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Comparative Pharmacokinetics of Coumarin Anticoagulants XLV: Pharmacokinetic and Pharmacodynamic Studies of Acute Interaction between Warfarin and Phenylbutazone in Rats

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Abstract □ A comprehensive investigation of the effect of phenylbutazone on warfarin pharmacokinetics and anticoagulant activity was carried out in rats to identify and quantify various aspects of the interaction between these drugs. Adult male rats received intravenous racemic warfarin alone and together with phenylbutazone in a crossover experiment. Prothrombin complex activity and the plasma concentrations of phenylbutazone and of free and total (free plus protein-bound) warfarin were determined repeatedly for up to 60 hr. The total plasma clearance, the apparent volume of distribution, and the disposition rate constant (β) of warfarin were significantly increased and the intrinsic plasma warfarin clearance was significantly decreased during phenylbutazone administration. Phenylbutazone decreased the serum protein binding of warfarin both *in vitro* and *in vivo*, but the *in vivo* effect was much more pronounced, apparently due to the displacing effect of phenylbutazone metabolite(s). Phenylbutazone alone had no apparent effect on prothrombin complex activity *in vitro* but caused a modest, yet statistically significant, anticoagulant effect *in vivo*. The anticoagulant effect–plasma warfarin concentration curves for total and free warfarin were shifted to a considerably lower concentration range during phenylbutazone treatment. Thus, the interaction between phenylbutazone and warfarin involves at least three processes: an inhibition of warfarin biotransformation (decreased intrinsic clearance); displacement of warfarin from plasma protein binding sites (increased free fraction); and apparent potentiation of the anticoagulant action produced by a given plasma warfarin concentration. The latter may have been caused, at least in part, by a direct anticoagulant effect of phenylbutazone and/or its metabolite(s). The net effect of decreased protein binding and decreased intrinsic clearance was an increase in the total plasma warfarin clearance. The results of this investigation demonstrate that drug interactions can be complex and multifactorial.

Keyphrases □ Warfarin—interaction with phenylbutazone, pharmacokinetics, *in vitro* and *in vivo*, rats, prothrombin complex activity □ Phenylbutazone—interaction with warfarin, pharmacokinetics, *in vitro* and *in vivo*, rats, prothrombin complex activity □ Anticoagulants—warfarin, interaction with phenylbutazone, pharmacokinetics, *in vitro* and *in vivo*, rats, prothrombin complex activity

There is a tendency to attribute most drug interactions to only one of several theoretically possible mechanisms. This approach is probably unrealistic and reflects the limited scope and the relative lack of pharmacokinetic quantitation characteristic of most drug interaction studies. For example, one drug may displace another from plasma protein binding sites and cause the displaced drug to be eliminated more rapidly. Unless the increased clearance of the displaced drug is *quantitatively* consistent with the increase of its free fraction in plasma, additional interaction mechanisms must be sought. These additional mechanisms may operate in the same direction (*i.e.*, increased clearance) or in the opposite direction from that attributable to reduced plasma protein binding. Moreover, pharmacokinetic as well as pharmacodynamic interactions may occur so that an assessment of the therapeutic implications of the interacting system requires consideration of the net effect resulting from both types of interactions. These potential complexities suggest that comprehensive model drug interaction studies are needed for the development of more effective strategies for exploring potential or suspected interactions involving new drugs.

The interaction between the anticoagulant warfarin and the anti-inflammatory agent phenylbutazone is phenomenologically well established, and its potentially disastrous clinical consequences are generally appreciated (1–3). The interaction between these two drugs has been studied in humans (4–8) and in dogs (9). However, the recent development of clearance concepts (10, 11) and the availability of an animal model exhibiting wide interindividual differences in the plasma protein binding of warfarin under normal physiological conditions (12) have provided a more rational basis and a promising means for better exploration of the interaction.

The investigation described here consisted of a rigorous quantitative determination of the phenylbutazone effect on the elimination kinetics and anticoagulant action of warfarin in a crossover study on rats specially selected for wide interindividual differences in serum protein binding of warfarin and, therefore, in warfarin clearance. The phenylbutazone effect on serum protein binding of warfarin was determined *in vitro* and *in vivo*. The relationship between anticoagulant effect and warfarin concentration in plasma was determined for free and total (free plus bound) drug. The phenylbutazone effect *per se* on the coagulation process was examined *in vitro* and *in vivo*.

It is believed that the results of these studies will demonstrate the potential complexity and multifaceted characteristics of drug interactions, will illustrate the practical limitations of many drug interaction studies in humans,

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Table I—*In Vitro* Phenylbutazone Effect on Protein Binding of Warfarin in Rat Serum [a]

Phenylbutazone, μg/ml	Warfarin Concentration 0.43 μg/ml	0.70 μg/ml	1.43 μg/ml	2.90 μg/ml	Mean ± *SD*
0	0.586	0.569	0.553	0.645	0.588 ± 0.040
25	0.830	0.846	0.752	0.821	0.812 ± 0.041
50	1.02	0.914	0.893	0.893	0.930 ± 0.061
100	1.01	1.03	1.08	1.16	1.07 ± 0.067
200	2.27	2.21	2.29	2.45	2.30 ± 0.103

[a] Pooled serum from eight rats. Reported are values of free fraction × 100.

and will suggest the effort required for a comprehensive and quantitative exploration of drug interactions in general.

EXPERIMENTAL

***In Vitro* Phenylbutazone Effect on Serum Protein Binding of Warfarin**—Eight randomly selected adult male Sprague–Dawley rats[1] were sacrificed under ether anesthesia by withdrawal of all blood from the abdominal aorta. The blood was converted into serum, and the latter was pooled. Two-milliliter portions of serum were spiked with racemic ^{14}C-warfarin[2] and with phenylbutazone[3] to produce concentrations of 0.4–3 μg of warfarin/ml and 0–200 μg of phenylbutazone/ml. ^{14}C-Warfarin was dissolved in ethylene dichloride. A portion of this solution was transferred to a glass vial and evaporated under nitrogen flow. Ten microliters of phenylbutazone in 0.1 *N* NaOH was added to the warfarin, and the mixture was shaken thoroughly and mixed with the serum. The serum binding was determined by equilibrium dialysis at 37° (12).

***In Vivo* Phenylbutazone Effect on Serum Protein Binding of Warfarin**—Ten randomly selected rats, 340–415 g, received phenylbutazone, 40 mg/kg ip, initially followed by 20 mg/kg ip every 4 hr for 10 doses. Immediately before the first injection and 2 hr after the last one, 2.5–3.0 ml of blood was withdrawn from the tail artery. Serum was separated and spiked with ^{14}C-warfarin for protein binding determinations.

Assay of Warfarin and Phenylbutazone—The concentrations of warfarin in serum, plasma, and buffer solution were determined by extraction, TLC, and scintillation counting (13). Phenylbutazone in plasma and serum was assayed by the method of Jähnchen and Levy (14). That assay was modified slightly to increase the sensitivity. To 0.1 ml of plasma, 0.1 ml of 3 *N* HCl and 2 ml of *n*-heptane were added. After shaking for 30 min, the phases were separated by centrifugation at 1000×*g* for 3 min. To 1.8 ml of the *n*-heptane phase, 2 ml of 1 *N* NaOH was added. After shaking for 5 min and centrifuging for the same time, the heptane phase was discarded. To 1.8 ml of the sodium hydroxide phase, 3 ml of buffered

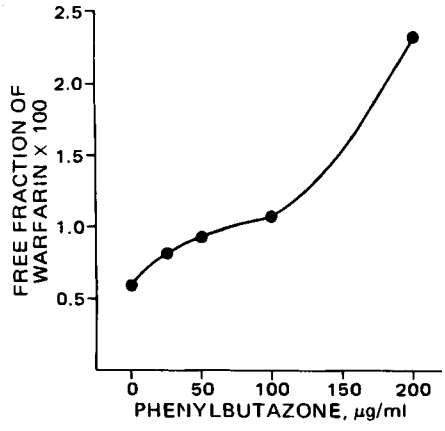

Figure 1—*Relationship between mean free fraction of warfarin in serum and phenylbutazone concentration. Both drugs were added to pooled rat serum* in vitro.

[1] Blue Spruce Farms, Altamont, N.Y.
[2] 3-[α-Acetonyl(benzyl-α-^{14}C)]-4-hydroxycoumarin, specific activity 71 μCi/mg, Amersham-Searle Corp., Arlington Heights, Ill.
[3] Geigy Pharmaceuticals, Ardsley, N.Y.

Table II—Phenylbutazone Effect on Warfarin Pharmacokinetics in Rats [a]

Pharmacokinetic Constant	Control	With Phenylbutazone
Total plasma clearance, ml/hr/kg	8.88 (2.92–24.0)	17.8[b] (6.74–41.2)
Half-life, hr	16.3 (6.31–31.6)	8.66[b] (4.55–14.6)
Volume of distribution, ml/kg	152 (117–219)	175[b] (128–271)
Serum free fraction × 100	0.908 (0.296–1.90)	2.92[b,c] (0.957–7.03)
Intrinsic plasma clearance, ml/hr/kg	985 ± 163 (764–1304)	651 ± 83[b] (497–796)

[a] Results are reported as mean values for 11 animals, with the range in parentheses. Standard deviations are omitted, with one exception, for reasons discussed in the text. [b] Statistically significantly different from the control value ($p < 0.01$) by paired *t* test. [c] The concentration of phenylbutazone in these serum samples was 67.1 ± 24.6 μg/ml.

permanganate solution (14) was added, and the mixture was incubated at 65° for 5 min. After cooling, 1 ml of *n*-heptane was added, and the procedure was continued as described previously (14). The assay was not affected by warfarin nor was the warfarin assay or recovery affected by phenylbutazone.

Warfarin–Phenylbutazone Interaction—Twelve dicumarol-screened (15, 16) male adult Sprague–Dawley rats, weighing 350–450 g and differing widely in dicumarol half-life, were selected from a group of 30 animals. They had unrestricted access to food[4] and water before and during the experiment. The rats were given intraperitoneal injections of phenylbutazone, 40 mg/kg, or saline solution at −2 hr and then 20 mg of phenylbutazone/kg or saline solution every 4 hr for 50–60 hr. The animals received warfarin, 0.6 mg/kg iv consisting of about 22 μCi of ^{14}C-warfarin[5]/kg and nonradioactive warfarin[6], except for three rats that received only 0.3 mg/kg in the experiment with phenylbutazone. Three weeks later, the rats that had received phenylbutazone received saline solution and *vice versa*. Blood samples were collected repeatedly from the tail artery for 40–60 hr after warfarin injection.

The warfarin injection solutions were prepared by separately dissolving the labeled and unlabeled drug in small volumes of 0.1 *N* NaOH. The

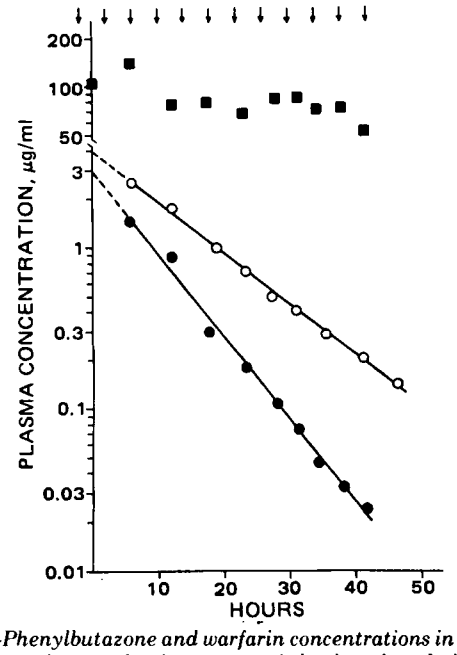

Figure 2—*Phenylbutazone and warfarin concentrations in the plasma of a representative rat after intravenous injection of warfarin alone* (○) *and with repeated intraperitoneal injections of phenylbutazone* (●). *The squares represent the phenylbutazone concentration in plasma, and the arrows indicate when phenylbutazone was injected.*

[4] Charles River Formula 4RF.
[5] About 8 μCi/rat, specific activity 71 μCi/mg.
[6] Endo Laboratories, Garden City, N.Y.

Table III—Phenylbutazone Effect on Serum Protein Binding of Warfarin in Rats Treated with Phenylbutazone Only [a]

	Warfarin[b], μg/ml		Phenylbutazone[b], μg/ml	Free Fraction of Warfarin in Serum × 100		
	Control	With Phenylbutazone		Control	With Phenylbutazone	Ratio[c]
Mean[d]	0.730	0.758	62	0.588	2.33[e]	4.0
SD	0.071	0.051	14	0.156	0.60	0.6
Range	0.630–0.861	0.665–0.846	37–78	0.260–0.767	1.32–3.26	3.3–5.1

[a] ^{14}C-Warfarin was added to the serum *in vitro*. [b] Measured after equilibrium dialysis. [c] Ratio of free fraction values, with phenylbutazone:control. [d] n = 10. [e] Statistically significantly different from the control value (paired t test, $p < 0.001$).

unlabeled drug solution was added to the labeled drug solution, and this mixture was diluted immediately with pH 7.4, 0.15 *M* phosphate buffer such that 1 ml of the final solution contained 0.29–0.34 mg of warfarin. Phenylbutazone was dissolved in 0.1 *N* NaOH, and the solution was diluted with saline; the pH was adjusted to ~8 with pH 7.4, 0.15 *M* phosphate buffer. The solutions were prepared just before injection.

Collection of multiple blood samples (0.45 ml) from rats, measurement of prothrombin time in 10-fold diluted plasma, conversion of prothrombin time to prothrombin complex activity (PCA), and calculation of prothrombin complex activity synthesis rates (R_{syn}) were carried out as described previously (16). Two 0.1-ml plasma samples were used for warfarin and phenylbutazone determinations, respectively. Two hours after the last phenylbutazone or saline injection, ~1.2 ml of blood was withdrawn, and the serum was separated. The latter was spiked with ~0.3 μg of ^{14}C-warfarin, and ~0.5 ml was used for serum protein binding determinations.

The apparent first-order elimination rate constant (β) and the warfarin biological half-life were determined from the least-squares slope of a plot of log plasma warfarin concentrations *versus* time. The apparent volume of distribution (V_d) was calculated by dividing the injected dose by the extrapolated zero-time plasma warfarin concentration. Total plasma clearance was calculated as the product of β and V_d, and intrinsic plasma clearance was calculated by dividing the total clearance by the serum free fraction of warfarin. The plasma warfarin concentrations at which the prothrombin complex activity R_{syn} was 0 and 50% of normal/day were determined from the linear portion of a plot of R_{syn} *versus* log plasma warfarin concentration.

RESULTS

Table I shows the *in vitro* effect of phenylbutazone (0–200 μg/ml) on the protein binding of warfarin (0.43–2.90 μg/ml) in rat serum. The warfarin concentrations were in the range of experimental interest. The free fraction of warfarin increased with increasing phenylbutazone concentration but was practically independent of warfarin concentration. A plot of mean free fraction value *versus* phenylbutazone concentration shows a shoulder in the 25–100-μg/ml range (Fig. 1). Therefore, the phenylbutazone dosage regimen was designed to maintain concentrations in this range to minimize fluctuations in the free fraction of warfarin in plasma during the *in vivo* experiments.

Figure 2 shows the effect of phenylbutazone administration on the warfarin concentrations in the plasma of a representative rat (Rat 3). Also shown are the plasma phenylbutazone concentrations. Blood samples were obtained at different times relative to the time of phenylbutazone injections (because blood sampling times were dictated primarily by the rate of the prothrombin complex activity change); this accounts for much of the fluctuation of the reported phenylbutazone concentrations in any one animal. The mean plasma phenylbutazone concentration of the 11 rats used in this study ranged from 45 to 90 μg/ml, with an average coefficient of variation of 29% in individual animals.

The phenylbutazone effects on warfarin pharmacokinetics in 11 rats are summarized in Table II. Phenylbutazone administration caused a significant increase in the total plasma clearance, apparent volume of distribution, and serum free fraction of warfarin and a significant decrease in the biological half-life and intrinsic plasma clearance of the drug. The animals used were selected by a screening test from a larger group of rats to obtain animals with widely different and relatively evenly distributed total clearance values. Consequently, all pharmacokinetic data in Table II, except those representing intrinsic clearance (which shows relatively small interindividual variation), are reported in terms of the mean and range of individual values rather than as the mean ± *SD*.

It is more informative to consider the ratio, phenylbutazone:control, than the absolute values of the pharmacokinetic constants. These ratios were determined individually for each animal and are reported as the mean ± *SD*, n = 11: total plasma clearance, 2.10 ± 0.40; intrinsic plasma clearance, 0.674 ± 0.120; and free fraction in serum, 3.16 ± 0.59. The relatively small coefficient of variation of these constants reflects a strong correlation between values obtained from individual rats in control experiments and during phenylbutazone administration. This correlation is shown in Fig. 3 for the biological half-life, in Fig. 4 for the apparent volume of distribution, and in Fig. 5 for the serum free fraction of warfarin.

There is no apparent relationship between the average plasma phenylbutazone concentration (indicative of the total clearance of that drug) in individual rats and the total or intrinsic warfarin clearance (alone or during phenylbutazone administration). On the other hand, there is some indication of a concentration–response relationship with respect to phenylbutazone inhibition of warfarin biotransformation. Specifically, there appears to be a negative correlation between average phenylbuta-

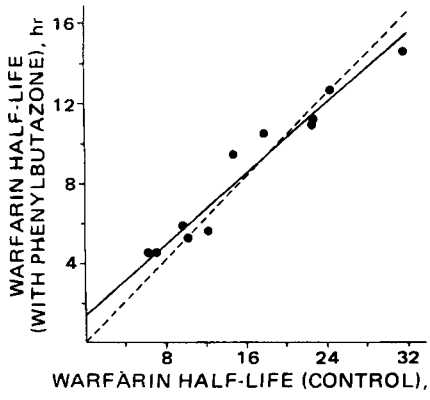

Figure 3—*Relationship between the warfarin biological half-life in control experiments and in the same rats during concomitant phenylbutazone administration. In this figure, as well as in Figs. 5 and 6, the continuous line was obtained by double-regression analysis and the stippled line was obtained by forcing the regression line through the origin. The correlation coefficient is 0.974, p < 0.001.*

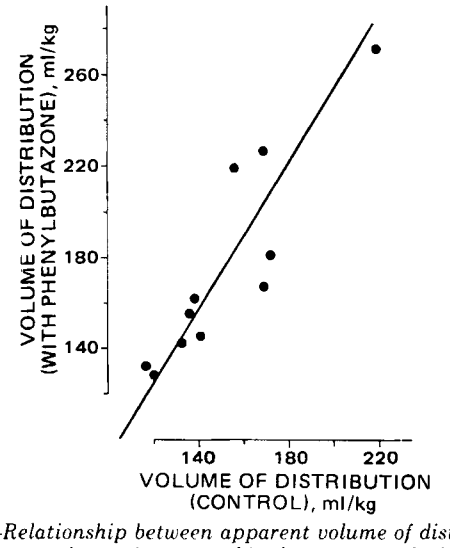

Figure 4—*Relationship between apparent volume of distribution of warfarin in control experiments and in the same rats during concomitant phenylbutazone administration. The correlation coefficient is 0.896, p < 0.001.*

Table IV—Effect of Phenylbutazone on Prothrombin Complex Activity (PCA) before Warfarin Administration and on the Maximum Anticoagulant Action of a Single Warfarin Dose in Individual Rats

Rat	PCA before Warfarin Administration, % of normal		Maximum Effect[a], PCA_{min}, % of normal	
	Control	With Phenylbutazone	Control	With Phenylbutazone
1	98.4	94.9	10.0	3.5
2	106.1	94.9	15.0	8.5
3	106.1	98.4	10.7	2.8
4	94.9	102.1	11.0	6.5
5	110.4	94.9	12.4	7.7
6	94.9	98.4	11.0	4.4
7[b]	102.1	96.6	13.8	12.2
8	102.1	100.2	12.8	2.3
9[b]	102.1	98.4	22.9	11.3
10	98.4	91.7	19.3	—[c]
11[b]	106.1	98.4	28.9	11.2
Mean	102.0	97.1[d]	11.8[e]	5.10[e,f]
SD	5.0	2.9	1.7	2.46

[a] Expressed as the lowest observed prothrombin complex activity value (PCA_{min}). [b] These rats received 0.3 mg of warfarin/kg with phenylbutazone (see text for explanation); the others received 0.6 mg of warfarin/kg with phenylbutazone. All rats received 0.6 mg of warfarin/kg in the control experiments. Even-numbered animals received phenylbutazone and warfarin in the first experiment; odd-numbered animals received only warfarin in the first experiment. [c] The experiment with this rat was discontinued because of severe bleeding. [d] Statistically significantly different from the control value (paired *t* test, $p < 0.05$). [e] Mean of the data for Rats 1–6 and 8 only. [f] Statistically significantly different from the control value (paired *t* test, $p < 0.001$).

zone concentration in plasma and the phenylbutazone:control ratio of the intrinsic warfarin clearances in individual animals (Fig. 6).

In view of the pronounced difference between the *in vitro* and *in vivo* effects of comparable phenylbutazone concentrations on serum protein binding of warfarin (free fraction values in Tables I and II), a group of rats was given a series of phenylbutazone injections (*i.e.*, no warfarin), and serum free fraction values were determined before and during phenylbutazone treatment. The results (Table III) show a magnitude of displacing effect similar to that observed in the *in vivo* warfarin–phenylbutazone interaction study and much greater than that observed in the *in vitro* experiments.

Theoretical considerations and previous experimental evidence (10) indicate that an essentially linear relationship between the total plasma clearance and the serum or plasma free fraction of warfarin should exist. Such correlations were obtained in the present study for warfarin in control experiments and during phenylbutazone administration (Fig. 7). The differences in the regression line slopes reflect the phenylbutazone effect on the intrinsic warfarin clearance. The shift of the free fraction values during phenylbutazone administration to a higher range than that observed without phenylbutazone is indicative of the displacing effect.

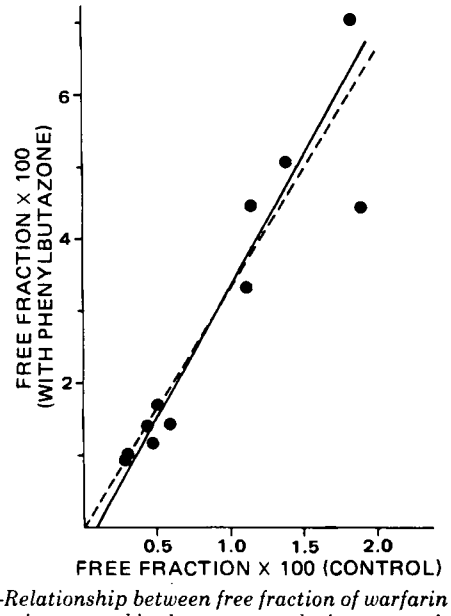

Figure 5—*Relationship between free fraction of warfarin in serum in control experiments and in the same rats during concomitant phenylbutazone administration. The correlation coefficient is 0.927,* $p < 0.001$.

Table V—Effect of Phenylbutazone on Relationship between Relative Prothrombin Complex Activity Synthesis Rate and Warfarin Concentration in Rat Plasma

Rat	$-m$[a], % of normal/day		C_{max}[b], μg/ml		Ratio, Control:With Phenylbutazone
	Control	With Phenylbutazone	Control	With Phenylbutazone	
1	1.16	1.32	0.298	0.0836	3.6
2	1.14	1.22	0.420	0.130	3.2
3	1.11	0.851	0.693	0.120	5.8
4	0.985	0.950	0.942	0.156	6.0
5	1.22	0.977	0.720	0.126	5.7
6	1.13	1.28	1.65	0.330	5.0
7	1.02	2.11	2.00	0.454	4.4
8	1.65	2.94	2.20	0.236	9.3
9	1.78	2.63	2.03	0.336	6.0
10	1.48	—[c]	2.72	—[c]	—[c]
11	2.72	1.58	3.14	0.754	4.2
Mean[d]	1.39	1.58[e]	1.41	0.272[f]	5.3
SD	0.535	0.731			1.7

[a] Slope of a plot of R_{syn}/R^0_{syn} *versus* log plasma warfarin concentration. [b] Intercept of the plot described in footnote *a* upon extrapolation to the concentration axis where $R_{syn}/R^0_{syn} = 0$. [c] The experiment with this rat was discontinued because of severe bleeding. [d] Without Rat 10. [e] No statistically significant difference between the two treatments. [f] Statistically significantly different from the control value (paired *t* test, $p < 0.001$).

Phenylbutazone, 100 μg/ml added *in vitro* to individual serum samples from 12 rats, had no effect on prothrombin complex activity. The prothrombin complex activity in serum (mean ± *SD*) was 98.2 ± 8.6% of normal without phenylbutazone and 98.4 ± 7.3% with added phenylbutazone. The ratio of prothrombin complex activity values, phenylbutazone:control, was 1.00 ± 0.03 (mean ± *SD*). On the other hand, phenylbutazone administered to rats caused a small but statistically significant prothrombin complex activity decrease (Table IV). This apparent anticoagulant effect was evident 2 hr after the first phenylbutazone dose, *i.e.*, at zero time relative to the time of warfarin injection when the first (before warfarin) blood sample was obtained (Fig. 2).

The maximum warfarin anticoagulant effect was significantly more pronounced during phenylbutazone administration than in the control experiments (Table IV). One animal (Rat 12), a slow eliminator given warfarin with phenylbutazone, was lost in the first part of the study due to hemorrhage. Therefore, the warfarin dosage in the second (crossover) part of the study was reduced to one-half in the three rats with the lowest total warfarin clearance. Even these three rats exhibited a more pronounced maximum anticoagulant effect during phenylbutazone administration, despite the reduced warfarin dose.

Repeated measurements of warfarin concentrations and prothrombin complex activity in the rats after warfarin administration permitted

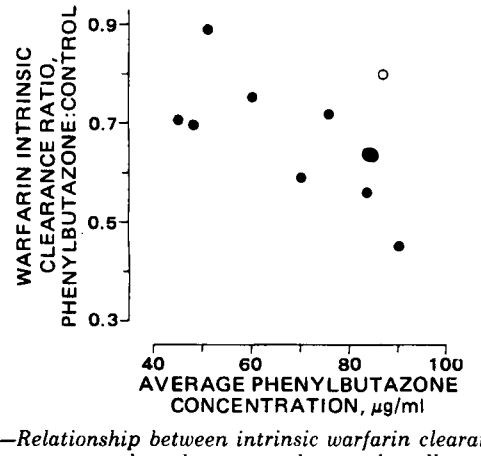

Figure 6—*Relationship between intrinsic warfarin clearance ratio, phenylbutazone:control, and average plasma phenylbutazone concentration. The correlation coefficient is −0.55* ($p < 0.1$) *for all 11 rats and −0.73* ($p < 0.02$) *for 10 rats (with O excluded).*

Table VI—Effect of Phenylbutazone on Total and Free Warfarin Concentrations in Plasma or Serum when the Anticoagulant Effect Is 50% of Maximum ($R_{syn} = 0.5\ R^0_{syn}$)

Rat	Total Concentration in Plasma, μg/ml		Control:With Phenylbutazone	Free Warfarin in Serum, μg/ml × 100		Control:With Phenylbutazone
	Control	With Phenylbutazone		Control	With Phenylbutazone	
1	0.110	0.0338	3.2	0.202	0.236	0.86
2	0.153	0.0507	3.0	0.291	0.222	1.3
3	0.246	0.0310	7.9	0.334	0.165	2.0
4	0.292	0.0463	6.3	0.330	0.154	2.1
5	0.280	0.0388	7.2	0.322	0.172	1.9
6	0.600	0.123	4.9	0.355	0.176	2.0
7	0.648	0.263	2.5	0.330	0.447	0.74
8	1.09	0.160	6.8	0.445	0.186	2.4
9	1.06	0.217	4.9	0.466	0.304	1.5
10	1.25			0.370		
11	2.06	0.364	5.7	0.612	0.348	1.8
Mean[a]	0.654	0.133[b]	5.2	0.369	0.241[b]	1.7[c]
SD			2.0			0.6

[a] Without Rat 10. [b] Statistically significantly different from the control value (paired *t* test, $p < 0.01$). [c] Statistically significantly different from the total concentration ratio (paired *t* test, $p < 0.001$).

determinations of the relationship between anticoagulant effect and drug concentration. Results obtained from a representative animal are shown in Fig. 8. The regression line of a plot of the relative prothrombin complex activity synthesis rate *versus* log plasma warfarin concentration can be characterized by its slope (m) and by the extrapolated intercept on the concentration axis at a synthesis rate of zero (C_{max}). The results obtained from all animals are summarized in Table V. The phenylbutazone effect is more readily apparent in Fig. 9, which is a schematic representation of the average results from all animals. There was a pronounced shift of the effect–log concentration regression line to a lower concentration range during phenylbutazone treatment, with no significant change in the slope.

A convenient index of anticoagulant effect is the warfarin concentration required to produce 50% inhibition of the prothrombin complex activity synthesis rate (referred to as the effective concentration). The effective concentrations of total as well as free warfarin were markedly reduced during phenylbutazone administration (Table VI). The average ratio of effective concentrations, control:phenylbutazone, was 5.2 for total drug and 1.8 for free drug. The difference in these ratios reflects the increased serum free fraction values of warfarin during phenylbutazone administration. There is no apparent correlation between the effective concentration ratios and the average phenylbutazone concentration in individual animals.

DISCUSSION

The results of this investigation demonstrate that the acute interaction between phenylbutazone and racemic warfarin in rats involves at least three mechanisms: displacement of warfarin from serum protein binding sites by phenylbutazone and its metabolites, inhibition of intrinsic warfarin clearance, and apparent potentiation of the anticoagulant action produced by a given plasma warfarin concentration. These effects were produced at average phenylbutazone concentrations in plasma (45–90 μg/ml) similar to those observed clinically. For example, 100 mg of the drug given three times a day to normal adult subjects resulted in plasma concentrations (mean ± *SD*) of 76 ± 7 μg/ml (7).

The phenylbutazone displacing effect on warfarin in serum was of similar magnitude in animals who received both drugs and in those who received only phenylbutazone (with warfarin added *in vitro*). On the other hand, *in vitro* phenylbutazone addition to serum from nonmedicated animals had a much less pronounced displacing effect at concentrations comparable to those obtained *in vivo*. These results indicate that the displacing effect is due not only to phenylbutazone but also to one or more of its metabolites. In fact, oxyphenbutazone, a major phenylbutazone metabolite, is strongly bound to albumin (17) and, therefore, may be a very effective displacer of warfarin from binding sites on the albumin molecule.

In theory, the more pronounced phenylbutazone displacing effect *in vivo* can be due also to accumulation of warfarin metabolites capable of competing with warfarin for albumin binding sites. There is indirect evidence that phenylbutazone inhibits the elimination of at least one warfarin metabolite in humans (7). However, the reduction of warfarin binding in serum from phenylbutazone-treated rats and from phenylbutazone- and warfarin-treated rats was comparable, indicating that warfarin metabolites contributed negligibly to the decreased protein binding of warfarin.

A recent report of a long-term multiple-dose study of the warfarin–phenylbutazone interaction in a single normal human subject stated that "changes in the degree of plasma binding of warfarin were the same as those found from *in vitro* experiments in which warfarin and phenylbutazone were added to control plasma samples" (8). Yet, the warfarin free fraction in that subject remained elevated after phenylbutazone

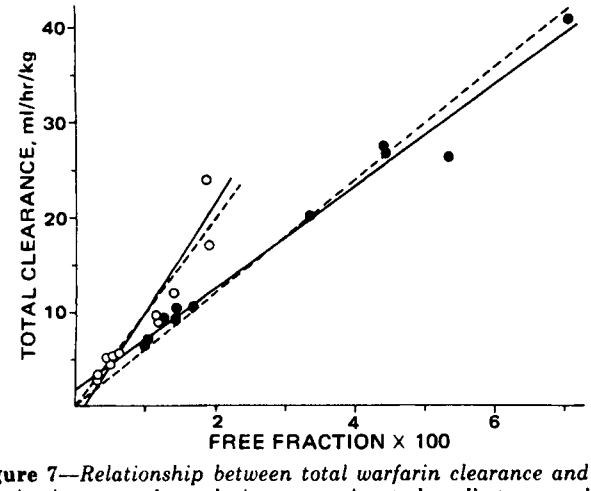

Figure 7—*Relationship between total warfarin clearance and free fraction in serum of rats during concomitant phenylbutazone administration (●) and in the same animals in control experiments (○). The correlation coefficient for the data with phenylbutazone is 0.997, p < 0.001, and that for the control data is 0.940, p < 0.001. There is a statistically significant difference (p < 0.001) in the slopes of the two regression lines.*

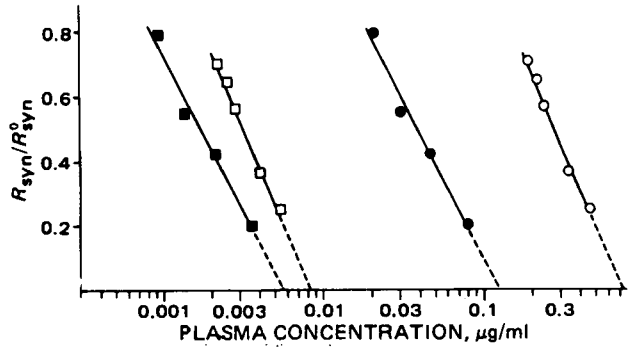

Figure 8—*Relationship between relative synthesis rate of prothrombin complex activity (R_{syn}/R^0_{syn}) and the total (free plus bound) concentration (circles) or the free concentration (squares) of warfarin in plasma of a representative rat. The closed symbols represent the relationship during phenylbutazone treatment, and the open symbols represent the relationship in the control experiment.*

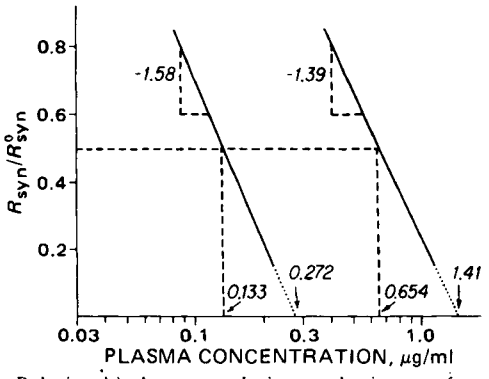

Figure 9—*Relationship between relative synthesis rate of prothrombin complex activity* (R_{syn}/R^0_{syn}) *and the total warfarin concentration in plasma of rats during treatment with phenylbutazone (left) and in control experiments (right) (based on the average data in Tables V and VI).*

administration was discontinued and plasma concentrations of this drug had decreased substantially, suggesting (as the authors acknowledged) that one or more phenylbutazone metabolites contributed to the displacing effect.

Phenylbutazone administration decreased the intrinsic warfarin clearance. This effect was less pronounced than the displacing effect, causing the total warfarin clearance to increase during phenylbutazone administration. Similar effects were observed in a chronic interaction study on one human subject (8). Whether chronic phenylbutazone administration would induce warfarin-metabolizing enzyme systems in rats remains to be determined. Since the total warfarin clearance is proportional to the drug's free fraction in plasma or serum, and since intrinsic drug clearance (*i.e.*, the proportionality constant in the relationship between total clearance and free fraction) differs relatively little between animals, a plot of total clearance *versus* free fraction in serum is essentially linear (10). If phenylbutazone treatment has a quantitatively similar effect on the intrinsic warfarin clearance in all animals, then the linear relationship between total clearance and the serum free fraction should remain. A decrease in intrinsic clearance and an increase in the serum free fraction should then cause the slope of the plot to decrease and the free fraction values to shift to a higher range. This is demonstrated strikingly by the present study (Fig. 7). The free fraction values were determined in serum rather than in plasma because we have found that addition of anticoagulants to blood affects the protein binding of warfarin.

The warfarin anticoagulant effect is a function of its free rather than of its total concentration in plasma (18). Consequently, decreased protein binding of warfarin due to phenylbutazone administration should shift the anticoagulant effect–plasma total warfarin concentration curve to the left, *i.e.*, to a lower concentration range. Such a shift is well demonstrated in this investigation (Fig. 9). However, a similar shift occurred in the anticoagulant effect–*free* warfarin concentration curve, albeit of considerably smaller magnitude. Such a shift may occur for at least two reasons. One is related to the fact that the phenylbutazone effect on warfarin pharmacokinetics is stereoselective. In humans, phenylbutazone decreases the total clearance of the more potent (*S*)-enantiomer and increases the total clearance of the (*R*)-enantiomer (7). The metabolic fate of the warfarin enantiomers in rats differs appreciably from that in humans (19), and there is no information about the possible stereoselectivity of the phenylbutazone interaction with warfarin in rats. A selective inhibition of the intrinsic clearance of (*S*)-warfarin would cause an increase in the (*S*)- to (*R*)-enantiomer concentration ratio in rat plasma after administration of racemic warfarin and a shift of the anticoagulant effect–free warfarin concentration curve to the left due to the higher potency of the (*S*)-enantiomer.

Another possible reason for such a shift is a direct effect of phenylbutazone (or its metabolites) on the blood-clotting process. Our studies have shown that phenylbutazone has no such effect when added *in vitro* to plasma but that even a single 40-mg/kg dose caused a small but statistically significant decrease of prothrombin complex activity 2 hr later (Table IV). It is possible that the effect was more pronounced during the interaction study, *i.e.*, following administration of several phenylbutazone doses.

The literature concerning the phenylbutazone effect on the blood-clotting process is conflicting. Some investigations in humans (6, 20) and a previous but very limited study on rats in our own laboratory (21) revealed no apparent effect. One investigator (22), however, reported observations concerning 44 patients on phenylbutazone of whom about one-third showed prolongation of the one-stage prothrombin time that was sometimes alarming. As explained by Koch-Weser and Sellers (2), a slight hypoprothrombinemic effect of a drug may escape detection in nonanticoagulated subjects but will be obvious in subjects whose prothrombin complex activity has already been depressed by coumarin anticoagulant therapy. Thus, technical difficulties of detection may account for the conflicting data concerning the effect of phenylbutazone administration on blood clotting.

This investigation, despite its relative comprehensiveness and the consequent technical difficulties and cost in time and effort, still falls far short of providing a complete picture of the phenylbutazone–warfarin interaction. The effect of warfarin on the disposition of phenylbutazone and its pharmacologically and physicochemically (with respect to displacing effect on warfarin) active metabolites has not been established; the dose or concentration dependence of the interaction is unknown (warfarin displacement from protein binding sites and warfarin metabolism inhibition may exhibit different effect–phenylbutazone concentration relationships, causing the net effect to be potentiation or reduction of anticoagulant response, depending on the doses used); time-dependent phenomena have not been explored; the mechanism of the increased anticoagulant effect of a given *free* warfarin concentration in plasma during phenylbutazone treatment has not been established. The latter effect could have been explored better if one warfarin enantiomer labeled with carbon 14 and the other labeled with tritium had been available. The alternative, separate interaction studies of phenylbutazone with (*R*)- and (*S*)-warfarin, could be helpful, but this would not reveal a possibly multifaceted interaction with results different from the sum of the interactions between phenylbutazone and each warfarin enantiomer separately.

The effort required to elucidate fully the mechanisms of a drug interaction can be enormous. If the drugs involved can produce potentially hazardous pharmacological effects (as is the case with phenylbutazone and warfarin), comprehensive studies in humans may be difficult or impossible. Cost–benefit and risk–benefit considerations rather than scientific curiosity will ultimately dictate how much we learn about many important drug interactions.

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Simultaneous Solubilization of Steroid Hormones III: Thermodynamic Evaluation

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Abstract □ The temperature effect on the solubilization of some androgens, estrogens, and C_{21}-steroids in aqueous polysorbate 40 and in tetradecyltrimethylammonium bromide was studied. Dialysis studies showed a linear relationship between micellar and nonmicellar steroids, which indicates that solubilization is governed by a distribution coefficient. With known water solubilities and solubilization capacities for the steroids at different temperatures, the changes of free energy (ΔG_s°), enthalpy (ΔH_s°), and entropy (ΔS_s°) for solubilization were calculated. All steroids studied had negative ΔH_s° values in polysorbate 40, except progesterone and ethisterone. The ΔS_s° values were positive for all of the actual steroids except for estradiol in both association colloids and for 17α-hydroxyprogesterone in polysorbate 40. The highest values were obtained for progesterone and testosterone. The steroids showed lower ΔS_s° values when they were solubilized simultaneously than when they were solubilized separately. No clearcut correlation between the entropy change of solubilization and the simultaneous solubilization behavior could be derived. Obviously, the solubilization mechanism also must be considered. The thermodynamic solubilization parameters are discussed, and the need for temperature-solubilization studies is stressed.

Keyphrases □ Steroid hormones—solubilization, thermodynamics, temperature effect, micellar structure, androgens, estrogens, C_{21}-steroids, in polysorbate 40 and in tetradecyltrimethylammonium bromide □ Solubilization—steroid hormones in polysorbate 40 and in tetradecyltrimethylammonium bromide, thermodynamics □ Thermodynamics—steroid hormone solubilization in polysorbate 40 and in tetradecyltrimethylammonium bromide

Two previous reports from this laboratory dealt with the simultaneous solubilization of estrogens, C_{21}-steroids, and androgens in aqueous solutions of association colloids (1, 2). Estradiol is solubilized independently of the C_{21}-steroids and testosterone, while the solubilization of ethinyl estradiol with progesterone and with testosterone is dependent. A plausible mechanism for simultaneous solubilization was discussed (2). However, mere solubilization capacities at one temperature are not a good basis for thermodynamic discussion of the solubilization mechanism. In this study, the temperature effect on solubilization was investigated to elucidate the contributions of enthalpy and entropy to the free energy of solubilization.

The thermodynamic parameters controlling micellization have been studied and discussed (3–5), but the corresponding parameters for solubilization have been comparatively neglected. One difficult question is the choice of a model for thermodynamic parameter calculations. Humphreys and Rhodes (6) found that the micellar pseudophase model seemed applicable because solubilization was governed by a form of the distribution law. Plots for the determination of enthalpic and entropic values were complex.

More straightforward results were obtained by Simons and Rhodes (7) with a linear relationship between the free energy of solubilization and temperature, despite much scatter due to experimental difficulties. Their results favor the pseudophase model.

EXPERIMENTAL

Materials—The purification methods and purity tests for the association colloids and steroid hormones[1] were described previously (1), except that the steroid purity was checked by silica gel TLC. The labeled steroids[2], ^{3}H-estradiol, ^{3}H-progesterone, and ^{3}H-testosterone, had a radiochemical purity of 98% by TLC, and they were used without further purification. The association colloids were tetradecyltrimethylammonium bromide[3] and polysorbate 40[4].

Solubilization—Solubility studies were carried out as described previously (1), but samples were filtered through a 0.45-μm filter membrane[5] before steroid quantitation. The UV absorbance was used to calculate the amount of unlabeled steroid solubilized; liquid scintillation counting, with a toluene-based scintillation cocktail, was used for the labeled steroids.

The equilibration temperatures were controlled to ±0.2°. The equilibrium dialysis was performed using dialysis tubing[6]. Complete equilibration of the solutions was ensured. All of the experiments were done at least twice.

RESULTS AND DISCUSSION

Dialysis studies were done with the three ^{3}H-labeled steroids: estradiol, progesterone, and testosterone. Figure 1 shows typical results. The linearity of such plots confirms that the relationship between the micellar and nonmicellar steroid concentrations is linear in both saturated and nonsaturated systems. This linearity indicates that solubilization in these systems is governed by a distribution coefficient.

The solubilization capacities at different temperatures between 293 and 323 °K of the two surfactants (tetradecyltrimethylammonium bromide and polysorbate 40) for the sex steroids (estradiol, ethinyl estradiol, progestone, 17α-hydroxyprogesterone, testosterone, and ethisterone) were calculated from saturation solubilization experiments (Table I).

The temperature effect on simultaneous solubilization was studied with the following combinations: ethinyl estradiol–progesterone, ethinyl estradiol–17α-hydroxyprogesterone, and estradiol–testosterone, all in tetradecyltrimethylammonium bromide; and ethinyl estradiol–proges-

[1] Fluka AG. Schweiz.
[2] The Radiochemical Centre, England.
[3] K & K Laboratories.
[4] Atlas Chemical Industries.
[5] Millipore Corp.
[6] Med Cell International Ltd., England.

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