Biochemical Pharmacology, Vol. 30, No. 14, pp. 1953–1958, 1981.
Printed in Great Britain.

0006-2952/81/141953-06 $02.00/0

INHIBITION BY WARFARIN ENANTIOMERS OF PROTHROMBIN SYNTHESIS, PROTEIN CARBOXYLATION, AND THE REGENERATION OF VITAMIN K FROM VITAMIN K EPOXIDE

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(*Received* 28 *September* 1978; *accepted* 8 *January* 1981)

Abstract—*S*(−)-Warfarin was about twice as effective as *R*(+)-warfarin at inhibiting the vitamin K_1 stimulated synthesis of prothrombin in vitamin K deficient rats. It has been proposed [R. G. Bell, *Fedn Proc.* **37**, 2599 (1978)] that warfarin blocks prothrombin synthesis by inhibiting the regeneration of vitamin K from its chief metabolite, vitamin K epoxide. Consistent with this idea, *S*-warfarin was more effective than *R*-warfarin at inhibiting the epoxide to vitamin K conversion *in vivo* with an anticoagulant dose of 50 μg/100 g body wt. *S*-Warfarin was 1.9 to 3.5 times more effective than *R*-warfarin at inhibiting the epoxide to K_1 conversion in hepatic microsomal and post-mitochondrial supernatant fractions. Similarly, *S*-warfarin was 1.9 to 3.3 times more effective at inhibiting epoxide-dependent carboxylation in these preparations. *S*-Warfarin was 1.4 to 3.0 times more effective than the *R*-enantiomer at inhibiting vitamin K dependent carboxylation in post-mitochondrial supernatant fractions. We have concluded that the greater anticoagulant potency of *S*-warfarin was due to two factors of almost equal importance: the slower turnover and the higher intrinsic activity of the *S*-enantiomer in inhibiting the regeneration of vitamin K from the epoxide.

The *S*(−)-enantiomer of warfarin is five to six times more potent than *R*(+)-warfarin as an anticoagulant in rats when tested over 24 hr [1–4]. This is due, in part, to the more rapid elimination of *R*-warfarin, but it has also been asserted that *S*-warfarin has about twice the intrinsic ability to inhibit clotting-protein synthesis [2, 4]. We wished to test the relative intrinsic effectiveness of the enantiomers more directly by measuring inhibition of vitamin K stimulated synthesis of prothrombin in vitamin K deficient rats. Vitamin K is required for the carboxylation of certain glutamic acid residues to γ-carboxyglutamic acid, which is necessary to convert the prothrombin precursor to the active clotting-protein [5].

Recent evidence supports the hypothesis that the principal mechanism of action of coumarin anticoagulants is the inhibition of the regeneration of vitamin K from its chief metabolite, vitamin K epoxide [6]. If this is true, the *S*-enantiomer should be more effective than *R*-warfarin in inhibiting the conversion of the epoxide to vitamin K. *S*-warfarin also should be more effective in inhibiting carboxylation that is stimulated by the epoxide.

MATERIALS AND METHODS

The warfarin enantiomers were gifts from Dr. William Trager, University of Washington, Seattle, WA. They were dissolved in 0.05% NaOH solution, and the pH was adjusted to 7.5 with HCl. [6,7-^{3}H]Vitamin K_1 (synthesized by Dr. Chuck Siegfried, Biochemistry Department, University of Nebraska School of Medicine, Omaha, NE) was oxidized to [^{3}H]vitamin K_1 epoxide [7]. These tritiated compounds were purified by chromatography [8].

Male Sprague–Dawley rats (10- to 15-weeks-old) from Charles River Laboratories (Wilmington, MA) were used in these experiments. Vitamin K deficiency was produced by feeding them, for at least 10 days, a vitamin K deficient diet prepared by Teklad Mills, Madison, WI, according to Matschiner and Taggart [9].

Liver preparations. A 25% homogenate (1 g liver +3 ml buffer) of liver from vitamin K deficient rats was prepared with a Teflon–glass homogenizer driven by a motor. The buffer was 0.25 M sucrose, 0.005 M Mg acetate, 0.1 M KCl, and 0.025 M imidazole-HCl (pH 7.2). The post-mitochondrial supernatant fraction was prepared by centrifuging the homogenate for 10 min at 15,000 *g*. One-ml aliquots of the 15,000 *g* supernatant fraction were frozen for use later. Microsomes were prepared by centrifuging the 15,000 *g* supernatant fraction at 100,000 *g* for 1 hr. The microsomal pellet was rinsed with buffer, resuspended in half the original volume of buffer, and frozen for use later.

RESULTS

Warfarin enantiomers and plasma prothrombin. Plasma prothrombin returned to normal much more rapidly in rats injected i.p. with *R*-warfarin than in animals treated with the *S*-enantiomer (Fig. 1). Racemic warfarin was intermediate in effect.

Inhibition by warfarin enantiomers of prothrombin

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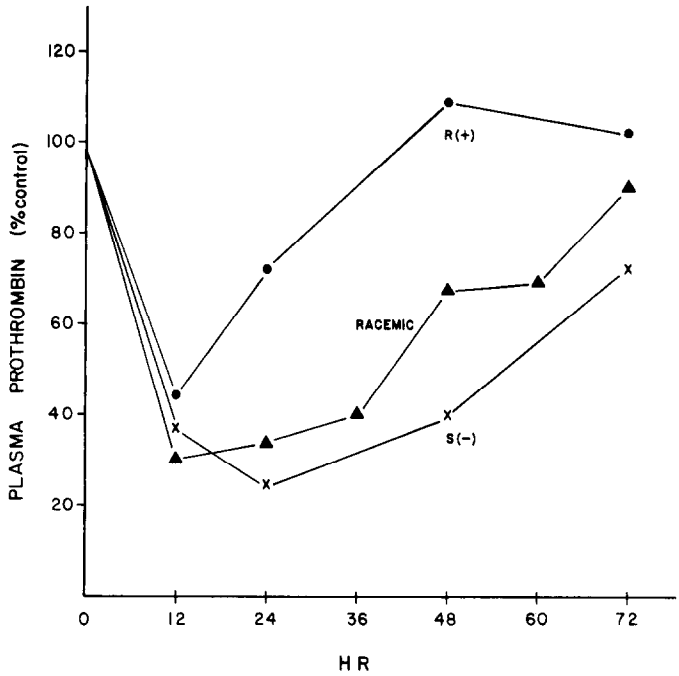

Fig. 1. Effects of warfarin enantiomers on plasma prothrombin. Rats were injected intraperitoneally (i.p.) with racemic warfarin or the enantiomers (35 μg/100 g body wt), and blood samples were taken for plasma prothrombin assay at the indicated times [10]. Each value is the average for five to seven rats.

synthesis. The effects of the enantiomers on the response to a near-minimal effective dose of vitamin K_1 (0.5 μg/100 g body wt) were measured in hypoprothrombinemic rats (Fig. 2). At a dose of 5 μg/100 g body wt, the enantiomers had little effect on the increase in plasma prothrombin that was stimulated by vitamin K_1, but at 20 μg/100 g body wt the *S*-enantiomer was clearly inhibitory. At 50 μg/100 g body wt, *S*-warfarin completely blocked the response, whereas increase in plasma prothrom-

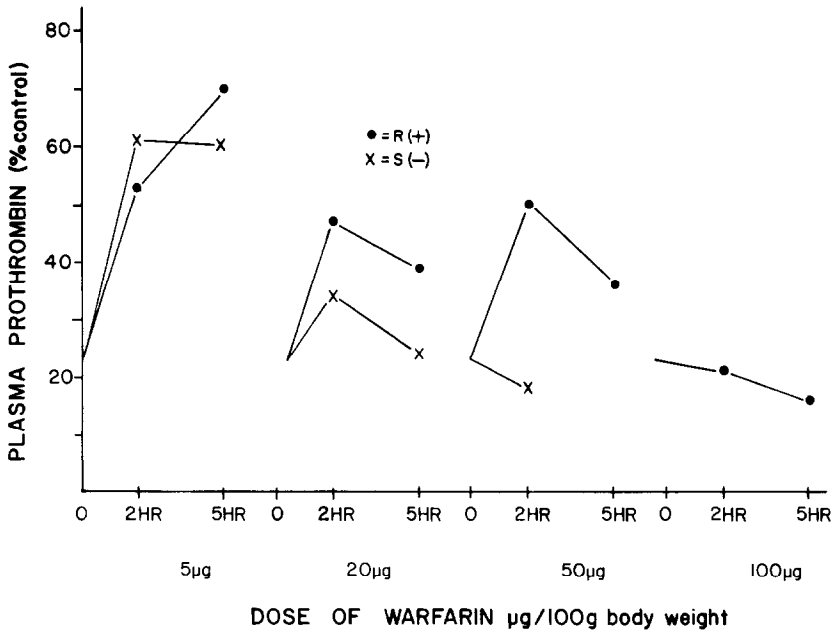

Fig. 2. Inhibition by warfarin enantiomers of the increase in plasma prothrombin stimulated by vitamin K_1 in vitamin K deficient rats. Rats were fed a vitamin K deficient diet for 10 days, and zero-time blood samples were taken. The animals were injected intracardially with vitamin K_1 (0.5 μg/100 g body wt) just after an i.p. injection of *R*- or *S*-warfarin at zero time. Blood samples were taken at 2 and 5 hr, and the plasma was assayed for prothrombin [10]. Each value is the average for three or more rats.

Table 1. Inhibition of the regeneration of vitamin K_1 from vitamin K_1 epoxide *in vivo**

	Dose (μg/100 g body wt)	[^{3}H]Epoxide:[^{3}H]K_1 ratio in liver
Control	0	0.24 ± 0.01
R-Warfarin	20	0.64 ± 0.04
S-Warfarin	20	0.53 ± 0.08
R-Warfarin	50	1.06 ± 0.10
S-Warfarin	50	1.62 ± 0.13

* Rats were injected i.p. with *R*- or *S*-warfarin and then immediately after injected intracardially with [^{3}H]vitamin K_1 epoxide (5 ng and 10^6 dpm per 100 g body wt) in a 0.5% Tween emulsion. The rats were killed 1 hr later, and livers were analyzed for [^{3}H]K_1 and [^{3}H]epoxide [12]. Each value is the average ± S.E.M. for seven rats.

bin was at a maximum during the first 2 hr after injection of the same dose of *R*-warfarin. A dose of 100 μg/100 g body wt of *R*-warfarin was required to completely block prothrombin synthesis, indicating that *S*-warfarin was about twice as effective as its enantiomer.

Inhibition by warfarin enantiomers of vitamin K_1 regeneration from the K_1 epoxide in vivo. To determine if the enantiomers differ in the ability to inhibit conversion of the epoxide to vitamin K_1, rats that had been injected i.p. with one of the enantiomers were injected intracardially with a tracer dose of [^{3}H] vitamin K_1 epoxide, and the relative amounts of [^{3}H]K_1 and the [^{3}H]epoxide in the livers were measured 1 hr later (Table 1). The [^{3}H] vitamin K_1 epoxide:[^{3}H] vitamin K_1 ratio is a measure of the inhibition of the epoxide to K_1 conversion [11]. With the lower dose of warfarin (20 μg/100 g body wt), the effects of the enantiomers were not significantly different, but at 50 μg/100 g body wt *S*-warfarin produced a significantly higher ratio ($P < 0.01$).

Inhibition by warfarin enantiomers of the conversion of vitamin K_1 epoxide to vitamin K_1 and of carboxylation stimulated by vitamin K_1 epoxide. To determine if inhibition by the enantiomers could be seen more clearly *in vitro*, their effects on the epoxide-to-K_1 conversion were measured in liver microsomes (Fig. 3). *S*-warfarin was significantly more effective at each of the concentrations tested ($P < 0.01$). The *S*-enantiomer inhibited the epoxide-to-K_1 conversion by 58–88 per cent at concentrations of 5–80 μM. When the logarithms of the warfarin concentrations were plotted against the percent

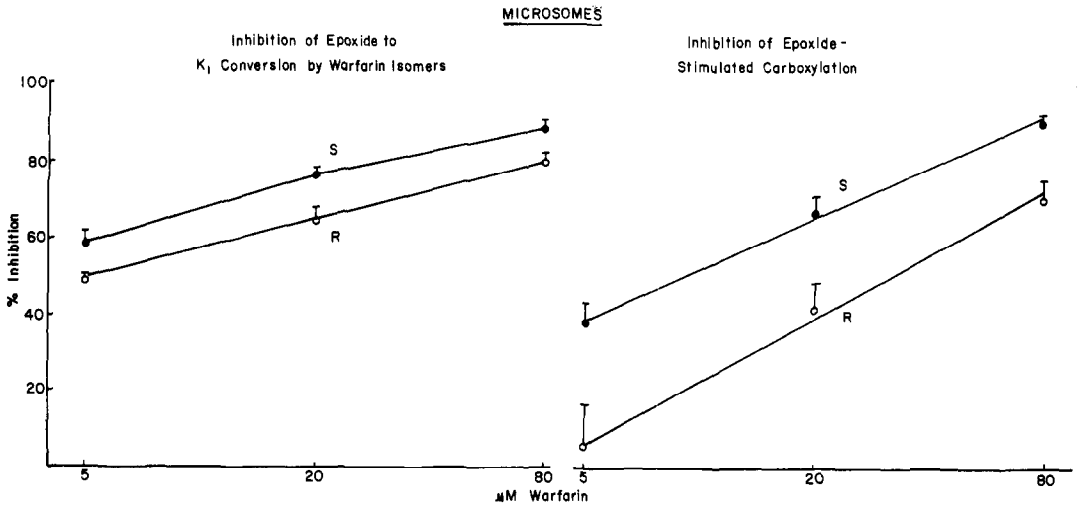

Fig. 3. Inhibition by warfarin enantiomers of the epoxide-to-K_1 conversion and epoxide-stimulated carboxylation in microsomes. The epoxide-to-K_1 conversion was assayed according to Zimmerman and Matschiner [13]. *S*-Warfarin (●) or *R*-warfarin (○) was added at the concentrations indicated. The incubation mixture contained 1 ml of thawed microsomes plus 1 mg dithioerythritol and 1 mg NADH. The reaction was initiated by the addition of 3 μg [^{3}H]vitamin K_1 epoxide (10^6 dpm in 0.015 ml ethanol), and the tube was sealed with plastic film. After incubation at 37° for 15 min with constant shaking, the reaction was terminated by adding 7 ml isopropanol–hexane (3:2). The mixture was transferred to a glass-stoppered centrifuged tube, shaken vigorously, and centrifuged for 10 min. An aliquot of the hexane layer was analyzed by reverse-phase chromatography on plastic thin-layer plates with carrier vitamin K_1 and vitamin K_1 epoxide [8]. The K_1 and epoxide spots were localized by ultraviolet light. The K_1 spot was cut out and added to scintillation fluid for ^{3}H-analysis. The average activity of the control incubations was 1.1 nmoles of [^{3}H]vitamin K_1 produced/g of liver in the 15-min incubation period. The amount of [^{3}H]vitamin K_1 produced was not affected by bubbling the assay mixture with nitrogen gas before incubation. Each point is the average for six to ten incubations; the vertical bars are the S.E.M. The incubation mixtures for the determinations of epoxide-stimulated carboxylation contained 1 ml of thawed microsomes, 1 mg dithioerythritol, 1 mg NADH, and 1.5×10^7 dpm of $Na_2{}^{14}CO_3$. *R*-Warfarin (○) or *S*-warfarin (●) was added at the indicated concentrations. The reaction was initiated by the addition of 3 μg vitamin K_1 epoxide in 0.015 ml ethanol, and the tube was sealed with plastic film. After incubation at 37° for 15 min with constant shaking, 0.1 ml of 1 M Na_2CO_3 was added, and the reaction mixture was cooled in ice. The [^{14}C]protein produced was determined according to Esmon *et al.* [14]. Each point is the average for eight incubations; the vertical bars are the S.E.M. Epoxide stimulated an average of 3.2×10^3 dpm of [^{14}C]protein/g of liver in control incubations.

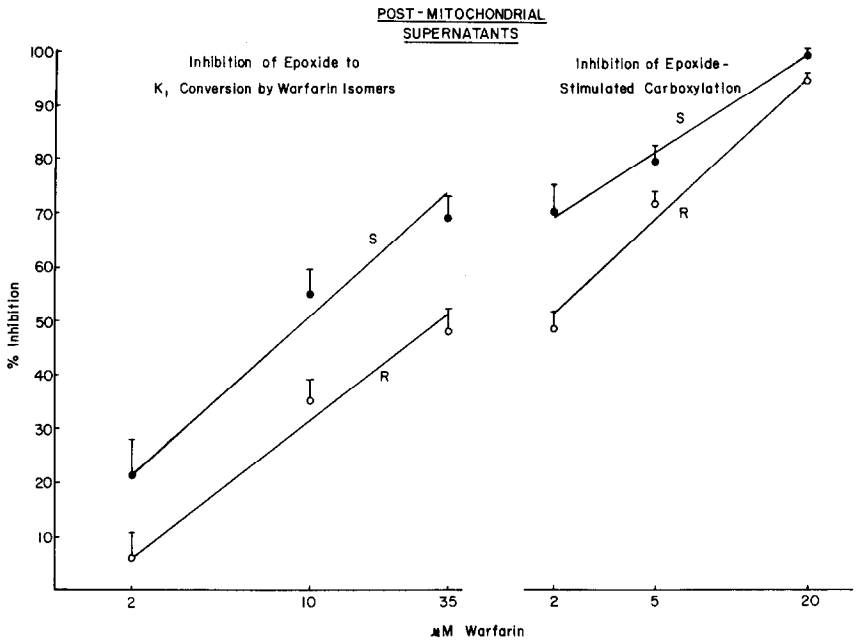

Fig. 4. Inhibition by warfarin enantiomers of the epoxide-to-K_1 conversion and epoxide-stimulated carboxylation in post-mitochondrial supernatant fractions. The epoxide-to-K_1 conversion was assayed as in Fig. 3. The incubation mixture contained 1 ml of thawed post-mitochondrial supernatant fluid, 2 ml buffer and 3 mg dithioerythritol. *R*-Warfarin (○) or *S*-warfarin (●) was added at the concentrations indicated. The reaction was initiated by the addition of 0.75 μg [^{3}H] vitamin K_1 epoxide (10^6 dpm in 0.015 ml ethanol). Each point is the average for seven or eight incubations; vertical bars represent the S.E.M. The average activity in control incubations was 0.43 nmole of [^{3}H]vitamin K_1 produced/g of liver in the 15-min incubation period. The incubation mixture for the determination of epoxide-stimulated carboxylation contained 1 ml of freshly prepared post-mitochondrial supernatant fluid and 5×10^7 dpm of $Na_2{}^{14}CO_3$. *R*-Warfarin (○) or *S*-warfarin (●) was added at the concentrations indicated. The reaction was initiated by the addition of 3 μg vitamin K_1 epoxide in 0.01 ml ethanol. The incubation and assay were carried out as in Fig. 3. Each point is the average for three to eight incubations; vertical bars represent the S.E.M. The average for the control incubations was 1.22×10^4 dpm of [^{14}C]protein produced/g of liver.

inhibition, nearly linear curves were obtained which were approximately parallel (Fig. 3). From these curves it was calculated that *S*-warfarin was 1.9 to 3.2 times more inhibitory than *R*-warfarin.

If the enantiomers differ in the ability to inhibit the reduction of vitamin epoxide to vitamin K, this should be reflected in the ability to inhibit carboxylation that was dependent on vitamin K_1 epoxide. To test this, the effects of the enantiomers on carboxylation were measured under the same conditions used to determine the epoxide-to-K_1 conversion except that $Na_2{}^{14}CO_3$ was added. Again, the semi-log curves were linear and nearly parallel (Fig. 3). *S*-Warfarin inhibited carboxylation by 37–89 per cent at concentrations of 5–80 μM and was significantly more effective than *R*-warfarin.

Since the difference between the enantiomers was small the epoxide-to-K_1 conversion was measured in post-mitochondrial supernatant fluid from liver (Fig. 4) to determine whether the cytosol could influence the inhibition. Again, *S*-warfarin was significantly more effective at the three concentrations tested ($P < 0.1$). It was calculated that *S*-warfarin was 2.7 to 3.5 times more effective than *R*-warfarin. The *S*-enantiomer inhibited the epoxide-to-K_1 conversion by 22–69 per cent at concentrations of 2–35 μM. The relative inhibition by the enantiomers of epoxide-dependent carboxylation was also studied in post-mitochondrial supernatant fractions (Fig. 4). In these experiments, dithioerythritol was omitted from the incubation so that the carboxylation would be more sensitive to warfarin inhibition [13]. Again the *S*-enantiomer, which inhibited carboxylation by 70–98 per cent over a concentration range of 2–20 μM, was significantly more potent at each of the concentrations tested ($P < 0.1$). From the curves it was calculated that *S*-warfarin was 1.9 to 2.4 times more effective than *R*-warfarin.

Warfarin enantiomers and the inhibition of vitamin K dependent carboxylation. Vitamin K dependent carboxylation in post-mitochondrial supernatant fractions was sensitive to warfarin only if the vitamin concentration was low (~0.5 μg/ml). This is presumably because warfarin inhibition of regeneration of vitamin K_1 from epoxide does not affect carboxylation if the vitamin concentration is already high. Again *S*-warfarin was significantly more inhibitory than *R*-warfarin at each concentration tested ($P < 0.1$) (Fig. 5). It was calculated that *S*-warfarin was 1.4 to 3.0 times more potent than *R*-warfarin.

Vitamin K dependent carboxylation in microsomes is inhibited by low levels of warfarin if a dithiol is the reducing agent [15]. The inhibition occurs even when the vitamin K concentration is high, indicating that warfarin does not block carboxylation by inhibiting the regeneration of vitamin K from the epoxide.

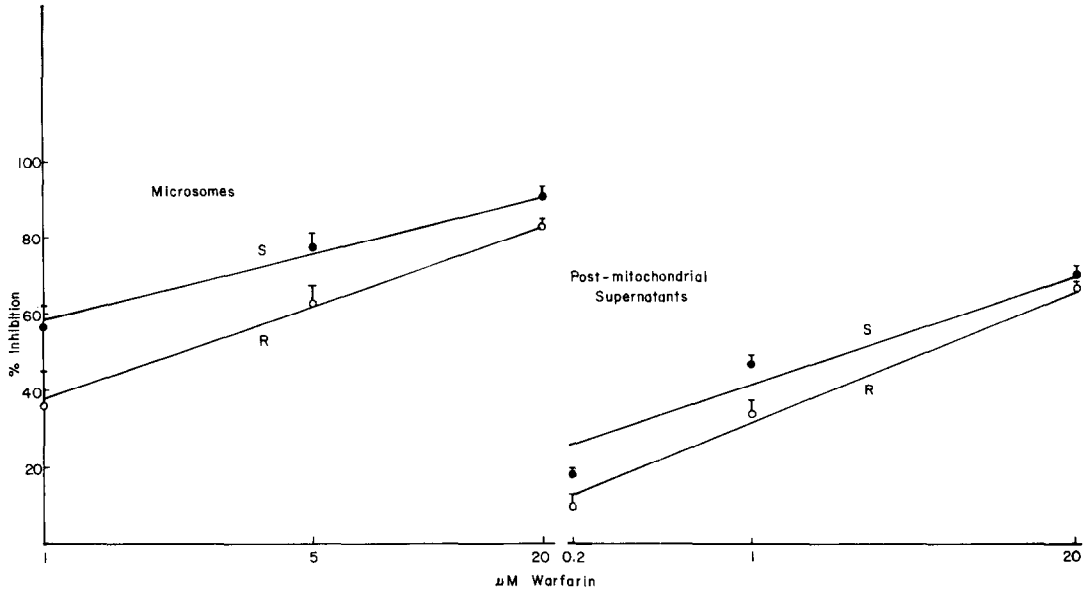

Fig. 5. Inhibition by warfarin enantiomers of vitamin K_1 dependent carboxylation. The incubation mixture for the determination of carboxylation in microsomes was 1 ml of thawed microsomes, 1 mg dithioerythritol and 2×10^7 cpm of $Na_2{}^{14}CO_3$. Carboxylation was initiated by the addition of 3 μg vitamin K_1 in 0.01 ml ethanol. The incubation mixture for carboxylation in post-mitochondrial supernatant fractions was 1 ml of freshly prepared supernatant fluid and 5×10^7 cpm of $Na_2{}^{14}CO_3$. Carboxylation was initiated by the addition of 0.5 μg vitamin K_1 in 0.01 ml ethanol. *R*-Warfarin (○) or *S*-warfarin (●) was added at the concentrations indicated. Incubations were for 15 min at 37°. Carboxylation in controls were an average of 1.18×10^4 dpm of [^{14}C]protein produced/g of liver for microsomes and 4.04×10^3 dpm/g of liver for post-mitochondrial supernatant fractions. Each result is the average for three to fourteen incubations; vertical bars represent the S.E.M.

It probably inhibits the reduction of vitamin K to vitamin K hydroquinone, a step necessary for carboxylation [16]. This afforded us an opportunity to test the relative inhibition by the enantiomers of a reaction different than the epoxide-to-K_1 conversion. *S*- Warfarin was significantly more inhibitory than *R*-warfarin at each of the concentrations tested ($P < 0.1$) (Fig. 5). It was calculated that *S*-warfarin was 2.2 to 4.0 times more potent.

DISCUSSION

We have demonstrated that *S*-warfarin is about twice as effective as *R*-warfarin in blocking prothrombin synthesis in vitamin K deficient rats. These results agree very well with those of Breckenridge and L'E Orme [2] and Yacobi and Levy [4] who calculated that the *S*-enantiomer was 1.9 and 2.2 times more effective, respectively. These results were based on studies relating plasma warfarin concentrations and prothrombin complex activities. If the epoxide reductase catalyzing the regeneration of vitamin K from vitamin K epoxide is the main target of 4-hydroxycoumarins, then *S*-warfarin should be more potent in inhibiting this reduction. *S*-Warfarin was more effective than *R*-warfarin in inhibiting the regeneration of vitamin K_1 from vitamin K_1 epoxide *in vivo* at a dose of 50 μg/100 g body wt, but there was no significant difference at a lower dose. The reason for this is not clear. However, *S*-warfarin was 1.9 to 3.5 times more effective than *R*-warfarin at inhibiting the conversion of vitamin K_1 epoxide to vitamin K_1 in hepatic microsomal and post-mitochondrial supernatant fractions. Similarly, *S*-warfarin was 1.9 to 3.3 times as effective in inhibiting epoxide-dependent carboxylation in these preparations. This was consistent with our hypothesis that the principal site of anticoagulant action is the epoxide-to-K_1 conversion [6]. Further, *S*-warfarin was 1.9 to 3.5 times more potent than *R*-warfarin *in vitro*, which was very close to its relative potency in inhibiting prothrombin synthesis *in vivo* (see Fig. 2; Refs. 2 and 4). *S*-Warfarin was 1.4 to 3.0 times more effective than *R*-warfarin in inhibiting vitamin K dependent carboxylation in post-mitochondrial supernatant fractions. These results are also consistent with inhibition by warfarin of the regeneration of vitamin K from epoxide that reduces the concentration of vitamin to an ineffectual level.

Fasco and Kaminsky [17] have also reported that *S*-warfarin was more effective than *R*-warfarin at inhibiting the epoxide-to-K_1 conversion in microsomes from Wistar rats. The difference was small but significant. Schmidt *et al.* [18] demonstrated that the *S*-enantiomer of both warfarin and phenprocoumon (a 4-hydroxycoumarin derivative) produced a higher ratio in plasma of vitamin K_1 epoxide to vitamin K_1 than the *R*-enantiomer. This indicates that *S*-phenprocoumon is a more potent inhibitor of the epoxide-to-K_1 conversion than *R*-phenprocoumon.

We have concluded that the greater anticoagulant potency of *S*-warfarin is due to two factors of almost equal importance: the slower turnover and the higher

intrinsic activity of the *S*-enantiomer. Drugs can alter the effect of warfarin by altering the metabolism of either or both enantiomers [19] or by affecting the action of warfarin on the vitamin K dependent carboxylation system in the liver. Drug interactions with warfarin are very important clinically.

In man, *S*-warfarin has a shorter plasma half-life than *R*-warfarin, in contrast to the rat. However, the intrinsic activity of *S*-warfarin in inhibiting the synthesis of the vitamin K dependent clotting-proteins has been estimated to be three to four times greater [20–22]. We would predict that the difference in the effectiveness of the enantiomers in interfering with the regeneration of vitamin K from vitamin K epoxide would be greater in man than in the rat. Shearer *et al.* [23] found that, when the enantiomers were administered to humans who had been injected with [^{3}H]vitamin K_1, similar plasma levels of [^{3}H]vitamin K_1 epoxide were found. The doses of the enantiomers, however, exceeded those required for the maximum accumulation of the epoxide [23].

Whitlon *et al.* [15] reported that, if a dithiol was used as the reducing agent in vitamin K dependent carboxylation in microsomes, the reaction was inhibited by low concentrations of warfarin. This indicated that there are two enzyme systems which reduce vitamin K to vitamin K hydroquinone, a step necessary for carboxylation. One vitamin K reductase system requires NADH and is insensitive to warfarin. The other requires a dithiol and is inhibited by warfarin. Whitlon *et al.* suggested that the latter may be the same enzyme system that reduces vitamin K epoxide to vitamin K. Our results are consistent with this idea since *S*-warfarin was 1.4 to 4.0 times more effective than *R*-warfarin in inhibiting carboxylation dependent on vitamin K_1 and dithioerythritol. The inhibition by warfarin of the reduction of vitamin K and hence of carboxylation and prothrombin synthesis does not appear to be important *in vivo* since warfarin does not inhibit prothrombin synthesis *in vivo* unless the hepatic vitamin K concentration is low (~0.75 μg/g of liver) [6]. Therefore, there appears to be only one principal site of action of 4-hydroxycoumarin anticoagulants—the vitamin K epoxide reductase system.

Acknowledgements—This study was supported, in part, by NIH Grant HL-14847. The authors thank Ms. Marilyn Panzica and Roxanne Johnson for excellent technical assistance.

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