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MECHANISM FOR POTENTIATION OF WARFARIN BY PHENYLBUTAZONE

INHIBITION OF VITAMIN K-DEPENDENT CARBOXYLATION AND PROTHROMBIN SYNTHESIS BY PHENYLBUTAZONE IN PREPARATIONS FROM RAT LIVER*

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Abstract—Phenylbutazone potentiated the anticoagulant effects of racemic warfarin and of the individual enantiomers to similar extents in the rat. This indicates that the phenylbutazone did not act stereospecifically on the enantiomers, as it does in humans. Phenylbutazone doubled the turnover rate of warfarin in plasma, but it did not increase the amount of the anticoagulant in liver or the amount excreted in urine. The drug had no effect on plasma disappearance of [^{3}H] or on hepatic levels of [^{3}H] vitamin K_1 or of its chief metabolite, [^{3}H] vitamin K_1 epoxide, after injection of [^{3}H] vitamin K_1. Phenylbutazone, however, at concentrations of 0.5 to 2.8 mM inhibited vitamin K-dependent carboxylation of a synthetic pentapeptide substrate in liver microsomes by 40–88 per cent. Vitamin K-dependent protein carboxylation was also inhibited by about 40 per cent in microsomes and post-mitochondrial supernatant fluid at drug concentrations of 2.8 to 4.8 mM. Most importantly, prothrombin synthesis was inhibited in post-mitochondrial supernatant fractions by 19 and 39 per cent at drug concentrations of 2.8 and 4.8 mM respectively. The inhibition of both carboxylation and prothrombin synthesis appears to have been of sufficient magnitude to account for the potentiation by phenylbutazone observed *in vivo*. The calculated hepatic level of phenylbutazone during potentiation was around 3 mM, a concentration that produced inhibition *in vitro*.

Phenylbutazone potentiates the decrease in plasma prothrombin that is caused by warfarin in humans [1–5], dogs [6], and rats [7]. Phenylbutazone displaces warfarin from plasma proteins [6–8] and decreases the half-life of the anticoagulant approximately 2-fold [2, 6, 7]. This suggests that phenylbutazone displaces warfarin from plasma to liver and increases the concentration of the anticoagulant at the site of clotting-protein synthesis. Phenylbutazone, however, also potentiates dicoumarol, a 4-hydroxycoumarin analog of warfarin, in rats without affecting the half-life of the anticoagulant in plasma [9].

Lewis *et al.* [4] and O'Reilly *et al.* [5] have provided evidence that phenylbutazone potentiates the effect of warfarin on plasma prothrombin in humans by inhibiting the turnover of *S*-warfarin, the more potent of the warfarin enantiomers. However, this may not be the mechanism in the rat since phenylbutazone also potentiates dicoumarol, which has no asymmetric center [9].

Studies of Levy *et al.* [7, 9–14] indicate that the rat is a good model for studies of drug interactions with warfarin. We wished to ascertain whether the potentiation of the effect of warfarin in the rat could be due to an effect of phenylbutazone on warfarin or on vitamin K metabolism, or to inhibition of vitamin K-dependent carboxylation and prothrombin synthesis.

MATERIALS AND METHODS

Chemicals. Two hundred milligrams of phenylbutazone (Sigma Chemical Co., St. Louis, MO) was dissolved in 15 ml of 0.2 N NaOH and diluted to 40 ml with water. The pH was adjusted to 7.5 with HCl. Vitamin K_1 (Sigma Chemical Co.) and menaquinone-3 (a gift from Dr. Paul Friedmann, Center for Blood Research, Boston, MA) were purified by silicic acid chromatography [15]. Sodium warfarin (Endo Laboratories, Inc., Garden City, NY) was dissolved in water. The warfarin enantiomers (a gift from Dr. William Trager, Department of Pharmaceutical Chemistry, University of Washington, Seattle, WA) were dissolved in 0.05% NaOH solution and the pH was adjusted to 7.5 with HCl. $Na_2{}^{14}CO_3$ (Amersham/Searle, Arlington Heights, IL) had a specific activity of 58.9 mCi/mmole. 5,6,7,8–[^{3}H]Vitamin K_1 (synthesized by Dr. Chuck Siegfried, Biochemistry Department, University of Nebraska, Omaha, NE; sp. act. 15 mCi/mmole) was dissolved in Tween 80 and diluted with water to a concentration of 5% Tween or less. The pentapeptide (phe–leu–glu–glu–leu) was obtained from the Vega-Fox Corp,, Tucson, AZ; 100 mg was dissolved in 10 ml of 0.1% NaOH.

Animals. Female Sprague–Dawley rats (10 to 35-weeks old) were obtained from the Charles River

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Laboratories, North Wilmington, MA, and were fed Purina rat chow.

Determination of warfarin half-life in plasma. Blood samples (1 ml) were taken at 3, 6, 12 and 26 hr after injection of 10^6 dpm of [^{14}C]warfarin (Amersham/Searle, 0.35 mg/kg body wt). [^{14}C]Warfarin in plasma was determined by modification of the procedure of Zimmermann and Matschiner [16]. Plasma (0.4 ml) was diluted with 6.0 ml water, acidified with 6 N HCl to pH 2–3, and extracted three times with diethyl ether. [^{14}C]Warfarin in the extracts was determined by thin-layer chromatography (t.l.c.) on silica gel plates developed with 2,3-dichloroethane–acetone (9:1) with carrier warfarin. Warfarin was visualized with u.v. light, and the spot was removed and assayed for ^{14}C by scintillation counting. The concentrations of warfarin in plasma were calculated and the half-life was determined by least squares analysis.

Determination of warfarin concentration in liver. Three milliliters of homogenate (1 g liver/15 ml buffer) was prepared in 0.25 M sucrose–50 mM potassium phosphate buffer, pH 7.5, which was then diluted with 6 ml water and acidified with 6 N HCl to pH 2–3. This suspension was extracted three times with diethyl ether, and [^{14}C]warfarin in the extract was determined by thin-layer chromatography as described above.

Post-mitochondrial supernatant fluid and microsomes. A homogenate (1 g liver + 3 ml buffer) from rats that had been injected with warfarin (1 mg/kg body wt) 14–18 hr previously was prepared in 0.25 M sucrose, 0.005 M Mg acetate, 0.1 M KCl and 0.25 M imidazole buffer, pH 7.2 (SIK buffer), with a Teflon–glass homogenizer driven by a motor. After centrifugation for 10 min at 15,000 *g*, the supernatant fluid was centrifuged at 100,000 *g* for 60 min, and the microsomal pellet was resuspended in its original volume.

Peptide carboxylation. One milliliter of the microsomal suspension was made 1% in Triton X-100 and incubated with 1 mg NADH, 1 mg dithioerythritol, 0.5 mg pyridoxal phosphate, 1 mg pentapeptide, 50 μg vitamin K_1 in 0.02 ml of ethanol and 1.4×10^7 cpm of $Na_2{}^{14}CO_3$ for 30 min at 27° with continuous shaking. The ^{14}C in the trichloroacetic acid supernatant fluid was determined according to the method of Suttie *et al.* [17].

Protein carboxylation in post-mitochondrial supernatant fluids. One milliliter of 15,000 *g* supernatant fluid was incubated with 5×10^7 cpm of $Na_2{}^{14}CO_3$ and 20 μg of vitamin K_1 in 0.01 ml of ethanol for 30 min at 27° with continuous shaking. In some assays 2 μg of menaquinone-3 was substituted for vitamin K_1. The ^{14}C in the trichloroacetic acid precipitate was determined according to the method of Esmon and Suttie [18].

Protein carboxylation in microsomes. One milliliter of microsomes was incubated with 1 mg NADH, 4×10^7 cpm of $Na_2{}^{14}CO_3$, and 50 μg of vitamin K_1 in 0.01 ml ethanol for 30 min at 37° with continuous shaking. The [^{14}C]-protein produced was assayed according to the method of Esmon and Suttie [1].

Prothrombin synthesis in vitro. Two milliliter samples of 15,000 g supernatant fluid were incubated with 4 μg of menaquinone-3 for 30 min at 27° with continuous shaking. The reaction mixture was cooled on ice and centrifuged at 100,000 *g* for 1 hr. The microsomal pellets were resuspended in 1 ml of SIK buffer containing 0.25% Triton and, then, assayed for prothrombin [19].

Phenylbutazone in liver. Phenylbutazone concentrations in whole liver homogenates and microsomes were determined according to the method of Jähnchen and Levy [10] for measurement of the drug in plasma. The recovery of phenylbutazone added to homogenates was 98 per cent.

RESULTS

Phenylbutazone and warfarin. Pretreatment with phenylbutazone for 4 days did not significantly alter the anticoagulant response to warfarin (Fig. 1). Phenylbutazone administration after warfarin injection, however, potentiated the response to warfarin at 12 and 24 hr. Phenylbutazone reduced prothrombin concentration to 23 per cent of normal by 24 hr after warfarin.

To determine if the potentiation was due to a stereospecific effect of phenylbutazone on the warfarin enantiomers, the effects on *R*- and *S*-warfarin were determined separately (Fig. 2). *S*-Warfarin alone rapidly reduced plasma prothrombin to 44 per cent of normal at 12 hr and to 18 per cent at 24 hr. If phenylbutazone was also administered to the anticoagulant-treated rats, prothrombin levels were reduced to 30 per cent at 12 hr and to 12 per cent at 24 hr. In rats injected with *R*-warfarin, plasma prothrombin was 52 per cent of normal at 12 hr but was not any lower at 24 hr. Phenylbutazone treatment of rats injected with *R*-warfarin caused prothrombin levels to decrease to 36 per cent at 12 hr and to 28 per cent at 24 hr. *S*-Warfarin was more potent than *R*-warfarin, but phenylbutazone potentiated the anticoagulant activities of both enantiomers to a similar extent. Although it did not appear to act stereospecifically, phenylbutazone might potentiate warfarin by altering the metabolism of both enantiomers.

Phenylbutazone did shorten the plasma half-life of warfarin from 17 to 8 hr (see Materials and Methods), but it had little effect on the amount of warfarin in the liver. The hepatic concentration of the anticoagulant at 12 hr was unchanged by phenylbutazone treatment and was slightly lower at 26 hr (Table 1). This indicates that phenylbutazone did not alter the response to warfarin by shifting warfarin from plasma to liver. Further, phenylbutazone did not alter the urinary excretion of ^{14}C after injection of [^{14}C]warfarin (Fig. 3).

Phenylbutazone and vitamin K metabolism. Each rat was injected with a tracer dose of [^{3}H]vitamin K_1 before the administration of warfarin. Phenylbutazone had no significant effect on the disappearance of ^{3}H from plasma (Fig. 4), indicating that liver uptake of the vitamin was not altered. Further, the drug had little effect on hepatic levels of [^{3}H]vitamin K_1 or on its chief metabolite [^{3}H]vitamin K_1 epoxide (Table 2), indicating that phenylbutazone did not affect the vitamin K–vitamin K epoxide cycle that is associated with prothrombin synthesis [15].

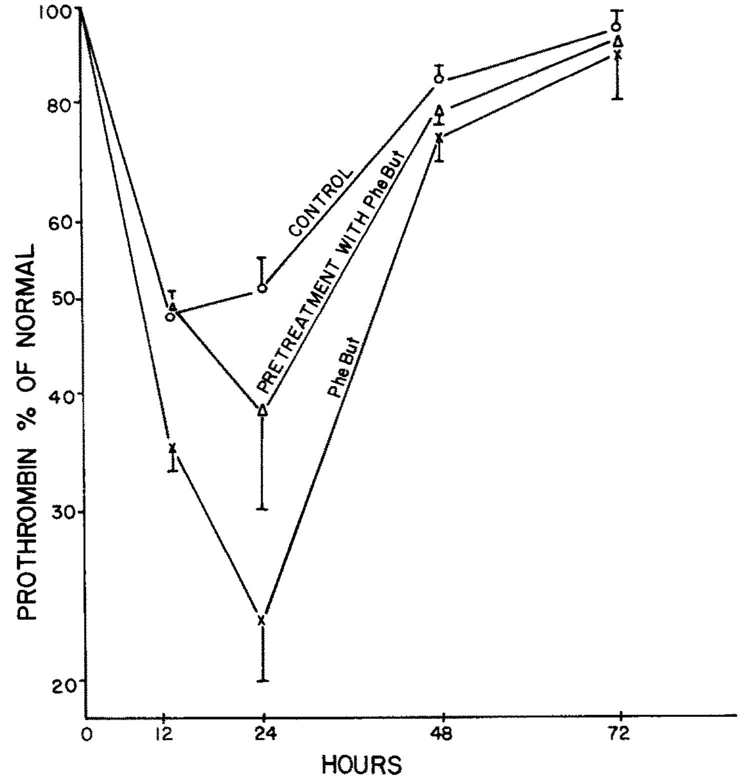

Fig. 1. Effect of phenylbutazone on the response of plasma prothrombin to warfarin. Three groups of rats were injected (i.p.) with 0.35 mg/kg body wt of sodium warfarin at zero-time. The rats of one group (×) were then injected i.p. with 40 mg/kg body wt of phenylbutazone (PheBut) every 4 hr for 12 hr and then every 12 hr for a total of 3 days starting at zero-time. Those in another group were first injected with phenylbutazone daily for 4 days and, then, warfarin was injected 2 days later (zero-time) (△). The control rats did not receive phenylbutazone (○). Plasma prothrombin was assayed by a two-stage assay [19]. Each experimental value is the average for three to six rats, and the control values are the averages for thirteen to sixteen rats. The vertical bars represent the S.E.M.

Phenylbutazone and vitamin K-dependent carboxylation. Phenylbutazone at concentrations of 0.5 to 2.8 mM inhibited vitamin K-dependent carboxylation of a pentapeptide substrate by 40–88 per cent in microsomes from warfarin-treated rats (Fig. 5). The inhibition was not reversed by increasing the concentration of vitamin K_1 nor by replacing vitamin K_1 with vitamin K_1 hydroquinone or menaquinone-3. Increasing the concentration of NADH five times or the peptide substrate three times also did not affect the inhibition by phenylbutazone. If pyridoxal phosphate was omitted from the incubation mixture, peptide carboxylation was decreased by about 70 per cent but the inhibition by phenylbutazone remained the same. If microsomes were prepared from normal rats, the inhibition by phenylbutazone was unchanged.

Table 1. Effect of phenylbutazone on [^{14}C]warfarin concentration in liver*

	[^{14}C]Warfarin (μg/g liver)	
	12 hr	26 hr
Control	1.17 ± 0.11	1.51 ± 0.05
Phenylbutazone	1.25 ± 0.08	1.18 ± 0.02

* Rats were injected i.p. with phenylbutazone (40 mg/kg body wt) at zero-time, 4, 8, 12 and 24 hr. [^{14}C]Warfarin (10^6 dpm, 0.35 mg/kg body wt) was injected i.p. at zero-time. The control rats received [^{14}C]warfarin alone. Livers were removed at 12 or 26 hr and analyzed for [^{14}C]warfarin (see Materials and Methods). The values are the averages for four to six rats ± S.E.M.

The inhibition of vitamin K-dependent protein carboxylation by phenylbutazone was not as great as the inhibition of peptide carboxylation. Phenylbutazone at a concentration of 2.8 mM inhibited protein carboxylation by 33 ± 9 per cent (average ±S.E.M.) in microsomes from warfarin-treated rats (see Materials and Methods). Phenylbutazone also inhibited vitamin K-dependent protein carboxylation in post-mitochondrial supernatant fluids of liver from warfarin-treated rats by about 40 per cent at 2.8 and 4.8 mM (Table 3). When menaquinone-3 was the source of vitamin K, the inhibition was less. More importantly, the drug inhibited prothrombin synthesis by 19 and 39 per cent at 2.8 and 4.8 mM respectively. The inhibition by phenylbutazone of carboxylation and prothrombin synthesis appeared to be of sufficient magnitude to account for the potentiation of warfarin seen *in vivo*.

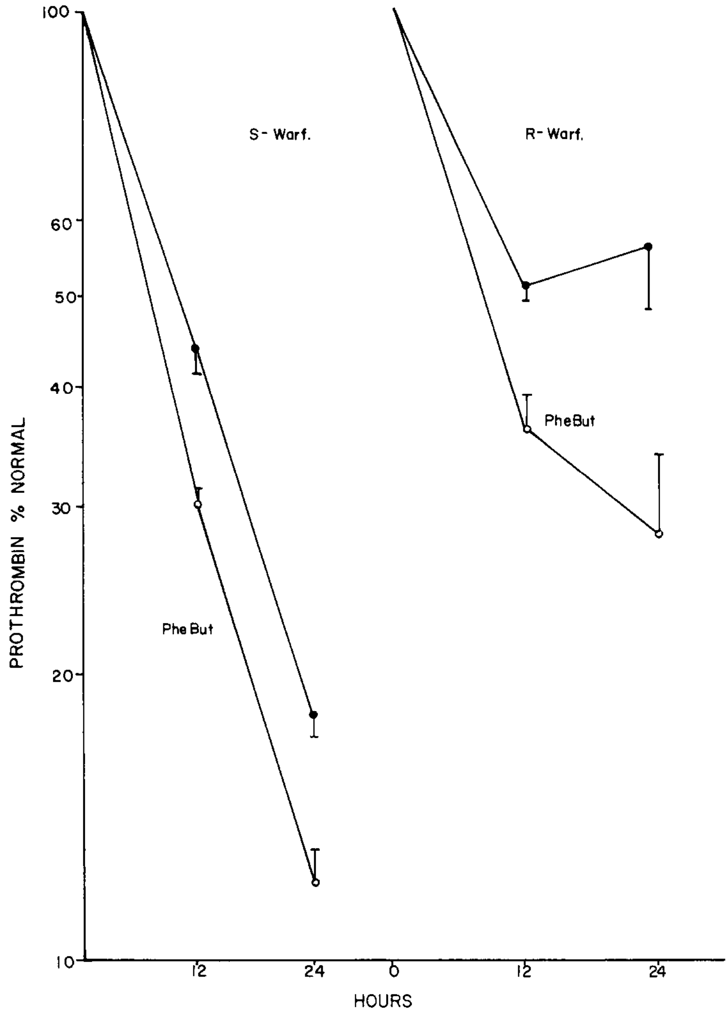

Fig. 2. Effect of phenylbutazone on the response of prothrombin to *R*- and *S*-warfarin. Rats were injected i.p. with 40 mg/kg body wt of phenylbutazone at zero-time, 4, 8 and 12 hr. *S*- or *R*-Warfarin (0.35 mg/kg body wt) was injected i.p. at the start of the phenylbutazone treatment (○). Other rats received *R*- or *S*-warfarin only (●). Blood samples were taken at the times indicated and plasma prothrombin was determined [19]. Each value is the average for four to eight rats. The vertical bars represent the S.E.M.

Table 2. Effect of phenylbutazone on [^{3}H]vitamin K_1 metabolism in liver of warfarin-treated rats*

	% Injected dose in liver at 12 hr	
	[^{3}H]Vitamin K_1	[^{3}H]Vitamin K_1 epoxide
Control	3.2 ± 0.3	1.3 ± 0.2
Phenylbutazone	2.8 ± 0.2	1.1 ± 0.1

* Each rat received an intracardial injection of a tracer dose of [^{3}H]vitamin K_1 (2×10^6 dpm) 15 min prior to i.p. injection of warfarin (0.35 mg/kg body wt). Phenylbutazone (40 mg/kg body wt) was injected i.p. every 4 hr. The control group received no phenylbutazone. Livers were removed after 12 hr and analyzed for [^{3}H]vitamin K_1 and [^{3}H]vitamin K_1 epoxide [20]. The values are the averages for three to eight rats ± S.E.M.

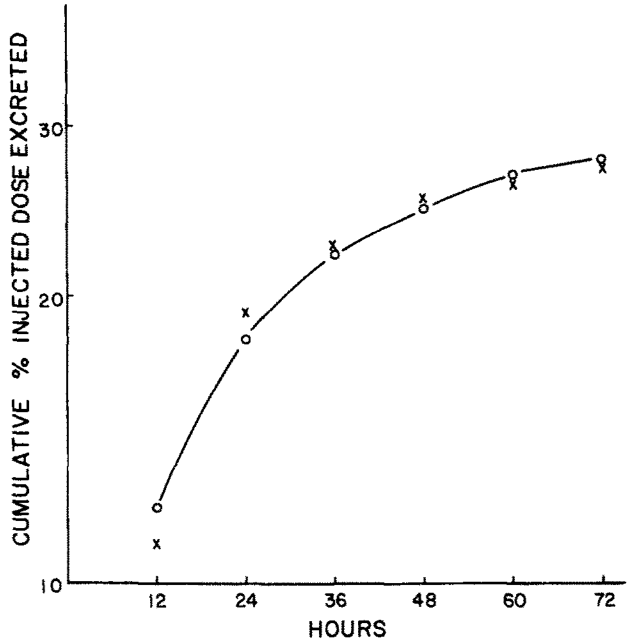

Fig. 3. Effect of phenylbutazone on urinary excretion of [^{14}C]warfarin and its metabolites. Rats were injected i.p. with 10^6 dpm of [^{14}C]warfarin (0.35 mg/kg body wt) at zero-time. Phenylbutazone (40 mg/kg body wt) was injected i.p. at zero-time, 4, 8, 12, 24, 36, 48 and 60 hr (×). The control group did not receive phenylbutazone (○). Urine samples were collected every 12 hr, and ^{14}C was determined. Each point is the average for four rats.

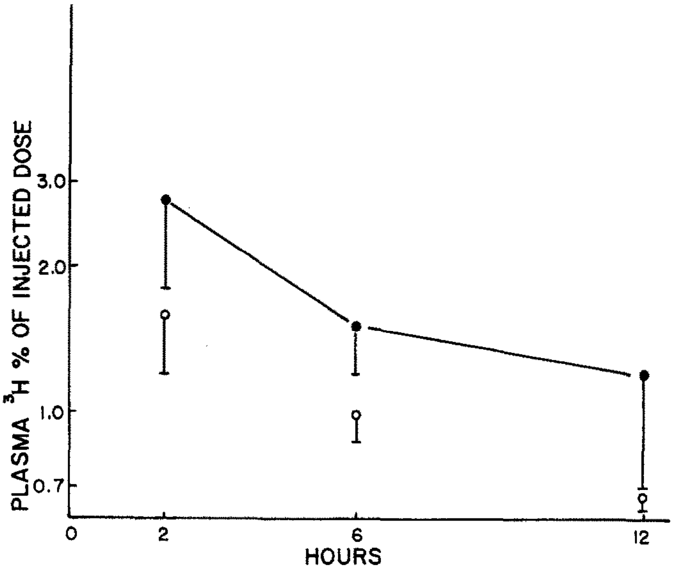

Fig. 4. Effect of phenylbutazone on plasma ^{3}H after administration of [^{3}H]vitamin K_1. Rats were injected intracardially with a tracer dose of [^{3}H]K_1 (2×10^6 dpm) 15 min prior to an i.p. injection of warfarin (0.35 mg/kg body wt). Phenylbutazone (40 mg/kg body wt) was injected i.p. at zero-time, 4 and 8 hr (○). The control group was not treated with phenylbutazone (●). Blood samples were taken at times indicated, and ^{3}H in plasma was determined. Each value is the average for four rats. The vertical bars represent the S.E.M.

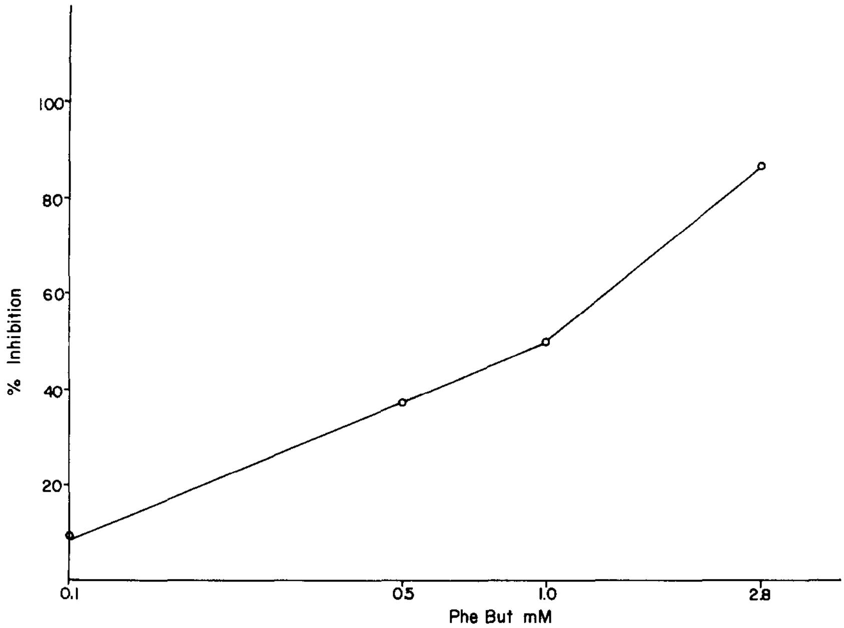

Fig. 5. Effect of phenylbutazone on vitamin K-dependent peptide carboxylation in liver microsomes. Peptide carboxylation was determined as described in Materials and Methods with the indicated amount of phenylbutazone added. The results are averages of four to eight determinations. The carboxylation in controls was 22,400–44,500 dpm of [^{14}C]peptide produced per g of liver.

To determine the phenylbutazone concentration *in vivo*, the drug and warfarin were administered as in Table 1 and the amount of phenylbutazone in liver was determined at 12 and 24 hr (see Materials and Methods). The hepatic concentration was calculated to be 3.1 ± 0.2 mM and 3.2 ± 0.4 mM respectively (the liver was assumed to contain 70 per cent water). The phenylbutazone in the microsomal fraction was 14.8 ± 1.3 per cent of the total amount in the liver at 12 hr. Thus, the liver concentration of phenylbutazone *in vivo* was comparable to that required to inhibit vitamin K-dependent carboxylation and prothrombin synthesis *in vitro*.

Phenylbutazone and prothrombin synthesis in vivo. To determine if phenylbutazone could inhibit prothrombin synthesis *in vivo*, rats were injected with warfarin (1 mg/kg body wt), which lowered prothrombin levels to 20 per cent of normal in 24 hr. Vitamin K_1 (intracardial injection of 0.75 mg/kg body wt) caused a rapid increase in plasma prothrombin that was unaffected by a series of phenylbutazone injections administered as in Table 1. However, it would be difficult to detect a small inhibition of prothrombin synthesis using this method. When rats were administered phenylbutazone as in Table 1, the concentration of plasma prothrombin was 97 ± 6 and 115 ± 10 per cent of normal at 12 and 24 hr respectively. Thus we could find no evidence of inhibition of prothrombin synthesis *in vivo* by phenylbutazone alone.

DISCUSSION

The potentiation of warfarin by phenylbutazone is well documented but the mechanism is still unknown [1–7]. In humans, phenylbutazone

Table 3. Effect of phenylbutazone on vitamin K-dependent protein carboxylation and prothrombin synthesis in post-mitochondrial supernatant fluids*

Phenylbutazone (mM)	% Inhibition of carboxylation activity		% Inhibition of Prothrombin synthesis
	K_1 added	MK-3 added	MK-3 added
1.0	17 ± 13	23 ± 8	
2.8	39 ± 4	22 ± 4	19 ± 5
4.8	41 ± 7	16 ± 6	39 ± 5

* Protein carboxylation was assayed as described in Materials and Methods. The average carboxylation in controls with vitamin K_1 (K_1) and menaquinone-3 (MK-3) was 3,000 and 10,600 cpm of [^{14}C]protein per g liver respectively. The results are the average of three to ten incubations ± S.E.M. Prothrombin synthesis was measured as described in Materials and Methods. The clotting times of controls were 32–54 sec. The results are the averages of three to four incubations ± S.E.M.

decreases the plasma clearance of the more potent *S*-warfarin while increasing that of *R*-warfarin. O'Reilly *et al.* [5], however, estimated that the inhibition of the turnover of *S*-warfarin is not great enough to account for the degree of potentiation caused by phenylbutazone.

We have confirmed that phenylbutazone also potentiates warfarin and decreases the half-life of the anticoagulant in rats. The drug, however, did not increase warfarin in liver. Similarly, Jähnchen *et al.* [79] found that phenylbutazone does not increase hepatic dicoumarol, indicating that phenylbutazone does not potentiate 4-hydroxycoumarins by displacing them from plasma to liver. It is possible that phenylbutazone displaces warfarin from liver proteins as well as from plasma proteins, but the drug did not displace [^{14}C]dicoumarol from proteins in a homogenate of rat liver [9].

If phenylbutazone potentiation of the response of prothrombin to warfarin in the rat were due to a stereoselective effect on warfarin metabolism, as in humans, then one would expect phenylbutazone to decrease the response to *R*-warfarin while potentiating the response to the *S*-enantiomer. However, phenylbutazone potentiated the response (Fig. 2) to both, thus making a stereospecific effect on one or the other of the enantiomers unlikely.

Phenylbutazone did not affect the metabolic disposition of vitamin K_1. The plasma clearance of ^{3}H and the hepatic levels of [^{3}H]vitamin K_1 and [^{3}H]vitamin K_1 epoxide were unchanged by the drug, which indicates that phenylbutazone did not inhibit the regeneration of vitamin K from vitamin K epoxide, the probable site of action of 4-hydroxycoumarins [21]. However, phenylbutazone inhibited vitamin K-dependent carboxylation and prothrombin synthesis *in vitro*. The concentration of phenylbutazone required to achieve *in vitro* inhibition was near the calculated hepatic concentration of drug during potentiation of warfarin.

The site of action of phenylbutazone is not clear. Vitamin K_1 hydroquinone did not reverse the inhibition, indicating that the site is not the reduction of vitamin K to the hydroquinone. Varying the concentrations of cofactors or the peptide substrate also did not alter the inhibition. Phenylbutazone may interact directly with the vitamin K-dependent carboxylase.

The results *in vitro* suggest that phenylbutazone alone should inhibit prothrombin synthesis *in vivo*. Yacobi *et al.* [7] reported that phenylbutazone slightly reduced plasma prothrombin complex activity, but we could find no effect of the drug on plasma prothrombin after repeated injections of phenylbutazone. It can be argued that inhibition by phenylbutazone of prothrombin synthesis *in vivo* may require warfarin. However, phenylbutazone inhibition of vitamin K-dependent peptide carboxylation in microsomes from normal rats was similar to that seen in preparations from warfarin-treated rats. Koch-Weser and Sellers [22] pointed out that it may be difficult to observe a small anti-coagulant response *in vivo* unless prothrombin complex activity is already diminished, as is the case in treatment with coumarin anticoagulants.

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