INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106
BACHMANN, Kenneth Allen, 1946-
PHENYLButAZONE AND WARFARIN: ASPECTS OF THEIR INTERACTION IN THE DOG.

The Ohio State University, Ph.D., 1973
Pharmacology

University Microfilms, A XEROX Company, Ann Arbor, Michigan
PHENYL BUTAZONE AND WARFARIN: ASPECTS OF THEIR INTERACTION IN THE DOG

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Kenneth Allen Bachmann, B.S.

The Ohio State University

1973

Reading Committee:

Allan M. Burkman, Ph.D.
Dennis R. Feller, Ph.D.
Michael C. Gerald, Ph.D.
Michael C. Gerald, Ph.D

Approved by

Allan M. Burkman
Adviser
Division of Pharmacology
College of Pharmacy
ACKNOWLEDGMENTS

I wish to express my sincere appreciation to

DR. ALLAN M. BURKMAN
for his counseling both in the laboratory and out, for his patience, and for his encouragement of independent thinking and decision-making

MY WIFE, MARY
for her sacrifices, encouragement, and understanding, and for her assistance in certain aspects of this undertaking

MY PARENTS
for many things

MR. ROBERT HODGES
for technical assistance
VITA

March 21, 1946.............................. Born - Columbus, Ohio

1969........................................ B.S., College of Pharmacy, The Ohio State University, Columbus, Ohio

1970-1972............................... Teaching Associate, College of Pharmacy, The Ohio State University, Columbus, Ohio

1970-1972............................... Fellow of the American Foundation for Pharmaceutical Education

1972-1973............................... Fellow of The Ohio State University, Columbus, Ohio

PUBLICATIONS

"Effect of Phenylbutazone on the Disposition of Warfarin in Dogs."

FIELDS OF STUDY

Major Field: Pharmacology

Drug-Drug Interaction Mechanisms

Drug Metabolism

Drug Disposition

Professor Allan M. Burkman
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vii</td>
</tr>
<tr>
<td><strong>Chapter</strong></td>
<td></td>
</tr>
<tr>
<td><strong>I.</strong> <strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>Drug-Drug Interactions</td>
<td></td>
</tr>
<tr>
<td>Coumarin Anticoagulants</td>
<td></td>
</tr>
<tr>
<td>Mechanism of Action of Oral Anticoagulants</td>
<td></td>
</tr>
<tr>
<td>Coumarin Anticoagulant Interactions</td>
<td></td>
</tr>
<tr>
<td>Coumarin-Pyrazolone Interactions</td>
<td></td>
</tr>
<tr>
<td>Statement of the Problem</td>
<td></td>
</tr>
<tr>
<td><strong>II.</strong> <strong>METHODS AND MATERIALS</strong></td>
<td>36</td>
</tr>
<tr>
<td>General Considerations</td>
<td></td>
</tr>
<tr>
<td>Determination of Plasma Warfarin (Total)</td>
<td></td>
</tr>
<tr>
<td>Determination of Plasma Warfarin (Unbound)</td>
<td></td>
</tr>
<tr>
<td>Prothrombin Determinations</td>
<td></td>
</tr>
<tr>
<td>The Effect of Acute Phenylbutazone</td>
<td></td>
</tr>
<tr>
<td>Administration upon the Pharmacologic Response to Warfarin <strong>In-Vivo</strong></td>
<td></td>
</tr>
<tr>
<td>The Effect of Acute and Chronic Phenylbutazone Administration upon the Plasma Half-Life of Warfarin <strong>In-Vivo</strong></td>
<td></td>
</tr>
<tr>
<td>Protein-Binding Inhibition of Warfarin by Phenylbutazone <strong>In-Vivo</strong></td>
<td></td>
</tr>
<tr>
<td>Platelet Aggregability</td>
<td></td>
</tr>
<tr>
<td><strong>In-Vitro</strong> Synthesis of Prothrombin Complex Activity</td>
<td></td>
</tr>
<tr>
<td>Determination of Prothrombin Complex Activity</td>
<td></td>
</tr>
<tr>
<td>Activity Synthesized</td>
<td></td>
</tr>
<tr>
<td>Drug Treatment for <strong>In-Vitro</strong> Metabolism of Warfarin by Canine Microsomal Enzymes</td>
<td></td>
</tr>
</tbody>
</table>
**Preparation of Enzyme for Warfarin Metabolism**

**Incubation of Warfarin with Liver Enzyme**

**Estimation of Warfarin Cytochrome P-450 Content**

### III. RESULTS

- Determination of Warfarin
- Effect of Chlordane Exposure Upon Warfarin Plasma Half-Life
- Plasma Coagulation Time and its Relationship to Prothrombin Complex Activity
- Effect of Warfarin upon the Availability of Plasma Prothrombin Complex Activity
- Prothrombin Complex Synthesis Rates and Plasma Warfarin Levels
- Protein-Binding Inhibition in Dog *In-Vivo*
- In-Vitro Synthesis of Prothrombin Complex Activity by Rat Liver
- Platelet Aggregability in the Dog
- Effect of Chronic Phenylbutazone Treatment upon Plasma Half-Life of Warfarin in the Dog
- In-Vitro Warfarin Metabolism by Canine Hepatic Microsomes

### IV. DISCUSSION

### V. SUMMARY AND CONCLUSION

**BIBLIOGRAPHY**
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Platelet Aggregating Agents</td>
<td>25</td>
</tr>
<tr>
<td>2.</td>
<td>Drugs Capable of Inhibiting Platelet Function</td>
<td>26</td>
</tr>
<tr>
<td>3.</td>
<td>Effect of Phenylbutazone upon the Degradation Rate Constant and the Relationship between Plasma Prothrombin Complex Activity Synthesis Rate and Plasma Warfarin Concentrations</td>
<td>66</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of Acute Phenylbutazone Administration on Warfarin Plasma Half-Life</td>
<td>67</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of Phenylbutazone (PBZ) on the Availability of Warfarin in the Dog</td>
<td>69</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of Chronic Phenylbutazone Administration upon Warfarin Plasma Half-Life</td>
<td>79</td>
</tr>
</tbody>
</table>
### LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Basic Clotting Reactions and Modulators</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td>Emission Spectra of Warfarin in N,N-di-methylformamide (DMF) and Water</td>
<td>51</td>
</tr>
<tr>
<td>3.</td>
<td>The Quenching of Warfarin Fluorescence in N,N-dimethylformamide by the Addition of HCl</td>
<td>52</td>
</tr>
<tr>
<td>4.</td>
<td>Warfarin Fluorescence Recovered from Plasma after Extraction with Ethylene Dichloride or Extraction and then Isolation by Thin-Layer Chromatography</td>
<td>53</td>
</tr>
<tr>
<td>5.</td>
<td>Warfarin Fluorescence Recovered from Plasma Ultrafiltrate after Extraction with Ethylene Dichloride</td>
<td>54</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of Exposure to Chlordane upon Warfarin Plasma Half-Life in the Dog</td>
<td>56</td>
</tr>
<tr>
<td>7.</td>
<td>Relationship between Canine Plasma Prothrombin Complex Activity and Coagulation Times</td>
<td>57</td>
</tr>
<tr>
<td>8.</td>
<td>Prothrombin Complex Activity after Warfarin (0.8 mg/Kg) Orally</td>
<td>59</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of PBZ upon Warfarin-Induced Plasma Disappearance of Prothrombin Complex Activity</td>
<td>60</td>
</tr>
<tr>
<td>10.</td>
<td>Rate of Prothrombin Complex Synthesis as a Function of Plasma Warfarin Concentration</td>
<td>61</td>
</tr>
<tr>
<td>11.</td>
<td>Rate of Prothrombin Complex Synthesis as a Function of Plasma Warfarin Concentration</td>
<td>62</td>
</tr>
<tr>
<td>12.</td>
<td>Rate of Prothrombin Complex Synthesis as a Function of Plasma Warfarin Concentration</td>
<td>63</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>13.</td>
<td>Rate of Prothrombin Complex Synthesis as a Function of Plasma Warfarin Concentration.</td>
<td>64</td>
</tr>
<tr>
<td>14.</td>
<td>Relationship between Prothrombin Complex Activity and Canine Plasma Coagulation Time.</td>
<td>70</td>
</tr>
<tr>
<td>15.</td>
<td>\textit{In-Vitro} Prothrombin Complex Synthesis in Rat Livers Derived from Animals Treated with Saline or Warfarin, or Pretreated with Phenylbutazone (PBZ) and then Treated with Warfarin.</td>
<td>72</td>
</tr>
<tr>
<td>16.</td>
<td>Effect of Phenylbutazone Treatment upon Platelet Aggregability in the Dog.</td>
<td>73</td>
</tr>
<tr>
<td>17.</td>
<td>Warfarin Plasma Disappearance in K-9 10306 before and after Chronic Phenylbutazone Treatment.</td>
<td>75</td>
</tr>
<tr>
<td>18.</td>
<td>Warfarin Plasma Disappearance in K-9 13134 before and after Chronic Phenylbutazone Treatment.</td>
<td>76</td>
</tr>
<tr>
<td>19.</td>
<td>Warfarin Plasma Disappearance in K-9 12990 before and after Chronic Phenylbutazone Treatment.</td>
<td>77</td>
</tr>
<tr>
<td>20.</td>
<td>Warfarin Plasma Disappearance in K-9 13407 before and after Chronic Phenylbutazone Treatment.</td>
<td>78</td>
</tr>
<tr>
<td>21.</td>
<td>Effect of Phenylbutazone Pretreatment on Liver Microsomal Metabolism of Warfarin ((2 \times 10^{-5} \text{M})).</td>
<td>80</td>
</tr>
<tr>
<td>22.</td>
<td>Effect of Phenylbutazone Pretreatment on Canine Hepatic Microsomal Metabolism of Warfarin.</td>
<td>81</td>
</tr>
<tr>
<td>23.</td>
<td>Effect of Phenylbutazone Pretreatment on Canine Hepatic Microsomal P-450 Content.</td>
<td>82</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

Drug-drug interactions. The potential for untoward reactions arising from the concurrent use of several drugs is well recognized. Indeed, the interactions between certain classes of clinically used drugs have resulted in fatalities or near fatalities. A potential for the most hazardous drug interactions appears to be associated with psychopharmacological and cardiovascular agents (Macgregor, 1965). Serious hypertensive crises have, for example, been attributed to the concomittant administration of tricyclic antidepressant drugs and monoamine oxidase inhibitors (Jarecki, 1963; Brachfeld et al., 1963; Goldberg, 1964; Simmon et al., 1970; and Sjoqvist, 1965). The review of O’Reilly and Aggeler (1970) documents the hazards of titrating a prothrombin response to anticoagulant treatment during ongoing barbiturate therapy and then withdrawing the barbiturate while continuing the anticoagulant.

However, not all drug-drug interactions can be characterized by fatal outcomes. Adverse consequences of many and perhaps most drug-drug interactions are considerably more subtle and are more apt to be reflected by inefficient therapeusis or exaggeration of side-effects as suggested by several published reviews (Conney, 1967; Macgregor,
1965; O'Reilly and Aggeler, 1970; Koch-Weser and Sellers, 1971; and Hansten, 1972). According to Koch-Weser and Sellers (1971) most patients in the United States can expect to receive more than five drugs concurrently during their general hospital stay. It is highly conceivable, then, that drug interactions may occur with a high frequency in clinical medicine, but are likely to remain undetected, with unsatisfactory therapeutics mistakenly attributed to patient idiosyncrasy and adverse reactions to the disease state itself (Hansten, 1972).

The need for experimental evaluation of drug interactions was aptly expressed by Modell (1964) who alleged that most drug interactions are evaluated in retrospective terms following the inadvertent coadministration of drugs clinically. Such experimentation, he suggested, is:

...carried out unwittingly by the physician on the unwitting patient, and, being unwitting, is too often witless.

The theoretical bases for the modification of expected responses to one drug by another have been described (Dayton and Perel, 1971; Meyer and Guttman, 1968; and Conney, 1967) and encompass alterations in absorption, distribution, metabolism, excretion, and receptor-interaction imposed by one drug upon another as well as frank pharmacologic antagonism or synergism. Since the pharmacologic response to coumarin anticoagulants is easily and reliably monitored, and the clinical responses to these agents can be altered by a diverse spectrum of pharmacological and toxicological agents, their interactions serve as useful examples of the drug-interaction phenomenon.
Coumarin anticoagulants. The rationale for the use of coumarin anticoagulants derives from their ability to depress clotting factor synthesis and thereby interfere with the coagulation process.

The entire coagulation process is describable in terms of three basic reactions (Seegers et al., 1968; Seegers, 1969; Seegers, 1971) as well as the more common "waterfall sequence" (Davie and Ratnoff, 1964) or "enzyme cascade" models (MacFarlane, 1964). The nomenclature used by Seegers differs from the more common Roman numeral designations (Wright, 1959) but is more descriptive and useful. In its most rudimentary form the coagulation process can be envisioned as three reactions involving two enzymes: 1) formation of autoprothrombin C (Factor X); 2) formation of thrombin; and 3) formation of fibrin. The enzymes involved are autoprothrombin C and thrombin. The former serves to autocatalyze its own formation to a limited extent, but is most notable for its conversion of a thrombin precursor to thrombin. The latter catalyzes the formation of fibrin from fibrinogen (Seegers, 1971). A great many variables condition the rate at which these processes proceed and can enhance (calcium, platelet cofactor, platelet factor 3, thromboplastin, cothromboplastin, autoprothrombin C, Ac-globulin) or impede (antithrombin) them (Seegers, 1971). The basic reactions and their modulators are depicted schematically in Figure 1.

Prothrombin (Factor II) is not a single simple entity but an aggregate of loosely bound subunits which can dissociate (Seegers, 1969). The two most notable subunits are autoprothrombin III
FIGURE 1

BASIC CLOTTING REACTIONS AND MODULATORS
(Factor X) and the thrombin precursor, prethrombin. Other subunits which derive from the prothrombin complex include cothromboplastin (Factor VII) and autoprothrombin II (Factor IX). Autoprothrombin III contains the enzyme, autoprothrombin C which is capable of catalyzing its own activation (Seegers, 1971). The rate of activation of autoprothrombin III to autoprothrombin C is accelerated markedly by the presence of the proenzyme, prethrombin (Seegers, 1971). Under physiologic conditions a number of other substances can enhance the activation of autoprothrombin III to autoprothrombin C. Among these are: autoprothrombin II, Hageman Factor (Factor XII), platelet cofactor, calcium ions, and platelet factor 3. The latter arises from platelet alterations (Seegers et al., 1968). Under pathophysiological conditions such as injury resulting in vascular trauma, the reaction is accelerated by tissue thromboplastin, calcium ions, and cothromboplastin which itself derives from the prothrombin complex. Autoprothrombin C—a peptidase—cleaves peptides from the thrombin precursor to form thrombin. To proceed at a rate compatible with physiological requirements the conversion of prethrombin to the active enzyme, thrombin, also requires the presence of calcium ions, Ac-globulin (Factor V), and platelet factor 3 (Seegers, 1971). The peptidase, thrombin, in addition to removing peptides from fibrinogen to form fibrin may also serve to deaggregate the prothrombin complex (Seegers, 1971). In addition to those regulators of the three reactions leading to the formation of fibrin which enhance the rate, substances have been identified which are impeding regulators. Most notable is anti-
thrombin which binds both thrombin and autoprothrombin C to form inactive complexes (Dombrose et al., 1971). Thus, the coagulation process can be envisioned in terms of three main reactions which are modulated in normal and pathophysiological circumstances by a variety of substances.

**Mechanism of action of oral anticoagulants.** It is generally accepted that four of the circulating proteins required for clot formation require vitamin K for their synthesis (Woolf and Babior, 1972). Prothrombin (Factor II), cothromboplastin (Factor VII), autoprothrombin II (Factor IX), and autoprothrombin III (Factor X) are referred to collectively as the vitamin K-dependent clotting factors (O'Reilly and Apgeler, 1970; Koch-Weser and Sellers, 1971; Deykin, 1970; and Woolf and Babior, 1972). Though an important role is attributed to vitamin K in the hepatic synthesis of procoagulants, very clearly, a precise role for the fat-soluble vitamin remains to be described.

Vitamin K is made available by diet and by the bacteria that comprise the normal gastrointestinal flora. However, the relative importance of each of these contributions is obscure (Deykin, 1970). The importance of bacterially synthesized vitamin K cannot be viewed lightly since it is extremely difficult to completely deplete the vitamin merely by removing it from the diet (Woolf and Babior, 1972). However, when a dietary deficiency is superimposed upon subjects whose gastrointestinal tracts have been sterilized with nonabsorbable
sulfonamides, vitamin K depletion is accomplished (Woolf and Babior, 1972). Vitamin K is absorbed from the gastrointestinal tract probably from the proximal small intestine. An oral dose of tritiated vitamin K\textsubscript{1} in man results in detectable radioactivity in the plasma chylomicron fraction after 30 minutes (Shearer et al., 1970). Once absorbed, the vitamin is concentrated in the liver before disseminating to other tissues (Woolf and Babior, 1972).

Cothromboplastin (Factor VII) activity in homogenates of rat liver slices appears upon incubation of the homogenates unless the animals receive prior treatment with bishydroxycoumarin or are subjected to a vitamin K-deficient diet (Babior, 1966). Rats made deficient in cothromboplastin were sacrificed and the elaboration of that glycoprotein by liver slices was estimated. Only after the intracardial administration of vitamin K prior to sacrifice was there detectable synthesis of cothromboplastin (Babior, 1966). Furthermore, administration of vitamin K restored the ability of liver slices to elaborate cothromboplastin even when homogenates were incubated in the presence of puromycin which successfully inhibited protein synthesis. The conclusion drawn was that the vitamin probably acts at a post-transcriptional level of cothromboplastin synthesis and is probably required for the synthesis of a peptide precursor of cothromboplastin (Babior, 1966). Contrariwise, while actinomycin D administration to intact vitamin K-deficient rats did not prevent the vitamin K-stimulated increase in plasma prothrombin nor did perfusion of an isolated rat liver with actinomycin D prevent the elaboration of
cothromboplastin into the perfusate, cothromboplastin production by the isolated liver was blocked by puromycin perfusion (Suttie, 1967). It was concluded that the effect of vitamin K is manifested beyond the production of a specific mRNA for clotting factor synthesis, and also that vitamin K does not activate the existing hepatic precursor of cothromboplastin.

Those levels in the synthesis of cothromboplastin at which the vitamin could act and not ruled out by Suttie (1967) include: removal of the peptide from the ribosome, attachment of a carbohydrate moiety to the completed peptide chain, and transport of the completed glycoprotein from the cell. Of these, the attachment of a carbohydrate moiety to the completed peptide portion appears to be the most likely level at which vitamin K acts. While the incorporation of radio-labeled amino acids into an electrophoretically pure prothrombin was unaffected by the administration of vitamin K to vitamin K-deficient rats, a marked increase in the incorporation of radiolabeled mannose and glucosamine was observed (Johnson et al., 1971). Demonstration of the accumulation in plasma of an antigenically active but biologically inactive prothrombin afterbishydroxycoumarin treatment in cows tends to reinforce the notion (Stenflo, 1970). The recent findings of Suttie (1973) in which rats made hypoprothrombinemic by ingestion of a vitamin K-deficient diet demonstrated increases in a prothrombin precursor in plasma which paralleled the disappearance of prothrombin from plasma provide a further indication that vitamin K is necessary for the conversion of a prothrombin precursor to active
prothrombin.

For many years following the discovery of bishydroxycoumarin in 1939 and related compounds thereafter, the anticoagulants were thought to be competitive antagonists of vitamin K (Collentine and Quick, 1951). Recent work by O'Reilly (1972) in humans supports the notion of competitive antagonism although the double reciprocal plots upon which the conclusion was based were constructed utilizing warfarin doses rather than warfarin plasma levels. The in-vitro work of Lowenthal and Birnbaum (1968) on the other hand, suggests that coumarin anticoagulant do not competitively antagonize the vitamin, but rather, that they inhibit the transport of the vitamin to its site of action, and that the inhibition can be surmounted only with high concentrations of the vitamin which can enter the cell by an alternate route, unimpeded by the presence of anticoagulant. Although additional insight into the nature of the anti-vitamin K effect of coumarin anticoagulants was not forthcoming, the work of Pereira and Couri (1971 and 1972) demonstrated that both agents act at the level of incorporation of a glycosyl moiety onto a completed core protein.

The existence of anticoagulant-resistant rats and humans has markedly contributed to the current understanding of the mechanism of action of anticoagulant drugs. In these resistant species it has been shown that the resistance arises not from altered metabolism or absorption of the anticoagulant, but from an inappropriate binding of the drug to microsomes which are presumably involved in the vitamin K-dependent clotting factors' synthesis (Woolf and Babior,
Furthermore, warfarin-resistant subjects exhibit an increased requirement for vitamin K although significantly less of the vitamin is necessary to overcome a drug-induced hypoprothrombinemia (O'Reilly, 1971). The findings in resistant rats (Thierry et al., 1970) and in humans (O'Reilly, 1971) led to the theory that anticoagulants and vitamin K affect a microsomal protein that regulates vitamin K-dependent clotting factor synthesis.

Extremely cogent evidence has been recently forthcoming which clearly characterizes the nature of the anti-vitamin K activity of warfarin. It begins with the observation that vitamin $K_1$ is oxidized to phylloquinone oxide in the livers of rats by a soluble enzyme and that the oxide is reduced back to $K_1$ in the microsomal fraction of rat liver (Zimmerman and Matschiner, 1972). In rats made deficient in vitamin K by diet, both the oxide and vitamin $K_1$ were effective in stimulating prothrombin synthesis, but in rats made deficient in prothrombin by warfarin treatment, only the vitamin itself could restore prothrombin synthesis (Bell and Matschiner, 1972). Thus it was proposed that phylloquinone oxide was an inhibitor of vitamin $K_1$, and that warfarin's anticoagulant effect derives from its ability to cause an accumulation of the oxide (Bell and Matschiner, 1972). Additionally, when rats were administered the oxide it was extensively converted to vitamin $K_1$ although this conversion was blocked by warfarin administration and the hepatic ratios of the oxide to vitamin increased indicating an accumulation of the oxide in the presence of warfarin (Bell et al., 1972). Finally, using warfarin-resistant rats
Bell and Caldwell (1973) demonstrated that the oxide could stimulate prothrombin synthesis in resistant rats treated with warfarin, but not in normal rats similarly treated; and that the oxide accumulated in the livers of normal rats treated with warfarin, but not in resistant rats. Their conclusion extended the notion of O'Reilly (1971) and Thierry et al., (1970) beyond the suggestion that warfarin resistance in rats and humans evolves from the synthesis of a mutant protein with lowered binding affinity for vitamin K and warfarin, to designate the mutated protein as the microsomal enzyme which reduces phylloquinone oxide to vitamin K₁.

It is therefore increasingly clear that coumarins and vitamin K are not competitive antagonists, rather: 1) phylloquinone oxide and vitamin K are competitive antagonists; 2) ordinarily the vitamin is readily converted to its oxide and the oxide is freely reduced back to the active vitamin; and 3) oral anticoagulants interfere with the microsomal enzyme which reduces the oxide thereby promoting its accumulation and the diminution of available vitamin K.

**Coumarin anticoagulant interactions.** Widely used for the management of thrombotic disorders coumarin anticoagulants are among those classes of drugs which possess a serious interaction liability. Extensive reviews describing the interaction potential of coumarin anticoagulants have been published in recent years (O'Reilly and Aggeler, 1970; Hansten, 1972; Formiller and Cohon, 1969; and Koch-Weser and Sellers, 1971).
As early as 1943 the hypoprothrombinemic effect of salicylates in man was reported (Shapiro et al., 1943) and shortly thereafter it was recognized that quinine sulfate possessed hypoprothrombinemic activity (Pirk and Engelberg, 1945). This particular pharmacodynamic characteristic, namely the ability to inhibit the synthesis of vitamin K-dependent clotting factors—referred to collectively as the prothrombin complex—has been the basis for the explanation of the potentiation of the response to oral anticoagulants in the presence of salicylates (Seegers, 1951) and quinidine, an enantiomer of quinine (Koch-Weser, 1968; Gazzaniga and Stewart, 1969). The demonstrated ability of salicylates to produce significant gastrointestinal lesioning (Smith, 1966; Emmanuel, 1971), however, may pose a more serious problem insofar as the tendency toward spontaneous hemorrhage in the face of combined salicylate and anticoagulant therapy is concerned (O'Reilly and Aggeler, 1970).

Many other agents—drugs and non-drug entities alike—are known to alter expected clinical responses to oral anticoagulant therapy. However, unlike salicylates and quinidine, the ways in which such alterations are effected appear to be independent of direct pharmacodynamic effects of such agents upon clotting factor synthesis.

Cholestyramine, an ion-exchange resin that binds bile acids and thereby promotes their fecal excretion, also has been shown to bind warfarin in the human gastrointestinal tract resulting in lower plasma warfarin levels and shortened prothrombin times (Robinson et al., 1971). Magnesium hydroxide administered with bishydroxycoumarin results in
the formation of a bishydroxycoumarin-magnesium ion chelate which is absorbed more readily than bishydroxycoumarin itself and leads to unexpectedly high plasma levels of the drug (Ambre and Fischer, 1973).

Myriad of drugs and other agents, for example pesticides, are now known to be capable of inducing hepatic microsomal enzymes which subserve the overall biodegradative processes involved in drug detoxification (Sher, 1971; Conney, 1967; Kato et al., 1964; Kuntzman, 1969; Conney et al., 1967; and Burns et al., 1965). Coumarin derivatives themselves, which enjoy widespread utilization as oral anticoagulants (Eipe, 1972), are also substrates for hepatic microsomal enzymes. Lorusso and Suttie (1972) demonstrated that the binding of radiolabeled warfarin to rat liver microsomal membranes was diminished if the animals had received prior treatment with unlabeled warfarin, and further, that the warfarin binding capacity was low in microsomes isolated from warfarin-resistant rats. Hydroxylated derivatives of warfarin have been isolated from plasma (Lewis and Trager, 1971) and urine in man (Lewis and Trager, 1970), and coumarin itself has been shown to undergo hydroxylation to 7-hydroxycoumarin (umbelliferone) by liver microsomes from rabbit, cat, pigeon, and guinea-pig (Creaven et al., 1965) and to 3-hydroxycoumarin by rat liver microsomes (Feuer, 1970). The 4-hydroxycoumarin derivatives, bishydroxycoumarin, warfarin, ethylbiscoumacetate, phenprocoumon, and acetanocoumarol, inhibited the N-demethylation of aminopyrine, hydroxylation of aniline, and the O-demethylation of p-nitroanisole by rat liver microsomes in-vitro (Christensen and Wissing, 1972). Bishydroxy-
coumarin metabolism in-vitro by rat liver microsomes has recently been characterized (Christensen, 1972). Indeed, pretreatment of human subjects or animals with agents characterized as enzyme inducers has resulted in marked alterations of the metabolic disposition of coumarin-type anticoagulants (O'Reilly and Aaggeler, 1970; Koch-Weser and Sellers, 1971; Hansten, 1972). Phenobarbital, perhaps the classic enzyme inducer, has been shown to hasten the disappearance of bishydroxycoumarin from plasma in pretreated dogs; a concomittant decrement in prothrombin times was observed (Welch et al., 1969). The in-vitro metabolism of bishydroxycoumarin by rat liver microsomes was also enhanced by pretreatment of rats with phenobarbital (Christensen, 1972). Ikeda et al. (1968) also reported the stimulatory effect of the halogenated hydrocarbon pesticide, chlorophenothane (DDT), as well as phenobarbital on the hepatic metabolism of warfarin in the rat in-vitro. The ability of phenobarbital pretreatment to lower bishydroxycoumarin levels in man and enhance its microsomal metabolism in the rat was demonstrated by Cucinelli et al. (1965), and to lower warfarin levels in man by MacDonald et al. (1969).

Other drugs which have been implicated as inducers of hepatic microsomal enzymes which mediate, in part, the metabolic degradation of coumarin-type anticoagulants include: glutethimide (MacDonald et al., 1969; Corn, 1966); carbamazepine (Hansen et al., 1971); griseofulvin (Catalano and Cullen, 1966; Udall, 1970; and Cullen and Catalano, 1967); and chloral hydrate (Cucinelli et al., 1966). In the case of chloral hydrate, however, recent reports suggest that the
enhanced disappearance rates of plasma bishydroxycoumarin in the dog and plasma warfarin in man are more consistent with changes in their distribution effected by chloral hydrate, inasmuch as the lower plasma levels of anticoagulant attained are accompanied by increased pharmacological responses (Weiner, 1971). Regardless of the mechanism of the interaction between chloral hydrate and oral anticoagulants, the clinical significance appears to be doubtful since chloral hydrate failed to evoke any significant alterations either in plasma levels or prothrombin times in patients undergoing long-term warfarin therapy (Griner et al., 1971). Of the non-drug microsomal enzyme inducers, halogenated hydrocarbon pesticides—notably DDT and chlordane—appear to be exquisitely effective in enhancing the degradation of coumarin anticoagulants (Conney et al., 1967; Ikeda et al., 1968).

In addition to various drugs altering the pharmacological effectiveness of oral anticoagulants through superimposition of their own hypoprothrombinemic activity upon that of the oral anticoagulants, interfering with their gastrointestinal absorption, or interfering with their hepatic degradation, it has been suggested that various agents are capable of altering the distribution of coumarin compounds by displacing the bound fraction from protein-binding sites, thereby enhancing the availability of the pharmacologically active unbound or free fraction (O'Reilly and Aggeler, 1970; Koch-Weser and Sellers, 1971; and Hansten, 1972). The theoretical basis for such an interaction has been provided (Meyer and Guttman, 1968), and several examples of protein-binding displacement between other agents can be
found. Anton has demonstrated both in-vitro (Anton, 1960) and in-vivo (Anton, 1961) that pyrazolone compounds, phenylbutazone and sulfinpyrazone, respectively, are capable of increasing the free fraction of sulfonamides. Furthermore, the ability of several sulfonamides, salicylates; and other acidic agents to decrease the binding of a number of penicillins to serum proteins was demonstrated by Kunin (1965 and 1966). Chlorphenoxyisobutyric acid, a compound with high affinity for plasma protein, has been shown to enhance the activity of androsterone by increasing the fraction of unbound steroid (Thorp, 1962).

Not unexpectedly, similar findings have accrued with respect to the ability of various compounds to displace coumarin congeners from plasma protein-binding sites and thereby enhance the hypoprothrombinemic activity of such agents. Diazoxide, ethacrynic acid, mefenamic acid, and nalidixic acid displace warfarin from human albumin binding sites in-vitro (Sellers and Koch-Weser, 1970). Decreased plasma levels of bishydroxycoumarin in rats fed acetylsalicylic acid and bishydroxycoumarin compared to plasma levels in rats ingesting only the anticoagulant have been attributed to the binding-displacement phenomenon (Coldwell and Thomas, 1971). That various pyrazolone compounds possess the capability to displace coumarin-type anticoagulants from serum albumin binding sites in-vitro was clearly demonstrated by Solomon and Schrogie (1967). Phenylbutazone, d-thyroxine, sulfaphenazole, and chlorphenoxyisobutyric acid demonstrated marked activity in-vitro (Solomon and Schrogie, 1967).
Coumarin-pyrazolone interactions. Of the plethora of agents purportedly capable of perverting expected responses to coumarin anticoagulants, one of the most interesting classes must indeed be the pyrazolone derivatives, most notably phenylbutazone and its hydroxylated metabolite, oxyphenbutazone. Assuredly, rather dire clinical consequences—ranging from exceedingly low prothrombin complex activity to hemorrhagic diatheses—have been observed (Eisen, 1964; Fox 1964; Hobbs et al., 1965; Hoffbrand and Kininmouth, 1967; Aggeler et al., 1967 and Udall, 1969).

The literature which deals with the interaction of pyrazolone analogs and coumarin oral anticoagulants contains some interesting, albeit contradictory features. One such outstanding contradiction resides in the reports of the influence of pyrazolone agents upon the hepatic microsomal metabolism of coumarin anticoagulants. An early communication dealing with this subject suggested that oxyphenbutazone slowed the disappearance of bishydroxycoumarin from the plasma of human subjects, and that the plasma bishydroxycoumarin changes were reflected by prothrombin responses (Weiner et al., 1965). The inhibition of hepatic microsomal enzymatic metabolism of coumarin anticoagulants by pyrazolone compounds was implicated as the mechanism whereby the apparent activity of the anticoagulants was potentiated (Weiner, 1964; Burns, 1965; and Cucinell et al., 1965). Interestingly, however, pyrazolone compounds appear to possess inductive qualities insofar as hepatic microsomal enzymes are concerned, rather than inhibitory ones. Conney et al. (1960) demonstrated that pretreatment
of rats with phenybutazone shortened the duration of zoxazolamine paralysis times and enhanced the liver microsomal metabolism of hexobarbital, aminopyrine, phenybutazone, and zoxazolamine in rats. Burns et al. (1963) also reported the ability of chronic phenybutazone pretreatment (25-100mg/Kg/day for 14 days) to induce its own metabolism in dogs, and further demonstrated the long-lived nature of the inductive effect reporting that such effects were observed for up to fifty days subsequent to the final dose of a chronic pretreatment regimen (Burns et al., 1965). That phenybutazone can be characterized as an hepatic microsomal enzyme inducer is further attested to by the work of Conney and Schneidman (1964) who observed that the treatment of immature rats and dogs with phenybutazone increased the activity of those hepatic microsomal enzymes that hydroxylate Δ₄-androstene-3,17-dione. Other investigators have shown that phenybutazone stimulates liver weight gain (Gershbein, 1966; Silvestrini et al., 1966).

Another approach to the problem of the pyrazolone-coumarin interaction involves the evaluation of distributive changes imposed upon coumarin anticoagulants by pyrazolone compounds; the notion here being that observed potentiation of the clinical response to coumarin anticoagulants may derive from the elevation of the unbound fraction of anticoagulant as a consequence of its displacement from plasma protein-binding sites by the pyrazolone drug. In a clinical study of the interaction Aggeler et al. (1967) initially found that the four day addition of phenybutazone to the drug regimen of a patient receiving 8 mg/day of warfarin, resulted in a potentiation of the
hypoprothrombinemic response to the anticoagulant as well as an apparent increase in the plasma levels of warfarin. Such findings are, of course, consistent with earlier reports that pyrazolone compounds possess the ability to inhibit the hepatic metabolism of coumarin anticoagulants (Weiner, 1964; Weiner et al., 1965; Burns, 1965; and Cucinell et al., 1965). However, Aggeler et al. (1967) subsequently noted that the spectrophotometric analysis of plasma warfarin was compromised by the presence of unseparated phenylbutazone, and suggested that the apparent increase in plasma warfarin levels during the four day interval of concurrent phenylbutazone therapy arose from sample contamination with phenylbutazone. Upon subsequent subtraction of the contribution of phenylbutazone to the net optical density of the warfarin samples, a decline in plasma warfarin levels was found to accompany the increase in clinical hypoprothrombinemia observed during the coadministration of the two agents. These studies were later repeated with a spectrophotofluorometric analysis of plasma warfarin—wherein phenylbutazone was no longer an interfering substance—and similar findings were reported, namely an enhanced plasma disappearance rate of warfarin and an elevated hypoprothrombinemia following coadministration of phenylbutazone and warfarin (O'Reilly and Aggeler, 1968).

The notion that such findings reflect the imposition of distribution changes upon warfarin by phenylbutazone, that is, protein-binding displacement, is reinforced by the in-vitro work bearing upon this subject. Utilizing an ultrafiltration procedure, Solomon
and Schrogie (1967) demonstrated that phenylbutazone could displace warfarin-$^{14}$C from human albumin binding sites. They further indicated that the displacement phenomenon was concentration-dependent. That is, higher concentrations of phenylbutazone effected greater percentages of unbound warfarin. Additionally, evidence was presented indicating that the displacement of warfarin by phenylbutazone was of a competitive nature. Other in-vitro work appears to corroborate these findings. A competitive binding-displacement of warfarin by phenylbutazone was suggested by the data of Aggeler et al. (1967) who examined the interaction on human serum albumin in-vitro by equilibrium dialysis. However, four of the six concentrations of warfarin utilized exceeded those which might be anticipated for production of clinical hypoprothrombinemia. Utilizing a fluorescent probe--1-anilinonaphthalene-8-sulfonate--the fluorescence of which increases markedly when bound to proteins and which binds to bovine serum albumin sites in a competitive manner with phenylbutazone (Jun and Luzzi, 1971) and presumably warfarin (Solomon and Schrogie, 1967; Aggeler et al., 1967), Jun et al. (1972) concluded that the two drugs bind competitively at identical sites on the bovine albumin molecule. It must be pointed out, however, that the concentrations of warfarin used greatly exceeded values that might be anticipated for adequate therapeusis.

In a further attempt to characterize those factors which influence the response to oral anticoagulants when a pyrazolone compound is included in the dosage regimen, Welch et al. (1969) noted in the dog
that the initial addition of phenylbutazone to the regimen effected responses (both in terms of bishydroxycoumarin plasma level changes and prothrombin complex activity changes) not unlike those reported in previous clinical studies (Aggeler et al., 1967; O'Reilly and Aggeler, 1968). That is, soon after the addition of phenylbutazone to the bishydroxycoumarin regimen, the plasma bishydroxycoumarin levels began to fall and the hypoprothrombinemic response intensified. However, as the phenylbutazone was continued, a reversal of the hypoprothrombinemia occurred, and the dose of bishydroxycoumarin required to maintain the prothrombin time within a "therapeutic" range had to be elevated (Welch et al., 1969). The investigators suggested that the diminution of plasma bishydroxycoumarin levels coupled with a decrement in pharmacologic response was simply attributable to the ability of phenylbutazone to induce the hepatic microsomal metabolism of bishydroxycoumarin.

Aside from activities which might alter the physiologic disposition of oral anticoagulants of the coumarin variety, and thereby diminish or enhance the observed hypoprothrombinemic response, pyrazolone compounds possess a distinct pharmacologic activity which may account, in part, at least, for the apparent potentiations of coumarin activity. Pyrazolone compounds may be capable of eliciting clinical responses during ongoing anticoagulant therapy, clinically indistinguishable from those which might result from increased hypoprothrombinemia associated with enhanced bio-availability of the pharmacologically active fraction of the anticoagulant, but which, in fact,
are independent of any pyrazolone-induced alterations of prothrombinemic activity. It has, in fact, been suggested that observed hemorrhagic episodes during the combined administration of coumarins and pyrazolones is a function of the hypoprothrombinemic activity of the anticoagulant (however it may be altered by the presence of the pyrazolone) and the independent inhibition of primary hemostatic mechanisms—specifically platelet aggregation—by the pyrazolone agent (O'Reilly and Aggeler, 1970; Koch-Weser and Sellers, 1971). The role of blood platelets in primary hemostatic processes has only recently come to light, and efforts to better understand the factors influencing platelet function have been the subject of recent reviews (Marcus and Zucker, 1965; Michal and Firkin, 1969; Mustard and Packham, 1970; Fields and Hass, 1971; Rossi, 1972).

Platelets are anucleate discoid cells, 2-3 micra in diameter, which arise from the fragmentation of megakaryocytes in bone marrow (Michal and Firkin, 1969). Normal mammalian platelets circulate with an average lifespan of approximately 10 days. Platelet cytoplasm contains numerous organelles including mitochondria and granules containing serotonin (5-Ht), phosphonucleotides (ADP and AMP), and lysosomal cathepsins.

A great deal has been learned about the manner in which primary hemostasis is effected by platelets. Subsequent to vascular trauma and exposure of platelets to collagen fibers of damaged vascular tissue, platelets are seen to adhere to collagen fibers. An enzymatic basis for platelet-collagen adhesion has been proposed (Jamieson et al.,
Collagen:glucosyltransferase mediates the transfer of glucose from uridine diphosphate glucose (UDPG) to incomplete heterosaccharide chains of collagen. The transferase which has been demonstrated in platelet membranes, thereby links the platelet to collagen fibers. Presumably, during such a bridge formation, structural changes occur within the platelet and are accompanied by the release of platelet constituents such as 5-HT, histamine, epinephrine, and ADP which are stored within osmiophilic storage granules (Evans et al., 1971). Platelet factor 3, a membrane-bound phospholipid procoagulant is also made available. The released ADP, epinephrine, and 5-HT are capable of promulgating further release from adjacent platelets (Salzman, 1971). The large pool of ADP made available by platelet adhesion to collagen stimulates the clumping or aggregation of platelets in the presence of ionized calcium (Zucker, 1972).

The initial platelet aggregate is reversible, and may disperse with the individual platelets returning to the circulation without loss of function (Michal and Firkin, 1969). However, platelets carry with them--adsorbed on their surface--a number of coagulation factors which, when surface activated, accelerate the formation of thrombin. The thrombin, while insufficient to cause clot formation, can, however, further stimulate the platelet release reaction.

The morphological changes in platelet ultrastructure which accompany the release reaction have been evidenced by electron microscopy in platelets aggregating in response to exogenous ADP, collagen, or thrombin, and have been attributed to an alteration in the
activity of a membrane-contained contractile protein, thrombosthenin. However, the precise mechanism of the viscous metamorphosis is unknown (Mustard and Packham, 1970). During viscous metamorphosis, platelets become closely packed and spherical and pseudopod projections can be observed. Furthermore, while the platelet unit membrane remains intact, most of the osmiophilic nucleotide-containing granules are lost (Rossi, 1972). With the platelet release reaction and loss of osmiophilic granules and lysosomal cathepsins platelet factor 3 is also made available. This phospholipid combines with other plasma factors adsorbed to the platelet surface to stimulate the formation of thrombin in sufficient quantities to contribute to the establishment of the fibrin clot (Michal and Firkin, 1969).

A decrease in platelet function— inability to form aggregates — could lead to hemorrhagic conditions while an increase in platelet function could give rise to thrombotic disorders (Hawkins, 1972). The methods for studying platelet aggregability have provided some insight into the contributions of various drugs toward hemorrhage and thrombosis with respect to their effects on platelet function. Some agents which have been observed to stimulate platelet aggregation are listed in Table 1. Numerous drugs, particularly those with antiinflammatory activity, possess remarkable platelet aggregation inhibiting potential. Some of these are listed in Table 2.

Two features in particular are notable with respect to platelet function in hemostasis; the influence of pyrazolone compounds on platelet function, and the interactions between coumarin oral
**TABLE 1**

**PLATELET AGGREGATING AGENTS**

<table>
<thead>
<tr>
<th>Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Epinephrine</td>
</tr>
<tr>
<td>Norepinephrine</td>
</tr>
<tr>
<td>Serotonin</td>
</tr>
<tr>
<td>Thrombin</td>
</tr>
<tr>
<td>Collagen</td>
</tr>
<tr>
<td>Trypsin</td>
</tr>
<tr>
<td>Papain</td>
</tr>
<tr>
<td>Dextran</td>
</tr>
<tr>
<td>Fluoride</td>
</tr>
<tr>
<td>Nicotine</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
</tr>
</tbody>
</table>

"Abridged from Mustard and Packham (1970)."
<table>
<thead>
<tr>
<th>Drug</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfinpyrazone</td>
<td>Packham et al., 1967; Mustard et al., 1967</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>Mustard et al., 1967; Packham and Mustard, 1969</td>
</tr>
<tr>
<td>Dipyridamol</td>
<td>Giromini et al., 1972; Zucker and Peterson, 1970</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Zucker and Peterson, 1970</td>
</tr>
<tr>
<td>Mefenamic Acid</td>
<td>Zucker and Peterson, 1970</td>
</tr>
<tr>
<td>Flufenamic Acid</td>
<td>Zucker and Peterson, 1970</td>
</tr>
<tr>
<td>Fenoprofen</td>
<td>Hermann et al., 1972</td>
</tr>
</tbody>
</table>
anticoagulants and pyrazolone derivatives. First, the pyrazolone agents have demonstrated marked activity in inhibiting platelet aggregability in various species in-vitro and in-vivo (Packham et al., 1967; Mustard et al., 1967; and Zucker and Peterson, 1970); and secondly, the combined impediment of both hemostatic processes—the reaction of platelets with vascular basement membrane to initiate the formation of the platelet plug, and the eventual formation of thrombin for the blood coagulation reaction—seriously compromises hemostasis (Evans et al., 1971). Thus it remains, as has been suggested, that the clinically observed hemorrhagic manifestations occurring when drugs of the coumarin and pyrazolone types have been coadministered, may have arisen from the independent activities of the coumarins and pyrazolones upon blood clotting and primary hemostatic processes, respectively.

Of those studies designed to evaluate the influence of other drugs upon the anticoagulant response to coumarins, most have appropriately sought to correlate the measured anticoagulant response with a temporally coincident plasma concentration of the anticoagulant itself. Unfortunately, however, the anticoagulant response is most often determined by methods which actually measure circulating plasma prothrombin complex activity or content such as the one-stage method of Quick (1966) or similar procedures such as Thrombotest (Owren, 1959), and is expressed as a prothrombin time, prothrombin content, or prothrombin activity. The difficulty with such an approach resides in the finding that anticoagulant responses expressed as prothrombin times,
content, or activity do not, in fact, correlate temporally with coumarin plasma levels (Nagashima et al., 1968). Nagashima et al. (1968) reported that peak hypoprothrombinemia succeeded peak warfarin levels by more than twenty-four hours. Peak drug levels occurred within about six hours after administration of a single oral dose of warfarin. Peak hypoprothrombinemia was not evidence until some thirty-six to forty-eight hours after drug administration. The reason for such a temporal disparity between drug plasma level and drug effect is that while the net effect of coumarin anticoagulants is manifested as a diminished availability of vitamin K-dependent clotting factors, the immediate effect of coumarins is suppression of the synthesis of these factors (O'Reilly and Aggeler, 1970; Koch-Weser et al., 1971; Eipe, 1972; Deykin, 1970; and Borden, 1972). Thus, simply measuring prothrombin time, content, or activity gives only an indication of the accrued net effect of prior anticoagulant treatment rather than a temporally relevant measure of the direct action of the anticoagulant upon prothrombin complex synthesis.

Having recognized the problem, Nagashima et al. (1968) also provided a means for dealing with it. The notion was posited that the measured plasma levels of vitamin K-dependent clotting factors at any given time represented the over-all status of their synthesis and degradation. Any change, therefore, in the rate of synthesis or degradation of the complex could be reflected by a change in the level of clotting factors. The net rate of change \( \dot{R}_{net} \) of the prothrombin complex \( P \) at any time could be described by:
\[ R_{\text{net}} = R_{\text{syn}} - R_{\text{deg}} \]

where \( R_{\text{syn}} \) represents the rate of P synthesis and \( R_{\text{deg}} \) represents the rate of P degradation.

The direct effect of coumarin anticoagulants is reflected by \( R_{\text{syn}} \) relative to its normal value, and this can be determined if \( R_{\text{net}} \) and \( R_{\text{deg}} \) are known. The net rate of change of P is simply determined by a gross evaluation of the change in P (determined by usual procedures such as the one-stage prothrombin time or Thrombotest) with time. Since the degradation of P is describable by first-order kinetics (Nagashima et al., 1968; Weintraub et al., 1973), \( R_{\text{deg}} \) can be represented by the expression, \( k_d P \), where \( k_d \) is the apparent first-order rate constant for P degradation. The value of \( k_d \) is determined experimentally by following the disappearance of P with time after a dose of anticoagulant has been given. By substituting \( k_d P \) for \( R_{\text{deg}} \) and experimentally determining \( R_{\text{net}} \), it becomes possible to obtain values for \( R_{\text{syn}} \).

As a result of the several inquiries into the nature and causes of the alteration of responses to coumarin anticoagulants effected by pyrazolone drugs, reasonable insight has been gained. Undoubtedly, however, a number of questions remain unanswered while new ones can be raised. The qualitative effect, for example, of pyrazolones upon the metabolic fate of oral anticoagulants has all but eluded investigators. Claims in this regard embrace both possibilities: that pyrazolones inhibit hepatic microsomal metabolism of coumarins (Weiner, 1964; Weiner et al., 1965; Burns, 1965; and Cucinell, 1965), or that they stimulate the metabolism of coumarins (Welch et al., 1969). Indeed,
biphasic effects of phenylbutazone upon the in-vitro metabolism of meprobamate and carisoprodol by rat hepatic microsomal enzymes have been reported (Kato et al., 1964). These investigators found that either the addition of phenylbutazone to the incubation flasks or in-vivo pretreatment of animals just prior to sacrifice would obtund the in-vitro metabolism of meprobamate and carisoprodol by hepatic enzymes obtained from naive or pretreated rats, respectively. They further reported that the metabolism of meprobamate and carisoprodol was enhanced by microsomal enzymes obtained from rats pretreated with phenylbutazone 48 hours prior to sacrifice. The N-demethylation of ethylmorphine by rat hepatic microsomes was inhibited by the inclusion of phenylbutazone in the incubation mixture (Rubin et al., 1964). It is noteworthy that the effect of pyrazolone pretreatment upon the in-vitro metabolism of coumarins has heretofore not been examined. Thus, in an effort to demonstrably characterize the qualitative effect of pyrazolones upon hepatic coumarin metabolism both in-vivo and in-vitro investigations have been undertaken and the results are reported herein. Furthermore, the pharmacologic consequences of pyrazolone influence upon coumarin metabolic disposition have been examined.

Considerable evidence has been amassed to suggest that the clinically observed hemostatic crises—retrospectively attributed to the interaction of pyrazolones and coumarins—evolved, in a mechanistic sense, from the elevation of the non-plasma protein-bound fraction of coumarin through a binding displacement phenomenon. However, such a notion must be tempered with the realization that the actual demonstrations of binding displacement were performed in-vitro, and that such
findings do not necessarily translate into the in-vivo situation (Meyer and Guttman, 1968; Holcenberg, 1969; Gordon et al., 1973). Furthermore, those in-vivo investigations which tend to corroborate the notion of binding displacement are ones in which only the changes in the total (bound and unbound fractions) plasma concentrations of coumarin were measured (Aggeler et al., 1967; O'Reilly and Aggeler, 1968; Welch et al., 1969; and O'Reilly and Levy, 1970). The data, for example, of O'Reilly and Levy (1970) indicate that subsequent to interjection of a single dose of oral warfarin into an ongoing phenylbutazone regimen in human subjects, the plasma levels of warfarin required to inhibit prothrombin complex synthesis are much lower than when warfarin is administered alone. Accompanying such findings is the additional evidence that warfarin disappearance rates are enhanced in the former instance (O'Reilly and Levy, 1970). Although, as the authors indicate, these results are consistent with the phenomenon of protein-binding inhibition of warfarin by phenylbutazone, another line of reasoning may equally well account for the data. For example, Schrogie and Solomon (1967) proposed that the potentiation of bishydroxycoumarin by d-thyroxine, clofibrate, and norethandrolone were effected through an increase in the affinity of an anticoagulant receptor for bishydroxycoumarin by those agents. Subsequently, evidence to reinforce that notion as it applied to the potentiation of the anticoagulant response to warfarin by d-thyroxine was presented (Solomon and Schrogie, 1967b). If, in fact, pyrazolone drugs are stimulators of the microsomal enzymes that degrade warfarin, it then
becomes reasonable to posit that data portraying enhancement of the pharmacologic response to warfarin concomittant with reduced plasma levels of warfarin may reflect an enhanced rate of metabolism of warfarin and an increased affinity of the anticoagulant receptor—whatever its nature—for warfarin.

By measuring the changes in total warfarin (bound and unbound fractions) in the plasma as well as the free fraction (unbound) as a result of in-vivo administration of warfarin with and without the in-vivo presence of phenylbutazone, it becomes possible to determine whether, in fact, the binding inhibition of warfarin by phenylbutazone already demonstrated in-vitro, can likewise be demonstrated in-vivo. The binding displacement of warfarin by phenylbutazone subsequent to the in-vivo administration of both agents, has therefore, been examined.

Not only have published reports failed to elucidate clearly the qualitative nature of the influence of pyrazolone compounds upon the metabolic disposition of coumarins in-vivo, but the in-vitro investigation of this particular aspect of their interaction remains to be reported. Furthermore, the enhanced rate of disappearance of warfarin from plasma in the face of phenylbutazone administration has, not unreasonably, been ascribed to the increased availability of the degradable (unbound) fraction of warfarin and its subsequent presentation to hepatic microsomal metabolizing enzymes through plasma protein-binding inhibition by phenylbutazone (O'Reilly and Levy, 1970). It therefore seems appropriate to determine which of the two suggested
influences are responsible for the phenylbutazone-imposed enhanced rate of warfarin disappearance—protein-binding inhibition, microsomal enzyme induction, or both. If, in fact, both mechanisms are operative it becomes important to be able to dissociate them.

Should phenylbutazone demonstrate the potential for protein-binding inhibition in-vivo as well as microsomal enzyme induction—the prothrombinemic consequences of which could tend to be offsetting—it would be useful to demonstrate whether or not another activity of phenylbutazone—-independent of the changes in hypoprothrombinemia effected by warfarin—might possess the potential for producing the kinds of hemorrhagic episodes recorded clinically. Sulfinpyrazone has already been shown to decrease platelet adhesiveness in patients being treated with that pyrazolone compound (Smythe et al., 1965). Furthermore, both sulfinpyrazone and phenylbutazone have impaired hemostatic plug formation at the ends of transected mesenteric vessels in rabbits, as well as inhibited platelet aggregation induced by collagen, antigen-antibody complexes, or gamma-globulin-coated surfaces (Fackham et al., 1967). The consequences of platelet-aggregability inhibition by phenylbutazone administered in-vivo with regard to primary hemostasis might be sufficient to seriously compromise over-all hemostasis in the face of ongoing anticoagulant therapy.

Statement of the problem. The foregoing introduction evidences the need for further research in the realm of interactions between coumarin anticoagulants and pyrazolone derivatives such as phenylbutazone. It is clear that coadministration of such compounds can lead, indeed has
led, to hemorrhagic diatheses. Although mechanisms whereby such pyrazolone-induced perturbation of responses to coumarins have been suggested, support for such notions is comprised chiefly of presumptive evidence.

The mechanisms whereby phenylbutazone, the parent pyrazolone anti-inflammatory agent, might influence the physiologic disposition of warfarin, the coumarin anticoagulant of choice, can be affirmed directly as well as presumptively. Furthermore, inasmuch as the consequences of the interaction between coumarins and pyrazolones become manifest during routine coadministration of the compounds, the study should be conducted within the context of a clinical model.

It is intended that the widely held notion of phenylbutazone-induced plasma protein-binding displacement of warfarin be evaluated in-vivo utilizing the dog as a model. By examining the anticoagulant-effected hypoprothrombinemic response as a function of the plasma concentration of anticoagulant it becomes possible to attribute changes in that relationship associated with phenylbutazone administration to alterations in anticoagulant distribution. Alterations of the anticoagulant plasma concentration-response relationship consistent with the phenomenon of protein-binding displacement can then be corroborated by direct determinations of changes in the free fraction of the anticoagulant in the plasma occurring during phenylbutazone coadministration.

The nature of pyrazolone-induced alterations of coumarin anticoagulant hepatic microsomal metabolism also requires additional study. Suggestions that pyrazolone pretreatment can enhance anticoagulant
metabolism appear in the literature with perhaps greater frequency than those of pyrazolone-induced inhibition of anticoagulant hepatic microsomal metabolism. In any case, the effect of pyrazolone pretreatment upon the in-vitro hepatic metabolic disposition of coumarins has not been evaluated in any species and remains a suitable object for investigation.

Pyrazolones have been alleged to contribute to hemorrhagic diatheses associated with their combined administration with oral anticoagulants in part, by a mechanism essentially independent of prothrombinemic alterations, presumably by inhibiting platelet aggregability and thus, primary hemostasis. Evidence for the in-vitro inhibition of platelet aggregability by phenylbutazone has, as yet, only been complemented by like evidence deriving from its in-vivo administration to rabbits. Further evidence arising from a more clinically relevant model, that the in-vivo administration of phenylbutazone might attenuate primary hemostatic mechanisms would lend credence to the notion that its contribution to spontaneous hemorrhaging during anticoagulant administration is, in part, divorced from changes in prothrombinemia.
CHAPTER II
METHODS AND MATERIALS

General considerations. Male, mongrel dogs (Blue Farms, Plain City, Ohio) weighing 10-20Kg and male, albino rats of the Sprague-Dawley strain (Harlan Industries, Cumberland, Ind.) weighing 400-500 g. were used. Dogs were sampled for blood by the technique of Hovell (1968) from the jugular vein. Whole blood was drawn into either oxalated or EDTA*-anticoagulated evacuated tubes (Vaccutainer, Becton-Dickinson, Rutherford, N.J.) to a final sample volume of either 3 or 10 ml., respectively. Venipuncture was accomplished with a siliconized 20 gauge, 1-1/2 inch Vaccutainer needle. Intravenous infusions were also made directly into the jugular vein. Those dogs infested with ectoparasites were dipped in a commercially available antiparasitic solution containing 40% chlordane as the active ingredient (Chlordaside, Nordon Laboratories, Lincoln, Neb.) at least 30 days prior to receiving drugs. Dogs utilized for in-vivo studies were maintained on a diet of Purina dog chow (laboratory grade) and fed once daily at 1:00 P.M. Water was available ad-libitum.

Determination of plasma warfarin (total). Warfarin (as the sodium salt) was determined by a procedure derived from the reported methods of Lewis et al. (1970) and Welling et al. (1970). Plasma was

*ethylenediaminetetraacetic acid
prepared from EDTA-anticoagulated blood by differential centrifugation (3,640g) in a Sorvall Superspeed centrifuge, type SS-1A (Ivan Sorvall, Inc., Norwalk, Conn.) at room temperature for 10 minutes. Plasma (0.2-2.0ml) was diluted to 2.0 ml with triple-distilled water and acidified with 1.0 ml 3N HCl. The acidified samples were then extracted with 3.0 ml of ethylene dichloride (EDC) (spectrophotometric grade). After shaking for 10 minutes on a heavy-duty horizontal shaker (General Electric, Ft. Wayne, Ind.) the samples were again centrifuged at room temperature for 10 minutes. Samples were resolved into three layers: upper (aqueous), middle (protein), and lower (warfarin-containing EDC).

After discarding the aqueous and protein layers, the solvent layer was transferred to a 5 ml glass vial and evaporated to dryness under a continuous nitrogen stream. The vessel walls were rinsed with 0.2 ml of acetone (reagent grade) and the acetone was also evaporated to dryness under nitrogen. The residue was taken up with 25 μl of acetone and quantitatively streaked with a micropipet onto 5 cm X 20 cm thin-layer chromatography (TLC) plates precoated with silica gel (impregnated with a fluorescent indicator) to a thickness of 250 microns (Brinkman Instruments, Inc., New York). The vial was additionally rinsed with another 25 μl of acetone, and the rinsings were again streaked onto the TLC plates. The prepared plates were then placed in appropriate development tanks (Desaga, Heidelberg, Germany) containing 100 ml of the developing solvent--EDC:acetone, 9:1. Plates were chromatographed by the ascending method until the solvent front had
traveled 15 cm, whereupon they were dried under nitrogen. Warfarin was localized by its quenching of fluorescence under a short-wave ultraviolet lamp (Mineralight UVS 12, Ultraviolet Products Inc., San Gabriel, Cal.) or by its anticipated R<sub>p</sub> value (0.42) and the localization of a warfarin standard which was run alongside each sample. The localized area of the plate containing warfarin was then scraped from the plate and eluted with 4.0 ml of acetone by shaking for 10 minutes on a horizontal shaker. The eluate was centrifuged for 10 minutes to sediment the silica gel, and 3.0 ml of the eluate were then transferred to a 5 ml vial and evaporated to dryness under nitrogen. The residue was dissolved in 1.0 ml of N,N-dimethylformamide. The solution was mixed on a vortex-type mixer (Lab-Line Super Mixer, A.H. Thomas, Phila., Penna.) for 5 seconds and transferred to a cuvette for fluorescence spectrometry reading.

Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, Md.) at excitation and emission wavelength settings of 330 nm and 408 nm, respectively. Sensitivity was adjusted with a quinine sulfate standard (1.0 mcg/ml in 0.1 N sulfuric acid) such that the standard produced a fluorescence reading of 80 with excitation and emission wavelengths of 350 nm and 450 nm, respectively.

The concentration of warfarin was determined by recording the difference in fluorescence before and after the addition of 3N HCl (0.1 ml) to the solution of warfarin in N,N-dimethylformamide, and adjusted for the fluorescence of a plasma blank carried through each assay. Warfarin
sodium (generously supplied by Endo Laboratories, Inc., Garden City, N.Y.) was added to normal plasma or an isotonic solution of electrolytes adjusted to pH 7.4 (Normosol-R pH 7.4, Abbott Laboratories, North Chicago, Ill.) in preparation of standards.

Determination of plasma warfarin (unbound). Plasma was obtained in the manner described. Ten ml were placed in an ultrafiltration cell (Amicon Corp., Lexington, Mass.). The sample was continuously stirred by a magnetic stirrer, and positive filtration pressure was achieved with nitrogen. Samples were filtered at ambient temperature through an ultrafiltration membrane retentive for molecules exceeding 10,000 in molecular weight (Diaflo PM-10, Amicon). Membranes were soaked in triple-distilled water overnight at 2-5°C prior to use. One ml of ultrafiltrate (10% of the plasma sample) was collected (approximately 30 minutes of filtration time) and assayed for warfarin as described with the following exceptions:

One ml of ultrafiltrate was acidified and extracted as described. After evaporation of the EDC layer, TLC was omitted, and the residue taken up in 1.0 ml of N,N-dimethylformamide. Determinations of warfarin concentration were the same as for total warfarin, and corrected for adsorbance of the drug to the membrane. The extent of warfarin adsorption by the membrane was determined by preparing aqueous solutions of warfarin sodium in Normosol-R pH 7.4, and measuring the concentration of warfarin prior to and subsequent to passing the solution through the ultrafiltration membrane.
Prothrombin determinations. Plasma prothrombin activity (prothrombin complex activity) was determined by the modified method of Quick (1966). Plasma, prepared as described from oxalated blood, was heated in 0.1 ml aliquots to 37°C on an automatic coagulation timer (Fibrometer, BBL, Cockeysville, Md.). Thromboplastin (Simplastin, Warner-Chilcott, Morris-Plains, N.J.) also heated to 37°C, was rapidly added in a 0.2 ml aliquot with a Fibrosystem pipetter (BBL), and the automatic timer bar was simultaneously depressed. The automatic timer was stopped once initial clot formation (development of fibrin strands) was detected, and the end-point registered on the digital readout in seconds and tenths.

Plasma prothrombin activity was expressed as a percent of normal. Normal values were derived from undiluted plasma samples. Each animal's experimental prothrombin activities were calculated on the basis of its own normal value. Standard curves for prothrombin content (activity) as a function of plasma coagulation times (seconds) were constructed in the usual manner by preparing dilutions of plasma with 0.9% sodium chloride solution.

The effect of acute phenylbutazone administration upon the pharmacologic response to warfarin in-vivo. Warfarin sodium as the commercially available tablet (Endo, Garden City, N.Y.) was administered orally to dogs at a dose of 0.8 mg/Kg. Plasma for both prothrombin activity and warfarin determinations was collected at the time of dosing and at 3, 6, and 12 hours subsequently. Thereafter plasma was collected at 6, 12, or 24 hour intervals until prothrombin
determinations approached pre-medication values. A three-week interval elapsed before the animal was subjected to any further treatment. At that time the animal was readministered oral warfarin (0.8 mg/Kg) and additionally oral phenylbutazone (50 mg/Kg). Phenylbutazone was also administered as commercially available 100 mg tablets (Geigy, Ardsley, N.Y.). Prothrombin and warfarin disappearance and prothrombin reappearance were again monitored.

The effect of acute and chronic phenylbutazone administration upon the plasma half-life of warfarin in-vivo. The plasma half-life of warfarin after oral administration of a single oral dose (0.8 mg/Kg) was determined from the slope of a semi-logarithmically plotted curve of plasma warfarin concentration versus time.

Four weeks subsequent to the combined single dose administration of warfarin and phenylbutazone a one week pretreatment with phenylbutazone was begun. Each animal was given phenylbutazone (50 mg/Kg) orally every twelve hours (9 A.M. and 9 P.M.). After seven days of pretreatment, all drug administration was terminated for an additional seven days. Once the seven day drug-free interval had elapsed, a single, oral dose of warfarin (0.8 mg/Kg) was re-administered, and its disappearance from plasma was monitored.

Protein-binding inhibition of warfarin by phenylbutazone in-vivo. Animals were initially administered sodium warfarin (1.0 mg/Kg) intravenously by jugular venipuncture. Infusion was slow (3-5 seconds) to prevent extravasation. After infusion, blood was aspirated back into
the syringe to ensure the intravenous placement of the needle throughout the procedure. Injections were made with a 2.5 cc plastic disposable syringe and a 1-1/2 inch 20 gauge stainless steel needle. The venipuncture site was clipped free of fur and swabbed with a benzalkonium chloride solution (Zephiran aqueous 1:750) prior to and after infusion. All solutions for injection were sterilized by membrane filtration through a Swinnex-25 filter unit (Millipore Corp., Bedford, Mass.) containing a membrane of 0.22 micron porosity.

Plasma levels of the unbound fraction and total fraction were determined. Sampling was performed 60 minutes after warfarin administration. The following modifications were made in the determination of total plasma warfarin:

One-tenth ml of plasma was acidified and extracted as described. Chromatography was omitted, and the residue of the dried EDC extract was taken up in 1.0 ml of N,N-dimethylformamide. Fluorescence was read in the manner already described.

After one week the animals were administered phenylbutazone (25 mg/Kg) intravenously. The solution was a commercially available preparation for veterinary use containing 200 mg/ml (Jensen-Salsbery Laboratories, Kansas City, Mo.). Sixty minutes subsequent to the infusion of phenylbutazone, sodium warfarin was reinfused at 1 mg/Kg. Sampling was again performed 60 minutes following warfarin infusion, and both total and unbound levels of plasma warfarin were estimated.
Platelet aggregability. The method for determining platelet aggregability was modified from Holdrinet et al. (1969). Platelet-rich plasma (PRP) was prepared from oxalated and EDTA-anticoagulated blood by centrifugation at 5°C in a refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn.) at 67g for 10 minutes. Two-tenths ml of a 0.3% collagen suspension was added to the oxalated blood shortly after it was collected. Collagen (Sigma, St. Louis, Mo.) was prepared by mixing the fibrous collagen with 0.9% sodium chloride in a Virtis mixer (Virtis, Gardiner, N.Y.) for 10 minutes, and then separating the coarse particles by centrifugation for an additional 10 minutes. The supernatant was decanted and stored until use at 2-5°C. Collagen suspensions were freshly prepared on each day of use.

After centrifugation of the blood samples, the optical density of both EDTA and oxalated PRP was determined turbidimetrically in a Spectronic-20 spectrophotometer (Bausch and Lomb) at 600 nm. The difference in optical density between the EDTA-PRP and oxalate-PRP to which collagen had been added was taken as a measure of platelet aggregability.

Dogs were sampled on three occasions on three successive days prior to drug treatment. Both EDTA-anticoagulated and oxalated blood were collected from the same venipuncture. This permitted the determination of a control for platelet aggregability in each animal. The animals were next subjected to the oral administration of phenylbutazone (50 mg/Kg) twice daily at 12-hour intervals (9 A.M. and 9 P.M.) for seven days. On days 5, 6, and 7 of treatment the animals were
again sampled 3 hours after the day's first dose, and platelet aggregability was re-estimated. Each animal served as its own control.

In-vitro synthesis of prothrombin complex activity. The method for producing and measuring in-vitro synthesis of prothrombin complex activity was modified from the procedures of Babior and Kipnes (1970) and Biezunski (1970). Rats were sacrificed and exsanguinated by decapitation. Livers were quickly removed and rinsed in cold, isotonic KCl (1.15%). The chilled livers were then blotted on filter paper, weighed, and homogenized in isotonic KCl such that 10 ml of the homogenate represented 1.0 g of liver. Homogenization was performed in a Potter-Elvehjem homogenizer with a teflon pestle. Samples were homogenized at approximately 5°C and during homogenization the pestle was raised and lowered 6 times. Ten ml of homogenate were placed in polycarbonate centrifuge tubes and centrifuged at 0°C for 20 minutes at 10,800g. One-half ml of the supernatant was then placed in a 25 ml Erlenmeyer flask containing 0.5 ml CaCl₂ (3.0 x 10⁻³ M) and 0.5 ml trisaminomethane (Tris) buffer (0.02 M) adjusted to pH 7.4. Samples were heated to 37°C for five minutes in air and then incubated in an atmosphere of 95% O₂-5%CO₂ in a Dubnoff metabolic shaker (Precision Scientific, Chicago, Ill.). Samples were removed at the onset of incubation and every five minutes thereafter up to twenty minutes.

Determination of prothrombin complex activity synthesized. Prothrombin complex activity was assayed by a modification of the one-stage method of Quick (1966) based on the correction of the defective
coagulability of plasma obtained from a dog deficient in vitamin K-dependent clotting factors. The dog was made hypoprothrombinemic by the prior administration of 1.0 mg/Kg warfarin sodium orally, 48 hours before sampling. Thromboplastin was heated to 37°C on an automatic coagulation timer. The incubation mixture (25 µl) was immediately transferred from the shaking incubator to a sample cup and heated an additional 60 seconds at 37°C with 0.2 ml of thromboplastin and 25 µl of Tris buffer, pH 7.4. After 60 seconds, 50 µl of prothrombin-deficient plasma was added to the reaction mixture and the coagulation time was monitored. The prothrombin status of the deficient plasma was evaluated just prior to the determination of in-vitro prothrombin complex synthesis by substituting 25 µl of Tris buffer for the incubation mixture. The difference in prothrombin activity of prothrombin-openic plasma itself, and that with incubate, was taken as a measure of prothrombin complex activity contributed by the incubate.

Rats were divided into three treatment groups. Each group contained three animals. All injections were made by the intraperitoneal route. One group received 0.9% sodium chloride (0.5 ml); a second group was injected with warfarin sodium (0.25 mg/ml) at a dose of 0.25 mg/Kg. The third group was pretreated orally for five days with a phenylbutazone suspension (25 mg/ml in 0.1% methylcellulose), 1.0 ml twice daily (9 A.M. and 9 P.M.). Five days elapsed prior to further treatment. At this time the animals were then administered sodium warfarin again (0.25 mg/Kg) by the intraperitoneal route. Two hours after warfarin or saline administration animals were sacrificed and
the in-vitro synthesis of prothrombin complex activity was analyzed.

Drug treatment for in-vitro metabolism of warfarin by canine microsomal enzymes. In experiments with phenylbutazone-induced dogs, the animals were pretreated with 50 mg/Kg twice a day (9 A.M. and 9 P.M.). Phenylbutazone was orally administered for five days. The last dose was given 12 hours prior to sacrifice.

Preparation of enzyme for warfarin metabolism. Animals were starved for 24 hours before sacrifice. Three animals received chronic phenylbutazone pretreatment and three animals were left drug-free. All animals were rendered instantly unconscious by stunning. This was accomplished by a "captive-bolt" pistol directed at the parietal suture of the skull. Once unconscious, the animals were exsanguinated by severing the carotid arteries and jugular veins bilaterally.

Portions of the liver were excised and rinsed in cold isotonic KCl (1.15%). Liver was then homogenized with 4 volumes of ice-cold isotonic KCl in 0.05 M Tris buffer (pH 7.4) in a Virtis homogenizer for 30 seconds and then a motor-driven teflon glass homogenizer for an additional 30 seconds. The teflon pestle was raised and lowered 6 times. The homogenate was centrifuged at 0°C for 20 minutes at 10,800g in a Sorvall RC2 centrifuge. Nine ml portions of supernatant were transferred to high speed centrifuge tubes and centrifuged at 104,000g for 60 minutes at 0-5°C in a Beckman Model L ultracentrifuge fitted with a 40,000 rpm rotor. The 104,000g supernatant was aspirated and the microsomal pellet resuspended in 2-1/2 volumes of isotonic
KCl by gentle manual homogenization with a teflon glass homogenizer.
The protein concentration was then determined by the method of Biuret
(Layne, 1957) with a Gilford spectrophotometer (Gilford Inst. Co.,
Oberlin, Ohio) using bovine serum albumin as a standard. Biuret
reagents were obtained from American Monitor (Indianapolis, Ind.).
The microsomal suspension was then diluted to a concentration of 10 mg/
ml with cold isotonic KCl in 0.05 M Tris buffer (pH 7.4). Enzyme thus
prepared was used immediately for the metabolism of warfarin.

**Incubation of warfarin with liver enzyme.** All incubations were
performed in duplicate in 25 ml Erlenmeyer flasks at 37°C for 10 minutes
in air. A Dubnoff metabolic shaking incubator oscillating at 120
cycles per minute was used for the incubation procedure. Each flask
contained: MgCl₂ (15 μmoles), glucose-6-phosphate (20 μmoles), NADP
(1.5 μmoles), and glucose-6-phosphate dehydrogenase (2 units).
Glucose-6-phosphate, NADP, and dehydrogenase were obtained from Sigma
Chemical Co. (St. Louis, Mo.). Warfarin (as the sodium salt) was
dissolved in isotonic KCl in 0.05 M Tris (pH 7.4) and added to make
final concentrations of 2.0, 4.0, 8.0, 16.0 and 30.0 X 10⁻⁵ M. Volumes
were adjusted with Tris-KCl to 3.0 ml. The reaction was initiated by
the addition of warfarin and stopped by the addition of 1.5 ml of
3 N HCl. In the case of tissue standards, 1.5 ml of 3 N HCl was added
prior to, rather than after incubation.

**Estimation of warfarin.** The extractive and TLC procedures for
warfarin were similar to those already described. Three ml of the
acidified incubate were extracted with 3.0 ml of spectral quality EDC by shaking for five minutes on a horizontal shaker. The mixture was then centrifuged for five minutes, and 2.0 ml of the EDC layer evaporated to dryness under nitrogen in a 5 ml glass vial. The vial walls were rinsed with 0.2 ml of acetone and the rinsings were evaporated. The residue was taken up with 25 μl of acetone and the solution was quantitatively streaked onto a glass TLC plate (5 cm X 20 cm) pre-coated with silica gel which was impregnated with an inorganic fluorescent dye. Chromatographic development, warfarin localization, and elution were performed as described. One-half ml of the acetone eluate was evaporated under nitrogen. The residue was dissolved with 2.0 ml of N,N-dimethylformamide, and warfarin was estimated spectrofluorometrically.

**Cytochrome P-450 content.** Cytochrome P-450 was determined in triplicate by a method similar to that described by Omura and Sato (1964). Fifteen mg of the microsomal preparation (10 mg/ml) was diluted with triple-distilled water to a total volume of 3.0 ml. An additional 3.0 ml of 0.3 M Tris buffer (pH 7.4) were added. One-half of each sample was placed into a cuvette. To the contents of one cuvette were added several milligrams of solid sodium dithionite. The contents of the other cuvette were bubbled with carbon monoxide for 30 seconds, after which sodium dithionite was added and carbon monoxide bubbled through for an additional 30 seconds. Both samples were read at 450 nm and 490 nm, and the difference in optical density...
at those wavelengths was recorded. The cytochrome P-450 content of control and phenylbutazone pretreated microsomal preparations was calculated using an extinction coefficient of 91 cm$^{-1}$M$^{-1}$. 
CHAPTER III

RESULTS

Determination of warfarin. The use of N,N-dimethylformamide as the solvent for detection of warfarin fluorescence afforded an enhancement of fluorescence compared to an aqueous solvent (Figure 2). N,N-dimethylformamide itself was devoid of significant fluorescence at the excitation and emission maxima for warfarin. Figure 3 illustrates the ability of 3.0 N HCl to quench warfarin fluorescence in dimethylformamide.

Warfarin fluorescence recovered from spiked canine plasma appeared linear over the concentration range studied. Recovery of warfarin from plasma averaged approximately 94% when samples were carried through the TLC operation. Figure 4 depicts the relationship between fluorescence recovered from warfarin-spiked plasma and the initial plasma warfarin concentration utilizing both extraction and separation procedures or extraction exclusively. Recovery from microsomal suspensions averaged 95%.

The relationship between fluorescence and warfarin concentration in ultrafiltered plasma is represented in Figure 5. When phenylbutazone was included in plasma ultrafiltrate samples "spiked" with warfarin, no alteration of warfarin fluorescence occurred.
FIGURE 2.

Emission spectra of warfarin in N,N-dimethylformamide (DMF) and water.
The quenching of warfarin fluorescence in N,N-dimethylformamide by the addition of HCl.

**FIGURE 3**

**INITIAL SAMPLE CONCENTRATION OF WARFARIN**

The quenching of warfarin fluorescence in N,N-dimethylformamide by the addition of HCl.
Warfarin fluorescence recovered from plasma after extraction with ethylene dichloride or extraction and then isolation by thin-layer chromatography. Vertical bars represent S.E.M.
Warfarin fluorescence recovered from plasma ultrafiltrate after extraction with ethylene dichloride. Vertical bars represent S.E.M.
Phenybutazone was included in ultrafiltrate samples in concentrations exceeding warfarin by 20-fold.

**Effect of chlordane exposure upon warfarin-plasma half-life.**

Upon evaluating the plasma half-life of warfarin after the administration of a single oral dose to dogs (0.8 mg/Kg) by monitoring the apparent first order disappearance of the drug from plasma it becomes readily apparent that the distribution of plasma half-lives among the initial group of animals studied (6) was bimodal. Two distinct groups of canine warfarin-metabolizers could be identified--rapid and slow--as indicated in Figure 6. The only distinction between the care, handling and treatment of the two groups was the topical chlordane treatment for ectoparasites which all of the rapid-metabolizers and which none of the slow-metabolizers received.

**Plasma coagulation time and its relationship to prothrombin complex activity.** An increase in the length of time required for detectable quantities of fibrin to form in oxalated plasma occurs as plasma prothrombin activity (content) is reduced. Figure 7 portrays the relationship between coagulation time and prothrombin activity or content. Undiluted plasma--assigned the value of 100% prothrombin activity--will produce electronically detectable quantities of fibrin within 7 seconds in the dog, whereas coagulation will not occur until nearly 45 seconds in plasma diluted to contain only 5% prothrombin activity.
FIGURE 6

Effect of exposure to chlordane upon warfarin plasma half-life in the dog. * = P<0.01 as per Student's t test.
FIGURE 7

Relationship between canine plasma prothrombin complex activity and coagulation times.
Effect of warfarin upon the availability of plasma prothrombin complex activity. The disappearance and subsequent reappearance of prothrombin complex activity as a consequence of warfarin administration (0.8 mg/Kg orally) to dogs is shown in Figure 8. The duration of hypoprothrombinemia correlates roughly with warfarin plasma half-life. (Also see Table 4). Peak hypoprothrombinemia occurs between 48 and 72 hours after warfarin administration, however, peak warfarin plasma levels occur within 3 hours of oral administration.

Figure 9 depicts the apparent first-order disappearance of prothrombin complex activity from the plasma of animals treated with a single, oral synthesis-blocking dose of warfarin (0.8 mg/Kg). The rate constant for the disappearance of prothrombin activity is derived from the slope of the disappearance curve. The disappearance of prothrombin activity from plasma when a single, oral dose of phenylbutazone (50 mg/Kg) is administered concurrently with warfarin is unaltered from the disappearance evoked by the administration of warfarin exclusively. Thus, the notion that phenylbutazone does not alter the rate of degradation of prothrombin complex activity is confirmed in the dog.

Prothrombin complex synthesis rates and plasma warfarin levels. The relationship between in-vivo synthesis rates of prothrombin complex activity and plasma warfarin levels is depicted for each of four dogs (Figures 10-13). Synthesis rates decrease with increasing plasma concentrations of drug. When warfarin is administered in a single oral dose (0.8 mg/Kg) the apparent plasma levels which are required to effect
Figure 8

Prothrombin complex activity after warfarin (0.8 mg/Kg) orally.
FIGURE 9

Effect of phenylbutazone (PBZ) upon warfarin-induced plasma disappearance of prothrombin complex activity.
Rate of prothrombin complex synthesis ($R_{syn}$) as a function of plasma warfarin concentration before and after coadministration of phenylbutazone (PBZ)

K-9 10306.
Rate of prothrombin complex synthesis ($R_{syn}$) as a function of plasma warfarin concentration before and after coadministration of phenylbutazone (PBZ). K-9 13407.
Rate of prothrombin complex synthesis ($R_{syn}$) as a function of plasma warfarin concentration before and after coadministration of phenylbutasone (PBZ).

K-9 12990.
Rate of synthesis of prothrombin complex ($R_{syn}$) as a function of plasma warfarin concentration before and after a single oral dose of phenylbutazone (PBZ) and the continued administration of PBZ every six hours after initial coadministration (PBZ chronic). K-9 13134.
inhibition of prothrombin complex synthesis are higher than when
warfarin is administered with phenylbutazone (50 mg/Kg). The potentia-
tion is evidenced by a shift-to-the-left of the plasma concentration-
response curve. In addition, the continued administration of phenyl-
butazone—every six hours until prothrombin complex activity returned
toward normal values—apparently even further reduced those concentra-
tions of warfarin required to inhibit prothrombin complex synthesis
in-vivo resulting in a dramatic shifting of the plasma concentration-
response curve (Figure 13).

The effect of a single oral dose of phenylbutazone upon the
relationship between plasma warfarin concentrations and the pharma-
cologic response to warfarin is further depicted in Table 3. While no
change in the degradation rate constant for prothrombin complex activity
is evidenced, a decrease in maximally effective plasma warfarin con-
centrations can be noted as well as a decrease in the slope of the
concentration-response line associated with phenylbutazone administra-
tion.

The reduction of slope value is even more marked when phenyl-
butazone is administered every six hours such that a high plasma con-
centration of the drug might be maintained (Figure 13). Such a regimen
of phenylbutazone decreased the slope value ($R_{syn}$ vs. plasma concen-
tration) from 207.5 to 21.4.

An apparent two-fold decrease in the warfarin plasma half-life
is also associated with administration of phenylbutazone with warfarin
(Table 4) and is consistent with the notion of protein-binding
TABLE 3

EFFECT OF PHENYL BUTAZONE UPON THE DEGRADATION RATE CONSTANT AND THE RELATIONSHIP BETWEEN PLASMA PROTHROMBIN COMPLEX ACTIVITY SYNTHESIS RATE AND PLASMA WARFARIN CONCENTRATIONS

<table>
<thead>
<tr>
<th>CONTROL</th>
<th>PHENYL BUTAZONE</th>
</tr>
</thead>
<tbody>
<tr>
<td>k&lt;sub&gt;d&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C&lt;sub&gt;p max&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.57</td>
<td>0.75</td>
</tr>
<tr>
<td>1.22</td>
<td>1.10</td>
</tr>
<tr>
<td>1.39</td>
<td>0.62</td>
</tr>
<tr>
<td>1.31</td>
<td>0.99</td>
</tr>
<tr>
<td>1.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.87&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Rate constant for disappearance of prothrombin complex activity (day<sup>-1</sup>).

<sup>b</sup>Extrapolated value for maximally effective plasma concentration of warfarin (mcg/ml).

<sup>c</sup>Mean of four animals.

<sup>d</sup>Values are S.E.M.

<sup>e</sup>Not significantly different from control p > 0.05.

<sup>f</sup>Significantly less than control (p < 0.05) by Student's t test.
TABLE 4

EFFECT OF ACUTE PHENYL BUTAZONE ADMINISTRATION ON WARFARIN PLASMA HALF-LIFE

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control</th>
<th>Phenylbutazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>13134</td>
<td>21.0</td>
<td>11.4</td>
</tr>
<tr>
<td>13407</td>
<td>13.0</td>
<td>7.2</td>
</tr>
<tr>
<td>12990</td>
<td>23.0</td>
<td>11.2</td>
</tr>
<tr>
<td>10306</td>
<td>16.5</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>18.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.6&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2.3&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Half-life is expressed in hours.

<sup>b</sup>Single oral dose of warfarin (0.8 mg/Kg).

<sup>c</sup>Single oral dose of warfarin (0.8 mg/Kg) and phenylbutazone (50 mg/Kg).

<sup>d</sup>Mean of four animals.

<sup>e</sup>Significantly different from control (p < 0.01) by Student's t test.

<sup>f</sup>Values represent S.E.M.
Protein-binding inhibition in dog plasma in-vivo. The intravenous administration of phenylbutazone (25 mg/Kg) just prior (60 minutes) to the intravenous administration of warfarin (1.0 mg/Kg) increased the apparent plasma concentration of the unbound warfarin fraction. The unbound warfarin fraction comprised 2.6% of the total warfarin concentration when no other drugs were present in the plasma. The prior administration, however, of phenylbutazone resulted in approximately a 3-fold increase in the concentration of unbound warfarin (Table 5).

Tabular values for unbound warfarin concentrations represent corrected values. The extremely low concentrations of free plasma warfarin were found to be appreciably retained by the ultrafiltration membrane (approximately 50%). However, the extent of membrane retention of free warfarin remained constant over a wide concentration range of warfarin. Furthermore, the addition of phenylbutazone in concentrations exceeding those of warfarin 50-fold had no effect upon the extent of warfarin retained by the membrane.

In-vitro synthesis of prothrombin complex activity by rat liver. The relationship between prothrombin complex activity and plasma coagulation time is shown in Figure 1. A value of 100% activity was assigned to plasma samples diluted 1:1 with Tris buffer.

When livers from saline-treated rats were removed and homogenized, and the 10,800g fraction incubated for varying intervals of time, the amount of prothrombin complex activity generated by the incubation
TABLE 5

EFFECT OF PHENYLButAZONE (PBZ) ON THE
AVAILABILITY OF WARFARIN IN THE DOG

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control(^a)</th>
<th>PBZ(^b)</th>
<th>%Free PBZ</th>
<th>%Free Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C(_t^c)</td>
<td>C(_f^d)</td>
<td>%Free</td>
<td>C(_t^e)</td>
</tr>
<tr>
<td>10306</td>
<td>6.76</td>
<td>0.12</td>
<td>1.7</td>
<td>6.38</td>
</tr>
<tr>
<td>1313(^4)</td>
<td>7.04</td>
<td>0.16</td>
<td>2.2</td>
<td>5.79</td>
</tr>
<tr>
<td>12990</td>
<td>7.65</td>
<td>0.18</td>
<td>2.3</td>
<td>7.51</td>
</tr>
<tr>
<td>13407</td>
<td>5.54</td>
<td>0.24</td>
<td>4.3</td>
<td>5.24</td>
</tr>
<tr>
<td></td>
<td>6.75e</td>
<td>0.18e</td>
<td>2.6e</td>
<td>6.23e</td>
</tr>
<tr>
<td></td>
<td>0.44e</td>
<td>0.03e</td>
<td>0.6e</td>
<td>0.49e</td>
</tr>
</tbody>
</table>

\(^a\)Controls were injected with sodium warfarin in saline (10 mg/ml) with a dose of 1 mg/Kg i.v. into the jugular vein. Plasma levels of total and free warfarin were determined from samples drawn 60 minutes after dosing.

\(^b\)PBZ animals were treated similarly to controls except for PBZ infusion (25 mg/Kg i.v.) which preceded warfarin by 60 minutes.

\(^c\)Total plasma warfarin (mcg/ml).

\(^d\)Free (unbound) plasma warfarin (mcg/ml). Values have been corrected for warfarin adsorbance to ultrafiltration membrane.

\(^e\)Mean of four animals.

\(^f\)Significantly different from control (p<0.005) by Student's \(t\) test.

\(^g\)Values represent S.E.M.
Relationship between prothrombin complex activity and canine plasma coagulation time. Plasma was mixed 1:1 with Tris-HCl (0.02 M, pH 7.4) prior to dilution with saline.
mixtures increased with time (Figure 15). Additionally, the increase appeared linear for only the initial fifteen minutes of incubation and began to plateau at approximately 20 minutes. The treatment of rats with warfarin (0.25 mg/Kg i.p.) reduced the in-vitro synthesis rate of prothrombin complex activity, however, synthesis still appeared linear with time for the initial 15 minutes of incubation. The livers of those animals pretreated with phenylbutazone prior to warfarin administration synthesized prothrombin complex activity at a rate comparable to livers from saline-treated animals. This indicated that the chronic phenylbutazone pretreatment offset the effect of warfarin on the in-vitro synthesis of prothrombin complex activity.

Platelet aggregability in the dog. The influence of chronic phenylbutazone treatment upon platelet aggregability is represented by Figure 16. The aggregability of platelets measured in platelet-rich plasma was decreased after the administration of phenylbutazone (50 mg/Kg) twice daily for 5-7 days in three dogs. The decreased aggregability is evidenced as a decrement in the difference between the turbidity of EDTA and oxalate-containing platelet-rich plasma. Changes in optical density ranged from none (K-9 1313) to a 43% reduction for K-9 12990. Optical density changes were diminished by 33% and 40% for K-9's 13407 and 10306, respectively.

Effect of chronic phenylbutazone treatment upon plasma half-life of warfarin in the dog. The rate of elimination of warfarin from the plasma is observed to be increased after chronic
FIGURE 15

In-vitro prothrombin complex synthesis in rat livers derived from animals treated with saline or warfarin, or pretreated with phenylbutazone (PBZ) and then treated with warfarin. Vertical bars represent S.E.M.
FIGURE 16

Effect of phenylbutazone treatment (PBZ) upon platelet aggregability in the dog. Vertical bars represent S.E.M.

* = P<0.05 as per paired t test.
phenylbutazone treatment (Figures 17-20). Warfarin is eliminated from plasma by an apparent first-order process both in the phenylbutazone-pretreated and in the untreated dog. The plasma half-lives are listed in Table 6.

In-vitro warfarin metabolism by canine hepatic microsomes. Microsomes obtained from the livers of untreated male, mongrel dogs were found to metabolize warfarin. Furthermore, the extent of warfarin metabolism was found to be greater in microsomes derived from livers of phenylbutazone-treated dogs (Figure 21). The Michaelis constant and $V_{\text{max}}$ were obtained from Lineweaver-Burk plots. Microsomes obtained from untreated dogs metabolized warfarin with a $K_m$ of $1.8 \times 10^{-4}$ M and $V_{\text{max}}$ of $23.2$ nmol/mg protein/10 min., whereas microsomes obtained from phenylbutazone treated dogs metabolized warfarin with an apparent $K_m$ of $2.6 \times 10^{-4}$ M and $V_{\text{max}}$ of $58.5$ nmol/mg protein/10 min. (Figure 22).

As a complementary indication that phenylbutazone with chronic administration is an inducer of microsomal warfarin metabolism, the hemoprotein content of microsomes derived from the livers of phenylbutazone-treated dogs showed a marked increase over the hemoprotein content in microsomes derived from livers of naive animals. Cytochrome P-450 content rose from $0.366$ nmol/mg protein in the microsomes from untreated dogs to $0.770$ nmol/mg protein in microsomes from phenylbutazone-treated dogs (Figure 23). Microsomal protein rose from $21.7$ mg/gm liver in naive animals to $33.3$ mg/gm liver in phenylbutazone-treated dogs.
FIGURE 17

Warfarin plasma disappearance in K-9 10306 before and after chronic phenylbutazone (PBZ) treatment.
Warfarin plasma disappearance in K-9 13134 before and after chronic phenylbutazone (PBZ) treatment.
Warfarin plasma disappearance in K-9 12990 before and after chronic phenylbutazone (PBZ) treatment.
Warfarin plasma disappearance in K-9 13407 before and after chronic phenylbutazone (PBZ) treatment.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Control^b</th>
<th>Phenylbutazone^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1313\textsuperscript{4}</td>
<td>21.0</td>
<td>11.6</td>
</tr>
<tr>
<td>13\textsuperscript{4}07</td>
<td>13.0</td>
<td>6.1</td>
</tr>
<tr>
<td>12990</td>
<td>23.0</td>
<td>8.4</td>
</tr>
<tr>
<td>10306</td>
<td>16.5</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>18.4\textsuperscript{d}</td>
<td>7.8\textsuperscript{d,e}</td>
</tr>
<tr>
<td></td>
<td>2.3\textsuperscript{f}</td>
<td>1.4\textsuperscript{f}</td>
</tr>
</tbody>
</table>

*Half-life is expressed in hours.

^b Single oral dose of warfarin (0.8 mg/Kg).

^c Chronic phenylbutazone pretreatment followed by single oral dose of warfarin (0.8 mg/Kg).

^d Mean of four animals.

^e Significantly different from control (p<0.01) by Student's t test.

^f Values represent S.E.M.
FIGURE 21

Effect of phenylbutazone (PBZ) pretreatment on liver microsomal metabolism of warfarin ($2 \times 10^{-5}$M). Vertical bars represent S.E.M. * = $P < 0.05$ as per Student's $t$ test.
Effect of phenylbutazone (PBZ) pretreatment on canine hepatic microsomal metabolism of warfarin.
FIGURE 23

Effect of phenylbutazone (PBZ) pretreatment on canine hepatic microsomal P-450 content. Vertical bars represent S.E.M. * = P < 0.05.
CHAPTER IV
DISCUSSION

The ability to measure small quantities of plasma warfarin spectrofluorometrically was initially reported by Corn and Berberich (1967). In their report two interesting features were remarked upon. It was noted that acetone elicited an auxochromic influence on warfarin, greatly intensifying warfarin fluorescence. In addition, acidification of warfarin extracts with HCl resulted in the quenching of warfarin fluorescence. Neither the mechanism for acetone's auxochromic influence nor the warfarin fluorescence quenching effect by HCl are known. However, the latter phenomenon was noted to be irreversible (Corn and Berberich, 1967). An abridged list of drugs not interfering with warfarin fluorescence was also reported. Though phenylbutazone was not included, O'Reilly and Aggeler (1968) subsequently reported the lack of interference of phenylbutazone in the spectrofluorometric assay of warfarin by the method of Corn and Berberich (1967). This was in marked contrast to their earlier findings with a spectrophotometric assay for warfarin in which phenylbutazone severely interfered (O'Reilly et al., 1962). Interference of phenylbutazone as well as of warfarin metabolites is also obviated by the use of thin-layer chromatography. The procedure outlined by
Lewis et al. (1970) is reported specific for unchanged warfarin. The use of N,N-dimethylformamide as the solvent in which to read warfarin fluorescence arose primarily from the reported findings of Chignell (1970). Dimethylformamide was observed to increase the quantum fluorescence yield of warfarin nearly twelve-fold when compared with water. The reason for such an auxochromic effect by dimethylformamide is also unexplained. However, this solvent provides a useful tool for increasing the sensitivity of determinations of warfarin in plasma and other tissues.

The plasma half-life of warfarin in naive dogs was in good agreement with an earlier report by Nagashima and Levy (1969) in which the plasma half-life of warfarin was found to be 22-23 hours in dogs. The slightly higher half-life in those studies compared to the 18.4 hour half-life reported herein is well within the realm of variability anticipated with use of mongrel dogs.

It is noteworthy that the half-life of warfarin in the plasma of dogs treated with topical chlordane for ectoparasites was approximately one-third of that observed for non-chlordane treated animals; this, despite the marked temporal lag between chlordane treatment and warfarin administration (over 30 days). Much has been learned about the stimulatory effect of halogenated hydrocarbons upon hepatic oxidative drug metabolism since the phenomenon was initially investigated by Hart and Fouts (1965). The salient lessons include species non-specificity and longevity of the effect due to gradual release of the insecticides from fatty storage depots (Conney, 1971). Conney
and co-workers (1967) reported that antipyrine metabolism in the dog was enhanced for as long as four months after the discontinuance of chlordane treatment.

It would appear as though animals exposed only once to chlordane applied topically are unsuitable candidates for subsequent biochemical or pharmacokinetic studies inasmuch as chlordane so applied appears to effect a potent and long-lasting induction of hepatic drug metabolism. Animals so treated were omitted from warfarin-phenylbutazone interaction studies described herein. The apparent slow release of the insecticide from fatty storage depots provides an interesting dilemma for experimentalists. In those situations in which ectoparacidal application is necessary or warranted, one must ask what interval of time must be permitted to elapse before the metabolic effects of such compounds become negligible? The practical need for such determinations is self-evident.

A normal plasma prothrombin time of approximately seven seconds is also in good agreement with an earlier report (Quick and Hussey, 1951). That the prothrombin activity of dog blood is greater than in human blood is probably a function of the form in which prothrombin is circulated in man and dog. In the dog essentially all of the activity is readily available, whereas in man, a relatively high level circulates as inactive precursor (Quick and Hussey, 1951).

The dog appears particularly well suited as a model for the study of coumarin interactions. Evaluations of interactions between chloral hydrate and bishydroxycoumarin (Weiner, 1971), phenobarbital,
tolbutamide, or phenylbutazone and bishydroxycoumarin (Welch et al., 1969), and d-thyroxine and warfarin in the dog (Weintraub et al., 1973) have affirmed this notion. Furthermore, human platelet reactivity to exogenous stimulators of platelet aggregation is more faithfully reproduced by canine platelets than rat, pig, or rabbit platelets (Macmillan and Sim, 1970).

The role of phenylbutazone in the alterations of pharmacologic responses to anticoagulants is at least tripartite. While devoid of any direct effects upon the normal formation and degradation of vitamin K-dependent clotting factors (Brodie et al., 1954) phenylbutazone undoubtedly alters the physiologic disposition of warfarin. Thus, phenylbutazone is capable, in single doses, of elevating the free fraction of warfarin in the dog. Single doses of phenylbutazone administered with single doses of warfarin also reduced the plasma concentrations of total warfarin necessary to effect a given hypoprothrombinemic response. This was observed as a distinct shift of the plasma concentration-response curve. Such shifts represent presumptive evidence for changes in warfarin distribution as a function of phenylbutazone coadministration, and influences of phenylbutazone upon the metabolism of warfarin are precluded from obscuring interpretations since alterations in response and plasma levels of the drug are considered only in terms of one against the other, and not with time. That metabolic changes imposed by one drug upon another do not complicate or contribute to the relationship between plasma concentration and response is demonstrated clearly by Levy et al.
(1970) for heptabarbital and warfarin.

The shift of the warfarin plasma concentration-response curve was also reported in man after a chronic pretreatment regimen of phenylbutazone (O'Reilly and Levy, 1970). Expectedly, the shift in slope of the curve in subjects receiving phenylbutazone was considerably more dramatic than in the dog receiving a single dose of phenylbutazone. In the former instance, a 72 hour half-life of phenylbutazone and multiple daily doses served to maintain phenylbutazone at high (and presumably constant) plasma levels. As discussed by Meyer and Guttman (1968), a constant plasma level of binding inhibitor will elicit an especially significant influence on the fraction of free drug at lower plasma concentrations of the binding substrate. Thus, as plasma levels of warfarin decline and those of phenylbutazone remain constant, the influence of phenylbutazone upon the free fraction of warfarin becomes more marked, and this is reflected in the slope of the warfarin (total) plasma concentration-response curve. After a single dose of phenylbutazone and warfarin administered together is given to the dog, however, phenylbutazone also disappears at a rapid rate (6 hour half-life) from plasma. Thus, binding inhibition at lower plasma levels of warfarin will not be nearly so remarkable as when high concentrations of phenylbutazone are maintained in the plasma. Consequently the decrease in slope of warfarin plasma concentration-response curves will appear less drastic. If, on the other hand, high concentrations of phenylbutazone are maintained in the plasma by multiple dosing, a much more dramatic decrease in the
slope can be effected. (See Figure 13.)

The potentiated response to warfarin associated with phenylbutazone administration was accompanied by a two-fold decrease in the plasma half-life of warfarin. The dissociation of drug from plasma protein-binding sites permits the presentation of higher concentrations of drug substrate both to hepatic metabolizing enzymes and to the glomerulus for filtration, the net result being an enhanced rate of elimination of drug from plasma. Woslait and Eisenbrandt (1972) have already demonstrated that oxyphenbutazone (75 mg/Kg) administered one-hour after warfarin in the rat markedly increases the excretion of warfarin in the bile.

By administering a single dose of phenylbutazone, the change in the rate of warfarin elimination more closely approaches an expression of the exclusive effect of phenylbutazone upon warfarin distribution and precludes, for the most part, its effect upon the induction of hepatic microsomal enzymes for which warfarin is a substrate. To the extent that this may be true, it becomes more evident that the decrease in plasma half-life of warfarin occurring after the combined administration of warfarin and phenylbutazone in a single dose is a reflection of an alteration in the availability of the filtrable fraction of the drug through protein-binding inhibition rather than a reflection of enzyme induction. Under these circumstances, then, the shift of warfarin plasma concentration-response curves to the left and the decrease in warfarin plasma half-life which accompanies such changes in pharmacologic responses, are most
likely attributable to the protein-binding inhibiting phenomenon. Strongly supportive evidence for this interpretation is found in the demonstration of elevated unbound warfarin levels in the dog after the administration of phenylbutazone. Interestingly, total plasma levels declined very little after phenylbutazone administration ($p > 0.05$) from 6.75 to 6.23 mcg/ml even though free levels nearly tripled. This observation could explain the notion that drugs such as clofibrate mediate potentions of anticoagulant responses by mechanisms other than protein-binding displacement. For example, O'Reilly et al. (1972) have suggested that the clofibrate-induced potentiation of the hypoprothrombinemic response to warfarin occurs mainly at the level of the biologic effect of warfarin through the enhancement of the degradation of prothrombin complex activity. That the potentiation might arise in part from changes in warfarin distribution is ruled out on the basis that no significant changes occurred in the plasma levels of (total) warfarin during clofibrate coadministration, although a slight decrease in the levels of warfarin was recorded. It is, however, conceivable that significant but unmeasured increases in the free fraction of warfarin accompanied the coadministration of warfarin as they did phenylbutazone.

Thus phenylbutazone possesses the ability to apparently enhance the response to warfarin simply by increasing the availability of the active fraction in the dog when administered in single doses with warfarin.

The chronic pretreatment of dogs with phenylbutazone results not
only in the induction of phenylbutazone-metabolizing enzymes (Burns et al., 1965), but in the induction of enzymes which metabolize warfarin as well. Phenylbutazone has a half-life in the dog of only six hours (Burns et al., 1965). By delaying the administration of warfarin to phenylbutazone pretreated animals for one week following the final dose of the pretreatment regimen, it becomes possible to examine the effects of phenylbutazone specifically with regard to warfarin's metabolic fate. Thus, at the time of warfarin administration phenylbutazone should no longer be present in the plasma, and alterations of warfarin disappearance must evolve from the long-lived effects of phenylbutazone upon warfarin hepatic metabolism. The enhanced rate of warfarin disappearance under these circumstances is ascribed to enzyme induction by phenylbutazone in the dog. Phenylbutazone given chronically has also been shown to maintain bishydroxycoumarin at exceedingly low plasma concentrations in the dog (Welch et al., 1969).

To corroborate the notion that phenylbutazone could enhance warfarin disappearance rates not only by displacing it from protein-binding sites but also by inducing its metabolism, the in-vitro metabolism of warfarin was examined in livers of dogs pretreated with phenylbutazone or left untreated. Again, evidence that phenylbutazone is indeed a stimulator of hepatic microsomal enzymes which metabolize warfarin was forthcoming. Not only did the $V_{\text{max}}$ increase for microsomes derived from pretreated animals, but the microsomal hemoprotein—cytochrome P-450—content markedly rose after pretreatment as did the amount of microsomal protein. Values for microsomal protein were in
excellent agreement with those reported by Conney and Schneidman (1964) in which control dogs exhibited 22 mg microsomal protein per gram of liver and phenylbutazone treated dogs exhibited 27 mg/gram. Not only is it apparent that phenylbutazone is capable of enhancing warfarin elimination by two independent mechanisms; it is also apparent that phenylbutazone does not serve as an inhibitor of the microsomal metabolism of warfarin (at least when given chronically) as suggested by Weiner et al. (1965). As has already been suggested (Aggeler et al., 1967), the decrease in disappearance rate of bishydroxycoumarin subsequent to oxyphenbutazone administration reported by Weiner et al. (1965) may have artifactually arisen from failure to correct for the interference of oxyphenbutazone in the assay procedure. Without such a correction, apparent warfarin levels are extremely higher than when no pyrazolone is present in the sample (Aggeler et al., 1967). However, the notion that acute administration of phenylbutazone might initially inhibit the metabolic oxidation of warfarin cannot be dismissed altogether. Cho et al. (1970) have demonstrated that a single dose of phenylbutazone (100 mg/Kg i.p.) administered to rats can inhibit the in-vitro ethylmorphine demethylase activity in microsomes from those rats for as long as 12 hours. By 24 hours after phenylbutazone administration however, an inversion of the inhibitory effect is seen, and the rate of ethylmorphine demethylation is enhanced. Vessel et al. (1972) have shown a triphasic action of GPA 1851—a congener of phenylbutazone—upon rat hepatic aniline hydroxylase and ethylmorphine N-demethylase activity in-vitro. Two hours after oral
GPA 1851 administration microsomal aniline hydroxylase and ethylmorphine N-demethylase activities were reduced. Twenty-four hours after oral GPA 1851 the activities and cytochrome P-450 were enhanced. But, after a four day GPA 1851 regimen, though the hemoprotein remained elevated, the hydroxylase and demethylase activities approached control values.

From the results of their studies on the influence of phenylbutazone upon aminopyrine plasma levels in man Chen et al. (1962) have also concluded that phenylbutazone administered acutely might block the metabolism of aminopyrine, but upon chronic administration (7-11 days) it stimulates aminopyrine metabolism. Even if, however, phenylbutazone administered acutely were shown to initially inhibit hepatic microsomal warfarin metabolism, it is apparent that quantitatively, the influence of such an inhibition would be insignificant inasmuch as the plasma half-life of the drug under just those circumstances is diminished as a consequence of protein-binding displacement.

Additional evidence for the ability of chronic phenylbutazone treatment to stimulate the activity of liver microsomal enzymes derives from the findings of Davies and Thorgeirsson (1971). These studies revealed that five of eight subjects treated for 5 days with only 5 mg/Kg of phenylbutazone had shorter phenylbutazone plasma half-lives than when receiving only a single 10 mg/Kg acute dose of phenylbutazone.

The pharmacologic consequences of an enhanced warfarin metabolism should be in direct contrast to those associated with binding...
inhibition. One might anticipate that as a result of decreased availability of warfarin a less pronounced inhibition of prothrombin complex synthesis might occur. The in-vitro prothrombin complex synthesis occurring in liver obtained from warfarin-treated rats was lower than that occurring in livers obtained from saline-treated rats, suggesting that warfarin does, in fact, inhibit the rate of synthesis of prothrombin complex in the liver. It would be desirable to extend these observations over a range of warfarin doses. Rats subjected to chronic phenylbutazone pretreatment prior to warfarin treatment did not synthesize prothrombin activity in-vitro at a slower rate than the control rate indicating that warfarin levels attained in the phenylbutazone pretreated rats were insufficient to inhibit the in-vitro prothrombin complex synthesis rate. Though the consequences of long-term phenylbutazone administration on the in-vivo pharmacologic response to warfarin in the dog were not evaluated, it already is apparent that such chronic phenylbutazone treatment markedly diminishes the pharmacological activity ofbishydroxycoumarin in the dog (Welch et al., 1969).

That single doses of phenylbutazone can enhance the response to a single dose of warfarin is clear. However, it is also apparent that the induction of its hepatic microsomal metabolism can be brought about through chronic phenylbutazone treatment. While the influences of phenylbutazone upon warfarin-evoked responses might be presumed to be mutually antagonistic—at least if both drugs are chronically coadministered—one is still confronted with clinical reports of apparent potentiation of coumarin responses associated with pyrazolone treatment.
This may simply be a manifestation of the predominance of the protein-binding displacement effect of phenylbutazone over the metabolic inductive effects, or inadequate length of pretreatment for significant inductive effects to have developed. Davies and Thorgeirsson (1971) suggest that in man, at least 5 days of phenylbutazone pretreatment is necessary for enzyme induction. It may also arise through a direct pharmacologic activity of phenylbutazone independent of alterations of the physiologic disposition of warfarin. In three of four dogs treated for 5-7 days with phenylbutazone, platelet aggregability was depressed. The compromising of both primary hemostasis and blood coagulation (the latter mediated by the anticoagulant) might be sufficient to account for the kinds of hemorrhagic diatheses attributed to the combined use of the two classes of drugs.

Platelet aggregation induced by ADP requires ionized calcium and platelet contiguity. Born and Cross (1963) demonstrated that increased rates of stirring of platelet-rich plasma (PRP) resulted in more rapid and complete aggregation. Inceman and Tangun (1969) demonstrated that centrifugation provided sufficient platelet propinquity to release platelet factor 3, the release of which accompanies platelet aggregation (Hardisty and Hutton, 1966). Further evidence suggesting that the platelet release reaction could be induced by centrifuging platelets was provided by Massini and Luscher (1971) who concluded that contact between platelets is sufficient stimulus for the initiation of the platelet release reaction and viscous metamorphosis of platelets leading to the formation of irreversible platelet aggregates.
Holdrinet et al. (1969) capitalized upon such findings in the development of a simple test for measuring aggregation. Noting that platelets in plasma centrifuged at slow speeds do not sediment with erythrocytes, leukocytes, or platelet aggregates they suggested and provided evidence for the following: The simple addition of an aggregating agent to plasma containing adequate ionized calcium for aggregation would be sufficient to produce aggregation if cell contact could be induced by centrifugation. Under these circumstances the aggregates sedimented leaving the PRP very low in platelet concentration. Addition of an aggregating agent without induction of cell contact will not give rise to the release reaction of aggregation (Massini and Luscher, 1971). If, however, calcium is present in insufficient concentrations to permit aggregation, centrifugation-induced cell contact in the absence of an aggregating agent will not induce aggregation and the resulting PRP will contain a high concentration of platelets. The difference, then, in the platelet concentration of PRP prepared in calcium-poor plasma and in calcium-available plasma to which an aggregating agent is added serves as a measure of platelet aggregability (Holdrinet et al., 1969).

In the foregoing studies collagen, rather than ADP was used as the aggregating agent. Pyrazolone compounds, while capable of blocking the aggregating action of collagen on blood platelets do not block the action of ADP (Packham et al., 1967). That is, pyrazolones are capable of blocking surface-induced platelet aggregation.

That inhibition of aggregation occurred only in three of four
animals tested with phenylbutazone may be attributed to the concentration of collagen necessary for the stimulation of aggregation. Initial attempts to demonstrate the inhibition of thrombin-induced aggregation by phenylbutazone were unsuccessful (Packham and Mustard, 1969). The effect could only be shown when minimum concentrations of thrombin were used (Packham and Mustard, 1969). Furthermore, a considerable variability of intrinsic reactivity of platelets to collagen has been demonstrated (Herrmann et al., 1972). Since the intensity of the collagen challenge conditions the extent of drug induced inhibition and the reactivity of platelets to collagen can differ from PRP to PRP, it is not unreasonable to presume that the possible inhibitory activity of phenylbutazone in one animal may have been obscured by exposing those platelets to a profound collagen challenge in relative terms.

The inability to demonstrate the inhibition of platelet aggregation in one animal notwithstanding, the results in the remaining animals tested suggested that the oral, in-vivo administration of phenylbutazone retains the potential for the inhibition of platelet aggregation in the dog. These findings are in agreement with others which indicate that pyrazolones might inhibit platelet aggregation (Mustard and Packham, 1970). The consequences of such an inhibition would tend to shift the balance of hemostatic processes in favor of hemorrhage. The superimposition of an impaired coagulation process by anticoagulation could then lead to hemorrhagic diatheses.
CHAPTER V

SUMMARY AND CONCLUSION

Apart from observations bearing directly upon the influences of phenylbutazone upon the expected responses to warfarin, it is evident that even a single topical application of chlordane to dogs provides sufficient exposure to induce the metabolism of warfarin even after a 1-2 month interval between chlordane exposure and warfarin dosing. It is therefore advised that treatment of dogs for ectoparasites with chlordane be avoided for experiments designed to evaluate warfarin hepatic metabolism in the dog. It is further suggested that the effects of topical exposure to chlordane be investigated such that a temporal profile of the extent of metabolic alterations might be constructed.

Coadministration of phenylbutazone (50 mg/Kg) in a single oral dose with warfarin (0.8 mg/Kg) potentiates the hypoprothrombinemic response evidenced as a shift-to-the-left of the plasma warfarin concentration-response curve. These findings are consistent with the phenomenon of protein-binding displacement and are supported by the additional findings: 1) a diminution of the warfarin plasma half-life after a single coadministered dose of phenylbutazone; and 2) a remarkable displacement and decrement in slope of the warfarin plasma concentration-response curve during the maintenance of higher
phenylbutazone plasma levels by multiple dosings. Furthermore, the administration of phenylbutazone (25 mg/Kg i.v.) just prior to warfarin (1 mg/Kg i.v.) resulted in a three-fold increase in the free fraction of warfarin in plasma.

Phenylbutazone pretreatment for a week, the final dose administered one week prior to the administration of warfarin, enhanced the warfarin plasma disappearance rate. Additionally, microsomes derived from phenylbutazone-treated dogs metabolized warfarin at faster rates than microsomes derived from naive animals. The small change in $K_m$ (1.8 to $2.6 \times 10^{-4}$M) relative to the large change in $V_{\text{max}}$ (23 to 58 nmol/mg protein/10 min) is suggestive of an increased quantity rather than a change in the nature of warfarin metabolizing enzymes. Cytochrome P-450 content was also elevated in microsomes isolated from phenylbutazone-treated dogs. The warfarin effected inhibition of in-vitro prothrombin complex synthesis by rat liver was reversed by pretreatment of rats with phenylbutazone. Thus, it appears that phenylbutazone administered chronically (5-7 days) induces the hepatic microsomal enzymes for which warfarin is a substrate in the dog and rat.

Pretreatment of dogs with phenylbutazone may inhibit collagen-induced platelet aggregation. It is therefore likely that hemorrhagic diatheses associated with the combined administration of phenylbutazone and coumarin anticoagulants might arise, in part, from the inhibition of primary hemostasis superimposed upon a compromised coagulation process.
BIBLIOGRAPHY


________ and Kipnes, R. S.: Vitamin K dependent formation of Factor VII by a cell-free system from rat liver. Biochem. 2: 2564-2569, 1970.


Gershbein, L.L.: Effect of various agents on liver regeneration and


and Peterson, J.: Inhibitor of adenosine diphosphate-induced secondary aggregation and other platelet functions by acetyl-
551, 1968.

and ______: Effect of acetylsalicylic acid, other non-