesulfonic acid (CAPS) 0·8% Deriphat 160, pH 10·8. After centrifugation at 135 000 g for 60 min, the supernatant was drawn off and the buffer changed by means of a Sephadex G-25 desalting column equilibrated and eluted with 0·25 M sucrose/0·1 M Tris-HCl/0·1 M KCl/0·8% cholic acid, pH 8·8.

Microsomal pellets from vitamin K-deficient rats were resuspended in SI/0·08 M KCl for measurement of carboxylation by the intact vesicles.

Cytosolic DT-diaphorase was purified by affinity chromatography as previously described (Hildebrandt & Suttie 1982). Microsomal DT-diaphorase was purified from vitamin K-deficient rat liver microsomes by a similar procedure. The thawed pellets were washed free of residual cytosolic diaphorase activity by resuspending with a Dounce homogenizer in SI buffer/0·08 M KCl then repelleting at 105 000 *g* for 45 min. With three repetitions of the wash procedure, the amount of diaphorase activity appearing in the supernatant fell to less than 0·7% of the activity in the original suspension. The pellet was then solubilized in SI buffer/0·08 M KCl/2% Triton X-100 and centrifuged at 135 000 *g*. The final supernatant was chromatographed on a menadione-Sepharose 4B column under the conditions described by Wallin et al (1978). Active fractions were dialysed and submitted to ion exchange chromatography as previously described (Hildebrandt & Suttie 1982). The specific activity of the microsomal enzyme thus obtained is low (1100 nkat $mg^{-1}$) compared to the cytosolic enzyme (14 000 to 27 000 nkat $mg^{-1}$); but the enzyme appeared pure upon 10% SDS-polyacrylamide gel electrophoresis.

*Enzyme assays*

Vitamin K epoxide reductase, vitamin K epoxidase, and vitamin K-dependent carboxylase activities utilizing a peptide substrate were measured in fixed-time experiments under conditions found to

give linear reaction throughout the incubation period as described by Hildebrandt & Suttie (1982). Vitamin K and vitamin K epoxide concentrations in extracts of enzymatic digests were analysed by h.p.l.c. rather than thin-layer chromatography. The residue was redissolved in methanol and an aliquot was injected onto an analytical μBondapak C18 reverse-phase h.p.l.c. column. 97·5% Methanol/ 2·5% water was used as the chromatographic solvent. Peaks were detected at 254 nm and integrated. Blank values, measured by incubations without microsomes, were subtracted from apparent values of fractional conversion of vitamin K epoxide to vitamin K. When whole microsomes were assayed for vitamin K-dependent protein carboxylase activity, the suspension equivalent to 0·38 g liver $ml^{-1}$ was incubated with 25 μCi $ml^{-1}$ $NaH^{14}CO_3$ and either 70 μg $ml^{-1}$ $KH_2$ or 70 μg $ml^{-1}$ of the quinone plus 1 mM dithiothreitol. After 10 min rotary mixing at 27 °C, the samples were stopped with 15 volumes of 10% trichloroacetic acid. The protein was redissolved in 0·2 M $Na_2CO_3$ and reprecipitated until no more $^{14}C$ appeared in the supernatant. The washed pellets were then dissolved in 0·7 ml of NCS, 3·9 ml of Econofluor were added, and radioactivity determined with external standardization. Purified DT-diaphorase activity was measured under steady-state conditions using dichlorophenolindophenol as an electron acceptor as previously described (Ernster 1967).

*Preparations of vitamin K and its derivatives*

Tritiated vitamin $K_1$ of high specific activity was purified by partitioning between hexane and water, followed by semi-preparative reverse-phase h.p.l.c. on μBondapak C18 with methanol as solvent. Chemical formation of vitamin $K_1$ 2,3-epoxide was carried out as described by Fieser et al (1941), and the epoxide was purified by semipreparative h.p.l.c. as above. Other syntheses and purifications of vitamin K derivatives were as described earlier (Hildebrandt & Suttie 1982).

*Materials*

Unless otherwise specified, scintillation fluid consisted of 18·9 g PPO, 1·1 g dimethyl-POPOP, and 38 ml methanol per gallon of toluene. Biochemicals and reagents were obtained from the following sources: sodium warfarin (Endo, Garden City, NJ); CAPS, vitamin $K_1$, β-NADH, dichlorophenolindophenol, and Tween-20 (Sigma, St Louis, MO); sodium salicylate (Fisher Scientific, Itasca, IL); diothiothreitol (Calbiochem, Los Angeles, CA); $NaH^{14}CO_3$ and NCS (Amersham/Searle, Arlington Heights, IL); Boc-Glu-Glu-Leu-OMe (Bachem, Torrance, CA); scintillation grade Triton X-100, PPO, and dimethyl-POPOP (R.P.I., Elk Grove Village, IL); Thromboplastin C (Dade Diagnostics, Miami, Fl); Konakion (Hoffman-LaRoche, Nutley, NJ); Deriphat 160 (disodium N-lauryl-β-iminodipropionate) (Henkel Corporation, Minneapolis, MN); Aquasol and Econofluor (New England Nuclear, Boston, MA). 6,7,8,9-3H-vitamin $K_1$ was synthesized by J. A. Sadowski, Marquette University, Milwaukee, WI. Sepharose 4B, DEAE-Sephadex A50, and Sephadex G25 were purchased from Pharmacia Fine Chemicals (Piscataway, NJ).

## RESULTS

The ability of a single dose of sodium salicylate to produce a hypoprothrombinaemic state was determined in the rat. As shown in Fig. 1, the anticoagulant effectiveness of the drug was directly related to dietary vitamin K intake. When no vitamin was supplied in the diet for 4 days, the decrease in clotting activity observed over a 10 h period corresponded to the loss expected for complete inhibition of clotting factor biosynthesis, assuming a half-life of prothrombin in the plasma of 6 to 7 h.

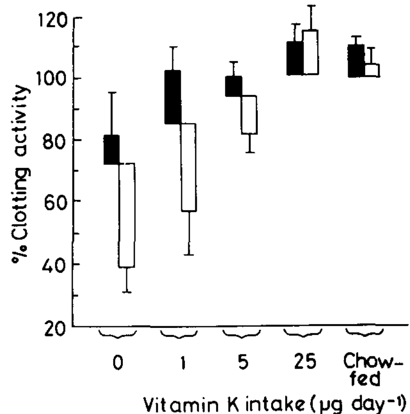


FIG. 1. Effect of salicylate treatment and vitamin K intake on clotting factor levels. The base of each bar indicates the 0 h clotting activity for each dietary group. The length of the bar indicates the magnitude of the increase or decrease in clotting activity after 10 h. Error bars are s.e.m. for 4–6 rats/group at 10 h. The s.e.m. for all 0 h groups was between 3 and 6 per cent. Closed bars are controls, and open bars are animals given 100 mg $kg^{-1}$ sodium salicylate. As measured by the Student's *t*-test, the control and treatment means at 10 h were significantly different at the $P < 0·05$ level at the 0 and 1 μg $day^{-1}$ intake level, different at the $P < 0·1$ level at the 5 μg $day^{-1}$ intake level, and did not differ in the 25 μg $day^{-1}$ and chow-fed groups.

As the amount of dietary vitamin was increased, the anticoagulant effect was diminished. Rats administered the dietary requirement of the vitamin, approximately 4 μg $day^{-1}$ in rats of this size (Suttie 1978), still retained some susceptibility to the hypoprothrombinaemic effect of salicylate. An excess supply of vitamin K such as that found in the commercial rat chow completely abolished the anticoagulant effect. The control rats in every nutritional group increased clotting activity about 10% over the 10 h period.

The percentage of plasma vitamin present as vitamin K epoxide has been reported to be elevated in methyl salicylate-treated rabbits (Park & Leck 1981). Pre-administration of sodium salicylate to rats also resulted in a statistically significant increase in the actual amount of epoxide found in the liver 3 h after injection of the labelled vitamin (Table 1). The magnitude of this effect of salicylate is small, however, when compared with the values found in warfarin-treated rats. The percentage of the injected [$^3$H]vitamin K recovered as vitamin K or vitamin K epoxide did not differ significantly among the treatment groups.

The in-vitro effects of salicylate on various enzymes involved in vitamin K metabolism were also studied. As can be seen in Fig. 2, sodium salicylate has only a slight effect on the vitamin $KH_2$-dependent carboxylation of a tripeptide substrate. When vitamin K quinone and 2 mM NADH were substituted for vitamin $KH_2$, however, the reaction was more strongly inhibited with 50% inhibition occurring at 0·6 mM. The [vitamin K + NADH]-supported carboxylation of endogenous microsomal proteins was also found to be more sensitive to inhibition by salicylate than the vitamin $KH_2$-dependent system. The [vitamin K + NADH]-dependent protein carboxylase activity was inhibited 50% by 2·5 mM salicylate, while vitamin

Table 1. Effect of administration of sodium salicylate on liver metabolism of [$^3$H] vitamin K in rates (n = 4).

| Pretreatment | % dose in liver | K[a] | % $^3$H in liver as KO | K+KO | KO / K+KO |
|---|---|---|---|---|---|
| Control | 29+1 | 56+5 | 3·2+0·3 | 60+5 | 5·3+0·1 |
| 500 μg $kg^{-1}$ Warfarin | 34+2 | 35+2 | 17·8+2·0 | 53+2 | 33·4+3·6[c] |
| 100 mg $kg^{-1}$ Salicylate | 32+2 | 49+2 | 6·4+0·5 | 55+2 | 11·7+1·1[b] |

The injected vitamin contained 4·0% epoxide by h.p.l.c. analysis. Control experiments have demonstrated that in the absence of salicylate or warfarin, injection of as much as 10% epoxide does not influence the normal liver distribution of vitamin K and vitamin K epoxide.
Values are reported as mean ± s.e.m. for each treatment group.
[a] Abbreviations used are K = vitamin K, KO = vitamin K epoxide.
[b] Significantly different from control at $P$ <0·005, by Students $t$-test.
[c] Significantly different from control at $P$ <·001, by Students $t$-test.

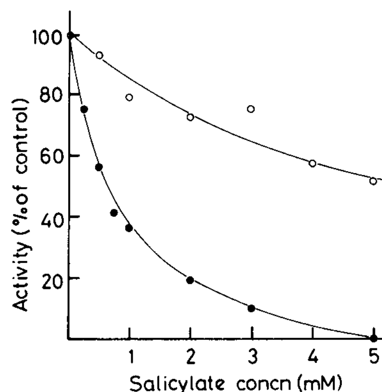


FIG. 2. Effect of salicylate on vitamin K-dependent carboxylase activity. Carboxylase activity in detergent-solubilized microsomes was measured with Boc-Glu-Glu-Leu-OMe as a substrate, and either 90 μg $ml^{-1}$ vitamin $KH_2$ (○—○) or [90 μg $ml^{-1}$ vitamin K + 2 mM NADH] (●—●) as a source of vitamin.

$KH_2$-dependent protein carboxylation was still about 86% as active as the control at this concentration of inhibitor (data not shown).

These results suggested that salicylate was acting primarily at the point of quinone reduction of vitamin $KH_2$ rather than at the carboxylase itself. In support of this hypothesis, it was found that DT-diaphorase purified from rat liver is quite sensitive to inhibition by sodium salicylate (Fig. 3). The enzyme was inhibited 50% by 80 μM salicylate. Equal sensitivity was seen with enzyme purifiable from liver microsomes. Liver microsomes also contain a dithiothreitol-dependent quinone reductase activity which is active only in intact microsomes and can be assayed by measuring the [vitamin K + DTT]-dependent carboxylation of microsomal precursor proteins. The data (Fig. 4A) indicate that this activity, but not vitamin $KH_2$-dependent protein carboxylase activity, is sensitive to salicylate. However, about three times higher concentrations of salicylate were needed to block this activity than were needed to inhibit the [vitamin K + NADH]-dependent carboxylation in solubilized microsomes (Fig. 2). This would suggest that the DTT-dependent enzyme is probably not an important site of action of salicylate in interfering with clotting factor biosynthesis. However, the [vitamin K + DTT]-dependent reaction in microsomes from the livers of warfarin-resistant rates was found to be insensitive to salicylate inhibition (Fig. 4B). The vitamin $KH_2$ epoxidase and the vitamin K epoxide reductase of rat liver microsomes were largely unaffected by sodium salicylate (Table 2). The

Table 2. In-vitro effect of sodium salicylate on vitamin $KH_2$ epoxidase and on vitamin K epoxide reductase.

| Salicylate Concn | Vitamin $KH_2$ epoxidase activity | Vitamin K epoxide reductase activity: Solubilized microsomes | Vitamin K epoxide reductase activity: Intact microsomes |
|---|---|---|---|
| 0 | 100 ± 4% | 100 ± 3% | 100 ± 4% |
| $10^{-4}$ M | 102 ± 6% | 102 ± 2% | 114 ± 3% |
| $10^{-3}$ M | 90 ± 4% | 99 ± 1% | 105 ± 10% |
| $10^{-2}$ M | 0% | 56 ± 2% | 30 ± 1% |

Values represent the mean ± s.e.m. for triplicate incubations, expressed as percent of control (no-salicylate) activity. Assays were conducted at 26 °C for 15 min (vitamin K epoxidase), 20 min (solubilized vitamin K epoxide reductase), or 40 min (microsomal vitamin K epoxide reductase).

epoxidase was inhibited at high concentration just as the $KH_2$-driven carboxylase was; the epoxide reductase was only partially inhibited at 10 mM salicylate.

## DISCUSSION

These data have demonstrated that the rat is an effective model for salicylate-induced hypoprothrombinaemia and have suggested a possible mechanism of action. Vitamin $KH_2$-dependent carboxylation/epoxidation was not sensitive to salicylate, but [vitamin K + NADH]-dependent carboxylation was. This suggests that inhibition of the reduction of vitamin K to its functional hydroquinone form represents the primary effect whereby salicylate interferes with the biosynthesis of vitamin K-dependent coagulation proteins. The enzyme DT-diaphorase, which has been suggested as an important enzyme in vitamin K reduction, was found to be sensitive to salicylate inhibition.

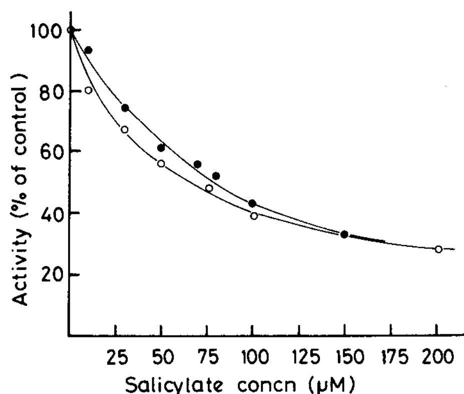


FIG. 3. Effect of salicylate on DT-diaphorase activity. Salicylate inhibition was measured with enzyme preparations purified from either rat liver cytosol (●—●) or rat liver microsomes (○—○) as described in the text.

Salicylate inhibition of several pyridine nucleotide-linked dehydrogenases has been reported (Hines & Smith 1964; Smith 1966; Smith & Dawkins 1971). The sensitivity of DT-diaphorase to salicylate is several times greater than any previously reported instance of enzyme inhibition by this drug.

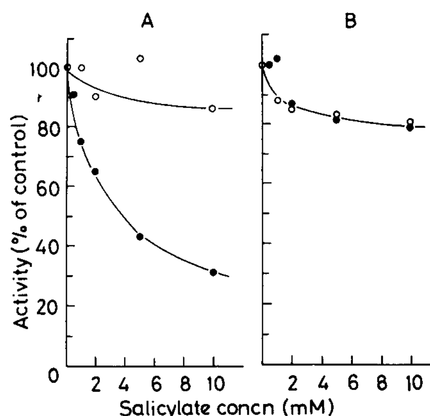


FIG. 4. Effect of salicylate on vitamin K-dependent carboxylase activity. Carboxylase activity in intact microsomes was measured with endogenous microsomal protein precursors as a substrate and either 70 μg $ml^{-1}$ vitamin $KH_2$ (○—○) or [70 μg $ml^{-1}$ vitamin K + 1 mM DTT] (●—●) as source of vitamin. Microsomal preparations were obtained from either normal (panel A) or a warfarin-resistant strain (panel B) of rats.

If this enzyme is in fact the important site of inhibition by salicylate, it might be expected that the hypoprothrombinaemia induced by salicylate would be unresponsive to administration of small amounts of vitamin K, although larger doses of the vitamin could conceivably overcome the enzyme inhibition. We have demonstrated that at least in the rat, the hypoprothrombinaemic effect of salicylate is fully preventable when a level of vitamin K intake in excess of the daily requirement is supplied. There have been various reports of clinical trials and animal experiments in which it was found that vitamin K could prevent the effect of salicylate (Meyer & Howard 1943; Shapiro et al 1943; Link et al 1943) or reverse it (Park & Leck 1981), and other reports in which the vitamin was not effective (Owens & Cimino 1980; Clausen & Jager 1946). It may be that the efficacy of vitamin K in overcoming salicylate inhibition of clotting factor synthesis is dependent on the dosage of vitamin and/or the time before or during salicylate treatment that the vitamin is administered.

The elevation of the ratio of vitamin K epoxide to vitamin K observed in-vivo by Park & Leck (1981)

and confirmed here cannot be explained on the basis of the in-vitro results presented in this study. The main enzyme involved in the recycling of vitamin epoxide, the epoxide reductase, was not salicylate sensitive. It is, however, possible that there is some subtle relationship between the vitamin K reductase and the vitamin K epoxide reductase that is not reproduced under our experimental conditions. Alternatively, salicylate could interfere with the epoxide reductase in an indirect manner. If, for example, it also acted to inhibit some component involved in supplying reducing equivalents to this enzyme (whose reductant in liver is unknown), then the prevention of recycling of the epoxide could cause some accumulation of vitamin K epoxide to be observed. The fraction of tritium present in the liver as the quinone or epoxide 3 h after vitamin administration was not significantly altered by salicylate treatment, demonstrating that an effect of salicylate on the rate of uptake or loss of the vitamin from the liver could not explain the observed effect.

An effect of warfarin on [vitamin K + DTT]-dependent carboxylation and vitamin $KH_2$ formation and an alteration of the sensitivities of this reaction in warfarin-resistant rats has also been observed (Fasco et al 1982). This is similar to the altered salicylate sensitivity of the [vitamin K + DTT]-dependent reaction observed in this study, which again points out that there may be more than one enzyme activity altered in this genetic resistance to warfarin.

*Acknowledgements*

The development by Linda J. Nyari, Jean L. Patterson, and Peter C. Presuch of certain procedures used in this study is greatly appreciated. The correspondence with Michael R. Owens was most valuable.

This work was supported by the College of Agriculture and Life Sciences of the University of Wisconsin-Madison, and in part by grant AM-14881 from the National Institute of Health, Bethesda, Maryland. Ellen Hildebrandt has been a recipient of a predoctoral fellowship from the National Science Foundation.

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