ASPECTS OF ANTICOAGULANT ACTION:
A Review of the Pharmacology, Metabolism and Toxicology of Warfarin and Congeners

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SUMMARY

Warfarin is the most widely used anticoagulant in the treatment of thromboembolism in man. It has also been used extensively as a rodenticidal agent. Insofar as its clinical use is concerned, it is now clear that many of the drug interactions observed in patients are mediated via metabolic or pharmacokinetic factors. An understanding of the disposition of warfarin is therefore essential if one is to predict the likely response in patients undergoing anticoagulant therapy with this compound. Warfarin-resistance has been reported in both man and rodents. Understanding resistance in both man and rodents is important for effective anticoagulant therapy, and in control of resistant strains of rodents. Warfarin resistance in rat strains does not appear to have a metabolic or pharmacokinetic basis; in this species, resistance is thought to be due to differences in permeability to, or affinity for a receptor. Apart from its clinical and rodenticidal uses, warfarin is an excellent substrate for probing the heterogeneity of cytochrome P.450, since its metabolic oxidation is mediated by this mixed function oxidase. This review draws together much of the current published literature on the pharmacology, metabolism and toxicology of warfarin and related congeners.

I. INTRODUCTION

Warfarin and other oral anticoagulants have been widely studied due to their therapeutic effectiveness in the treatment of thromboembolism and as rodenticidal agents. In many cases, patients undergoing anticoagulant therapy are either under or overtreated with warfarin, due to drug interactions or inherent differences in receptor sensitivity to the drug, resulting in serious complications /1/. Because of this problem, an understanding of the metabolism and pharmacokinetics of warfarin is essential in order to predict the response of each patient to a particular warfarin dosage regimen.

The oxidative metabolism of warfarin to multiple products is catalysed by cytochrome P-450 /2/. Studies of the regio- and stereoselective metabolism of warfarin have proved useful in probing the heterogeneity of cytochrome P-450, since it has been suggested that the functional multiplicity of cytochrome P-450's can account for the

metabolic differences occurring between animal species, genetic strains and organs.

Changes in the metabolism and pharmacokinetics of warfarin have also been studied in an attempt to elucidate the cause of warfarin resistance at the biochemical level. Comparing the absorption, distribution, metabolism and excretion of warfarin in warfarin-sensitive and warfarin-resistant rat strains, showed no differences between the strains. In this species, warfarin resistance is thought to be due to differences in permeability to, or affinity for a receptor /3,4,5/. The biochemical manifestation of warfarin resistance in the mouse has not been elucidated.

Thus, aspects of the pharmacology, metabolism and toxicology (with respect to anticoagulant resistance) of warfarin and related anticoagulants will be discussed in this review.

II. PHARMACOLOGY AND TOXICOLOGY OF WARFARIN AND RELATED ANTICOAGULANTS

2.1 Synthesis of vitamin K-dependent clotting factors and the vitamin K cycle

Vitamin K has various biological functions, but the most important with respect to blood coagulation is the post-translational modification of precursor proteins to form clotting factors /6,7/. This involves the γ-carboxylation of glutamic acid (GLU) which is transformed to γ-carboxyglutamic acid (GLA) in the presence of vitamin K and molecular oxygen. The biotransformation is important because it enhances binding of Ca++ by the clotting factors, which is essential for the concentration of blood clotting activity at the surface of platelet phospholipid micelles. GLA residues on the clotting factors enhance the binding of Ca++ by chelating phosphate on the phospholipid, thus accelerating, and providing a template for, the blood clotting mechanism /8/.

The vitamin K-dependent clotting factors which have been fully characterised are factors II (prothrombin), VII, IX and X, and are synthesised in the liver. Proteins containing GLA are present in many tissues, such as the kidney /9/, spleen /10/, lung /11/, pancreas /12/, placenta /13/ and bone /14/. These proteins are typified by osteocalcin, isolated from bone, which contains four GLA residues /15/, and binds

two Ca++ ions per molecule. The protein is carboxylated in the same manner as prothrombin. All of these GLA-containing proteins are vitamin K-dependent and administration of oral anticoagulants will have an effect on them. Responses due to extrahepatic vitamin K antagonism must therefore be considered when evaluating anticoagulant therapy and rodenticidal actions.

Concomitant with the γ-carboxylation of glutamic acid residues to form clotting factors, an epoxidation reaction occurs, converting the active form of vitamin K, the hydroquinone, to vitamin K 2,3-epoxide /16/. The epoxide is subsequently converted to the quinone form of vitamin K, forming the vitamin K cycle (Fig. 1). Both the metabolism of vitamin K and the γ-carboxylation of GLU occur on the rough endoplasmic reticulum of the liver. The epoxidation and carboxylation reactions of the vitamin K cycle are linked /17/, both processes

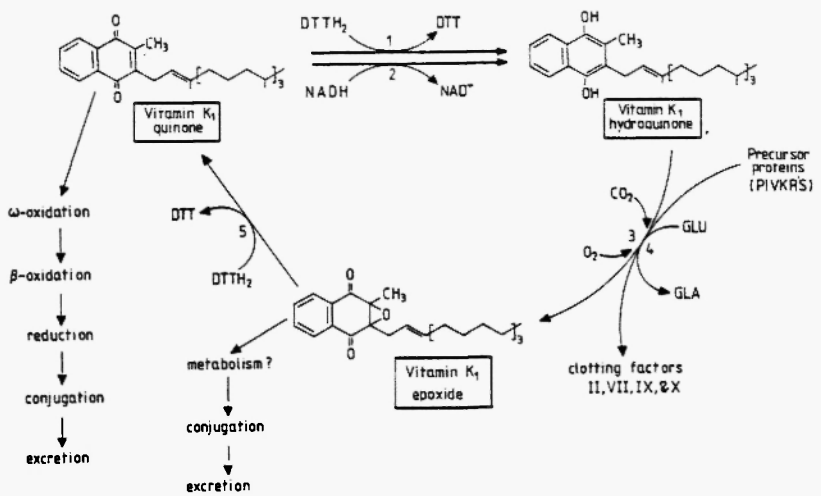

Fig. 1: The vitamin K_1 cycle
Abbreviations are:
GLU, glutamic acid
GLA, γ-carboxy glutamic acid
$DTTH_2$, dithiothreitol
1, vitamin K_1 reductase
2, dehydrogenase (NAD(P)H reductase)
3, epoxidase
4, carboxylase
5, vitamin K_1 epoxide reductase

occurring via a common intermediate, thought to be a hydroperoxide of vitamin K /18/. Although linked, the epoxidation reaction can occur, without the carboxylation reaction taking place, however, the reverse is not true. Vitamin K 2,3-epoxide may exist as two enantiomers, the enzyme vitamin K epoxide reductase is 15% stereoselective for the R(+)-enantiomer, and converts the epoxide to the more stable quinone form of the vitamin K. The physiologically predominant isomer of vitamin K 2,3-epoxide has been tentatively assigned the (2R, 3S) configuration /18/.

It is therefore clear that the formation of vitamin K-dependent clotting factors relies on the normal functioning of the vitamin K cycle. Interference with any part of the system will give abnormal clotting factor formation or function, thus causing incoagulable blood.

2.2 Antagonism of the physiological role of vitamin K by anticoagulants

The first anticoagulant, subsequently identified as dicoumarol, was discovered in the mid-1920's, and associated with the development of a haemorrhagic disease in cattle due to eating mouldy sweet clover silage. Dicourmarol (3,3-methylene-bis (4-hydroxycoumarin)) (see Fig. 2), was isolated by Link /19/ and used subsequently for the treatment of thromboembolic disorders. In the 1930's, Dam and Doisy /20,21/ discovered the link between vitamin K deficiency, decreased blood clotting activity and hypoprothrombinaemia. Hypoprothrombinaemia was at first thought to be solely due to a decrease in clotting factor II (prothrombin), but is much more complex, involving the depression of three of the vitamin K-dependent clotting factors II, VII and X /22/ normally measured by a bioassay – "the one-stage prothrombin time".

Since these early studies, research has led to the development of a large group of drugs, the oral anticoagulants. Their pharmacological properties allow their use as therapeutic agents in thromboembolic disorders, and also as rodenticides. The oral anticoagulants may be divided into four main groups on the basis of their chemical structures. These are:

i) compounds with two coumarin rings, the "dicoumarols",
ii) 3-substituted 4-hydroxycoumarins, the "mono-coumarols",
iii) cyclic acetals, the "cyclocoumarols",
iv) indandiones.

The indandiones are not considered in this review. The anticoagulant

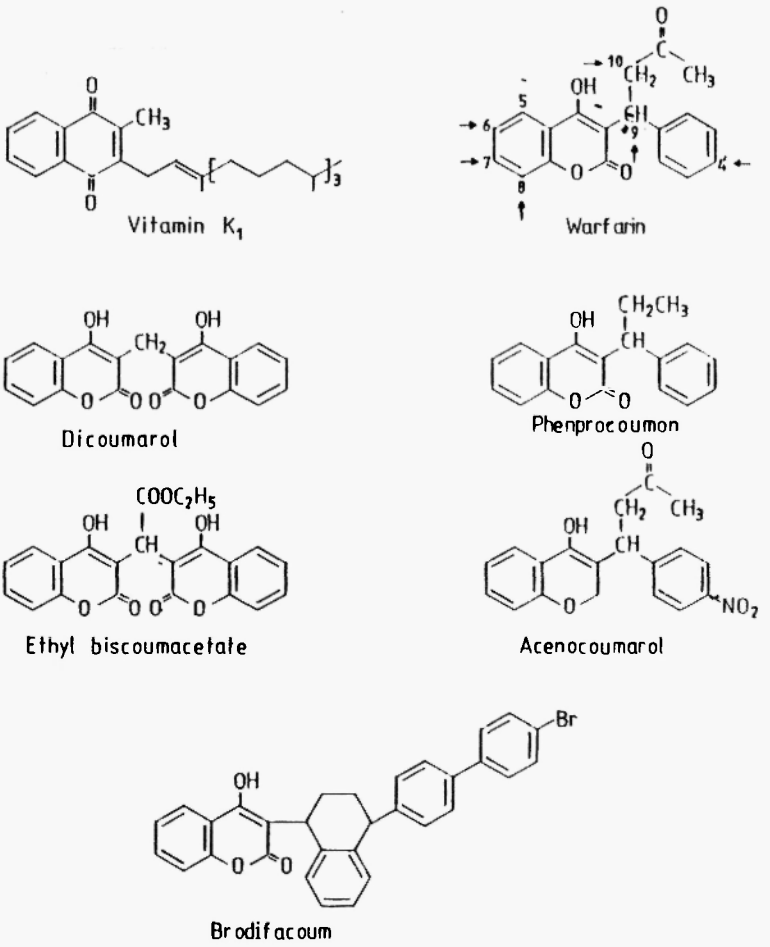

Fig. 2: Structural comparison of warfarin and related anticoagulants with vitamin K_1 *, chiral centre
→, site of cytochrome P-450-dependent hydroxylation

activity of the coumarins is thought to be governed, not by individual structural features, but by a combination of several including, molecular shape, increased activity with six-membered heterocyclic rings with a substituent in position 8 and with a methoxy rather than a free hydroxyl group /23/ (Fig. 2).

Warfarin inhibits the synthesis of activity of the individual vitamin K-dependent clotting factors II, VII, IX and X, and the degenerative

half-lives of these clotting factors can be related to plasma levels of warfarin. The half-life of each of the clotting factors is different as indicated below.

	Man /24/	Rat /25/
II =	72-96 hours	9.0 hours
VII =	4-6 hours	–
IX =	20 hours	4.7 hours
X =	48-70 hours	5.9 hours

Early work showed that the release of clotting factor VII by vitamin K in liver slices from vitamin K-deficient rats was inhibited by coumarin anticoagulants /26/, demonstrating that coumarins antagonise the physiological role of vitamin K. Although clotting factor synthesis is reduced on anticoagulant administration, there is no evidence to suggest inhibition of clotting factor release, or any increased rate of utilization or degradation /27/.

In the absence of vitamin K, or an anticoagulant administration, proteins named PIVKA's (proteins induced by vitamin K antagonism) accumulate in many mammals. These are immunologically identical to clotting factors but lack their activity /28/ due to the fact that the final γ-carboxylation of GLU has not occurred. Thus, the PIVKA's have fewer GLA residues than are normally present in the clotting factor. For example, factor II (prothrombin) has 10 GLA residues but its PIVKA, PIVKA II has less. The conversion of PIVKA II into clotting factor II is thought to be controlled by a carboxylating enzyme system unique in that it does not require biotin /29/. PIVKA's are therefore the precursor proteins previously described which are carboxylated to give clotting factors. The enzyme(s) responsible has (have) been partially purified /30/ and called factor II synthetase, and is thought to be similar to carboxylase isolated from vitamin K-deficient rat livers. Further purification of this enzyme system is needed before its mechanism of action can be fully elucidated.

PIVKA's have been purified from the plasmas of patients on chronic oral anticoagulant therapy. After gel filtration, all of the normal prothrombin was bound to phospholipid, whereas only half the coumarin-induced prothrombin (PIVKA II), was bound /31/. Furthermore, a wide spectrum of abnormal prothrombins with varying quantities of GLA per mol of zymogen is suggested. Levy and Lian /32/

measured the urinary excretion of GLA in warfarin-anticoagulated patients. Urinary GLA was decreased in patients on warfarin therapy, and the urinary GLA level correlated with the plasma prothrombin time. Therefore urinary GLA excretion may be clinically useful to monitor coagulation status and in general the vitamin K status of the patient undergoing warfarin therapy.

In the rat, ribosomes isolated from liver microsomes were found to bind warfarin, the *in vitro* warfarin-binding site is localised in microsomal membranes /33/. This binding is essentially irreversible, although determination of the exact nature and localization of the warfarin-binding proteins await their isolation /33/. Another consequence of vitamin K antagonism by anticoagulants is the depletion of liver concentrations of the vitamin. This is thought due to the fact that vitamin K_1 is metabolised by two different routes:

i) in the mitochrondrion by sidechain reactions (fast) and
ii) via the vitamin K cycle in the endoplasmic reticulum (slow) as described in reference 34.

The latter conservative process maintains a small pool of vitamin K_1 in the liver. On anticoagulant administration the vitamin K cycle no longer functions, but the mitochrondrial metabolism continues, resulting in a depletion of vitamin K.

It was therefore suggested that an altered vitamin K_1 metabolism may result from the action of oral anticoagulants. Caldwell *et al.* /35/ showed that warfarin did not increase the excretion of radioactive metabolites of [6,7 ^{3}H] vitamin K_1 and [phytyl ^{14}C] vitamin K_1. However, in both humans and rats, anticoagulants cause a relative increase in vitamin K_1-epoxide and metabolites of the vitamin more polar than the epoxide. The involvement of inhibition of the vitamin K cycle in the mechanism of action of warfarin therefore was suggested. Following therapeutic anticoagulation with warfarin, the pattern of urinary excretion of vitamin K_1 conjugates is changed and the excretion of normal hydroxylated aglycones of the vitamin is blocked /36/. These hydroxylated metabolites found in warfarin-treated subjects are consistent with the hypothesis that oral anticoagulants may interfere with the synthesis of vitamin K-dependent clotting factors by inhibiting the regeneration of vitamin K_1 from its 2,3 epoxide.

Thus, the effects of various coumarins on prothrombin clotting activity (PCA) synthesis, and the conversion of vitamin K-epoxide to vitamin K quinone were measured. There is a good correlation in

rabbits between the inhibition of prothrombin synthesis and of the epoxide to K_1 conversion both *in vivo* and *in vitro* /37/, contrary to the findings of Caldwell *et al.*/35/. Other studies have shown that there is an excellent correlation between plasma dicoumarol concentration and the inhibition of PCA synthesis rates in the rat and in man /38/. The much more rapid reduction of PCA synthesis in the rat as compared to man is consistent with the known differences in the half-lives of the vitamin K-dependent clotting factors of the two species.

The general hypothesis that warfarin exerts its effect on clotting factor synthesis by inhibition of the enzyme vitamin K-epoxide reductase is well supported /36-39/. In addition to its action here, it is suggested that there is a warfarin-sensitive, dithiothreitol (DTT) and NADH-dependent reduction of vitamin K_1 occurring in microsomal preparations /40-43/. However, inhibition of the vitamin K-epoxide reductase is thought to be the biologically important site of action of warfarin.

As has been stated previously, the mode of antagonism of the vitamin K-epoxide reductase is unclear. However, Olson /44/ has proposed that warfarin allosterically alters a vitamin K_1 receptor protein, such that its affinity for vitamin K is reduced and the affinity for warfarin is decreased to a greater extent. It has also been suggested that the coumarins are mechanism-based suicide inactivators of vitamin K-epoxide reductase /45,46/. The anticoagulant is converted by the epoxide reductase to hypothetically active diketo forms by tautomerisation. These active molecules then undergo attack by active-site nucleophiles, which become acylated, and thereby inactivate the epoxide reductase.

2.3 Sex and species differences in anticoagulant effect

A major problem in the clear definition of the pharmacological properties of oral anticoagulants is a marked variability in responsiveness between species, between individuals and in the same individuals under differing conditions. Factors such as sex and age can also be important in the variations. Due to these differences, considerable caution is needed in extrapolation of effects in one or even several types of animals to man /47/.

Marked sex differences were shown in the anticoagulant effect of warfarin in rats. The LD_{50} values were three times greater in males than

in females after single intraperitoneal dosing /48/. The response in the rat is modified by two sex-dependent differences: i) higher plasma levels of prothrombin complex clotting factors and faster restoration of these after administration of coumarins, in females compared to males, ii) slower metabolism of coumarins in females than in males. However, levels of the circulating anticoagulant vary between individuals. In addition, it has been amply demonstrated in dogs that the level of factor VII may be greatly decreased without any sign of haemorrhages /49/. The increased sensitivity of male rats is due to a true sex difference under hormonal control. Similarly, in mice, sex differences in anticoagulant response have been shown, females being more resistant to the effects of dicoumarol and warfarin. This sex difference may be explained by the fact that both female mice and rats are considered to be less susceptible to vitamin K_1 deficiency than males /50/. Male mice became more susceptible to warfarin poisoning with maturity, but the reverse situation is seen in the rat, where females became more resistant with age. A marked sex difference in susceptibility to warfarin was found in 6 month old LAC-grey mice, whereas no such difference was evident in 6 week old mice /51/.

Species differences in anticoagulant response to warfarin were shown by Shah and Suttie /52/, who reported that the hamster is much less susceptible to warfarin than the rat. In fact, the vitamin K-epoxide reductase from the hamster was 2-4 times less sensitive to warfarin, compared to the rat /53/. Vitamin K-epoxide reductase activities in the rat and hamster were similar without warfarin but the rat preparation was significantly more susceptible to the anticoagulant. This increase in resistance in the hamster relative to the rat was concluded to be due in part to an epoxide reductase less sensitive to warfarin.

In man and the rabbit, warfarin apparently acts by inhibiting the vitamin K-epoxide reductase as in the rat and hamster. The differences in the anticoagulant effect of warfarin may be compared in these species. In the rat, 1 mg/kg of warfarin blocked PCA synthesis completely over eight hours, whereas ten times this dose was needed to inhibit PCA synthesis by 80% in the hamster /54/. In the rabbit, maximum inhibition of PCA synthesis occurred at 63 mg/kg, however, this may not be the lowest dose needed to give 100% inhibition /55/.

Therefore, differences in the anticoagulant effect of one dose of an anticoagulant occur amongst species and in rare instances marked sensitivity or resistance to oral anticoagulants is evident and may be

related to genetic factors. The mechanisms of such resistance in man, rat and mouse are discussed later.

III. PHARMACOKINETICS AND METABOLISM OF WARFARIN AND CONGENERS

3.1 Absorption

One of the most important assumptions made in the use of mathematical models is that the pharmacological effect is a function of the amount of drug in the body at that time. However, sometimes the pharmacological effect is delayed, as is the case for warfarin, where the pharmacological response does not appear until 2-4 days after drug administration, which represents the half life of decay of the clotting factors.

The systemic availability of a drug depends upon the degree of absorption of that drug into the blood stream and the absorption of drugs may be affected by solubilizing effect of intestinal fluids, gastrointestinal tract (G.I.T.) motility, gut wall metabolism, pH and the anatomical site of absorption. The percentage absorption of warfarin and anticoagulants may be found by comparison of the relative areas under the plasma-time curves after oral and intravenous dosing /56/. For 100% absorption these areas are identical. Both warfarin and sodium warfarin are well absorbed from the gut /57/, and subsequent to oral or intravenous administration no difference in the time of onset of pharmacological effect is observed when prothrombin response is measured /58/.

The oral anticoagulants are weak organic acids, warfarin having a molecular weight of 308 and a pKa of 5.05 /57/. The absorption of many drugs follows the pH-partition theory where the degree of ionization of an organic electrolyte in solution appears to be one of the most important factors determining its absorption. In the stomach and rectum unionized warfarin alone is absorbed, in agreement with the pH-partition theory. However, in the duodenum at pH 8, absorption of warfarin is rapid, peak plasma concentrations occuring within 10 minutes in the rat /59/. Since warfarin is virtually totally ionized at this pH, absorption appears to occur in the dissociated form. Absorption of drugs in their dissociated forms has been documented previously, although this process is contrary to the usually accepted pH-partition theory. Kakemi *et al.* /60/ showed that in general both ionized and

unionized drugs can pass across the mucosal membrane of the small intestine. The absorption process of ionized drug can be fitted to first order kinetics, and therefore a carrier system is not suspected. Only in the intestine does ionized warfarin become absorbed, in the stomach and rectum unionized drug alone is absorbed, as would be expected according to the pH-partition theory.

In man the absorption rate of warfarin is rapid (complete by 120 minutes) when a two compartment pharmacokinetic model is used. However, a more complex model may be needed to give a more compatible picture with respect to the physiological situation. Maximum plasma concentration of warfarin occurs between 25 and 60 minutes /53/. Similarly, other oral anticoagulants are mostly well absorbed. These include, ethylbiscoumacetate /61/, and acenocoumarol /62/, whereas in contrast, the absorption of dicoumarol is less predictable /61/. Dicoumarol is also more slowly and less completely absorbed when in tablet form, rather than in solution, or as a powder. The response of two preparations may differ by as much as 50% for the same anticoagulant, and is an important clinical aspect of drug absorption. Poor absorption of various anticoagulants has occurred in man, although this is mainly due to errors in compounding or compression of tablets /63,64/. Warfarin is the only oral anticoagulant available in forms for i.v. or i.m. administration (available in the USA but not the UK).

There are few accounts of fatal intoxication with oral warfarin in man /65/ but transcutaneous intoxication has been recently investigated. It was found that significant transcutaneous uptake of the anticoagulant does occur, and has been seen to cause intoxication in two reported cases /66,67/.

The fact that the enantiomers of warfarin have differing biological activity, S(-) being 5.5 – 6.6 times more active in rats, and 3.8 times more active in man than R(+) warfarin, has been shown to be due to some factor(s) other than differential stereoselective absorption of the enantiomers. R(+1) and S(-) warfarin are absorbed from the GIT to the same extent /68,69/.

3.2 Distribution

The rate of distribution of a drug is an important determinant of its rate of onset of action. The tissue distribution of a drug also determines

how much of the drug is available at the biological site of action, and differences in drug distribution between species also depend on variations in drug binding to plasma proteins.

The tissue distribution of warfarin has been documented in the rat /70/ and the guinea-pig /71/. In the rat, after intravenous (i.v.) administration, warfarin was distributed quickly throughout the plasma and the liver showed an affinity for the uptake of warfarin as early as 1 hour after administration. The liver showed the greatest affinity of any tissue studied and this affinity was maintained for the entire 48 hours studied. Tissues such as heart and skeletal muscle showed some uptake during the first hour, and probably reflected blood distribution rather than actual uptake of warfarin, but this was not as great as that by the liver /70/.

This uptake of warfarin by tissues other than the liver is interesting, since vitamin K_1-dependent clotting factor synthesis occurs solely in the liver. There are however vitamin K-dependent proteins containing GLA, other than clotting factors /9-15/. Consequently, warfarin may have an effect on these proteins by antagonising the action of vitamin K in these extrahepatic tissues. The distribution and tissue uptake of other coumarin anticoagulants in the rat (e.g., dicoumarol) has been shown to be essentially the same as warfarin /72/.

Thus, hepatic uptake has a major quantitative effect on warfarin distribution. This uptake decreases with increasing drug concentration and this may cause the apparent volume of distribution (Vd_{app}) of warfarin to decrease with increasing dose in rats /73/. Warfarin pharmacokinetics are dose-dependent, the Vd_{app}, intrinsic clearance (Cl_{INT}) and total clearance (Cl_T) decrease with increasing dose in the 0.1-1.0 mg/kg range. At least one of these parameters, probably the Vd_{app}, is due to decreased hepatic uptake of the drug /74/.

The disposition of warfarin may be changed by various factors such as fasting, repeated administration, and dose. Plasma protein binding is altered in the fasted rat, the concentration of unbound warfarin in the liver supernatant of fasted rats given warfarin was significantly increased at 6 and 24 hours. It is concluded that short periods of fasting influence the disposition of unbound warfarin without apparently modifying its biotransformation /75/. On chronic dosing with warfarin, dogs show decreased plasma warfarin concentrations, and lengthened prothrombin times. There is, however, no increase in the clearance of warfarin. It is proposed that the decrease in warfarin may result from

increased tissue binding of the drug due to induction of additional binding sites /76/.

Many studies on protein binding of warfarin and other oral anticoagulants have been undertaken to elucidate the nature and extent of plasma protein binding. Sodium warfarin is bound solely to albumin of plasma protein /77,78/. Albumin has a negative charge at the pH of serum, but can interact with both negative and positive charges on drugs, and there is little correlation between the net charge on albumin and the degree of binding of most drugs. The net negative charge on albumin is increased by complex formation with the anionic warfarin. This is a co-operative effect, the initial binding of warfarin allowing more binding sites to become available. The initial electrostatic attraction between the binding site on albumin and the drug is reinforced by hydrogen bonds, dipole-dipole and hydrophobic (Van der Waal's) bonds. Warfarin and congeners have an ionizable acidic group at physiological pH and are hydrophobic. Therefore, hydrophobic binding is important and the overall binding of warfarin and other coumarins is high. However, interspecies differences in anticoagulant binding occur. In the rat, only 3% of warfarin and 0.2% of dicoumarol are free in plasma, the rest being bound to albumin /79/. In comparison, at warfarin concentrations ranging from 0.6-7.0 x 10^{-6} M, 5 and 7% of warfarin is unbound in plasma of the human and dog respectively /83/.

Warfarin has a small volume of distribution (Vd) which is equal to the albumin space, indicating the strength of warfarin binding. Albumin binding sites are formed during the process of binding, rather than being preformed as in antigen-antibody reactions /77/.

The most striking difference between precursor coumarin compounds (with very little anticoagulant activity) and the coumarin anticoagulant drugs in binding to plasma albumin, is the number of binding sites. The precursor compounds have an average of 0.9, while anticoagulants have an average of 1.9 binding sites. Both monocoumarins (e.g., warfarin, acenocoumarin and phenprocoumon) and dicoumarins (ethylbis-coumacetate and dicoumarol) exhibit this bivalency in binding.

The major source of the energy of binding of the coumarins to human plasma albumin is the coumarin nucleus itself. The addition of the hydroxyl group at position 4 of the nucleus, or the substitution at position 3, necessary for anticoagulant action, contribute only a little to the binding energy /78/. The side-chains of the coumarin molecule provide anticoagulant activity not through an increase in the binding

energy, but by the formation of another binding site. The increase in binding strength of the warfarin-albumin interaction with increasing pH is consistent with the uncovering of new binding sites on the albumin molecule /80/.

Species differences in the affinity of binding of anticoagulants to plasma albumin have been described. There is also considerable variation in the anticoagulant effect of coumarins among animal species /81-83/. The human, rat and mouse are most sensitive, guinea-pigs, cats and dogs intermediate, and the rabbit, cow and chicken least affected. It seems that there is no inverse relationship between the degree of albumin binding and anticoagulant sensitivity. In fact, the species with the most extensive binding (human and rat) are most sensitive, suggesting that difference in affinity at the site of action between species and not plasma protein binding account for differences in the pharmacological response.

Binding of warfarin to serum albumin is high in the rat, followed by sheep, dog and horse /84/. A six-fold greater binding to human, compared to canine plasma albumin has also been documented /78/. Also interesting is the fact that the number of apparent sites involved in binding are different among albumins. For some drugs it can therefore be seen that differences in serum protein binding and free drug concentrations may account for variation within and between species, but does not seem to be the case for warfarin.

Although it is now widely recognised that warfarin and congeners have two binding sites /78/, other studies disagree with this conclusion. For example, it has been shown that human serum albumin (HSA) has three strong affinity sites for dicoumarol as well as a number of weaker sites, and one strong bivalent binding site for warfarin /85/. In addition, the binding of the monocoumarin acenocoumarol to HSA shows the presence of two classes of binding sites with two different affinities for this compound /54/.

Pharmacologically, the S(-) isomer of warfarin is 6 times as active as R(+) warfarin in the rat and 3-4 times more potent than R(+) warfarin in man. A simple explanation of this difference would be a lower degree of S(-) isomer binding to albumin with higher free concentrations of S(-) warfarin available at the site of action, although the S(-) isomer shows greater binding to HSA than the R(+) isomer. This should give a slower rate of drug clearance, and less anticoagulant activity, since the free warfarin concentration in equilibrium with the receptor sites of

drug action and biotransformation is reduced. Alternatively, this increased binding may reflect a greater affinity of S(-)-warfarin for the receptor site. Differences in potency of the two isomers are not due primarily to differences in plasma protein binding /86/.

Since the oral anticoagulants are very tightly bound to serum albumin, this decreases the amount of free drug available for uptake by the liver. There is therefore a competitive relationship between serum albumin and the soluble fraction of the liver in the binding of warfarin. In the soluble fraction of the liver, 84% of warfarin and 72% of dicoumarol are bound to multiple sites on either the same or different proteins, the latter appearing more likely /87/. Although various other cell fractions of the liver contribute to anticoagulant binding, the soluble fraction plays a predominant role in the intracellular binding of these drugs *in vivo*.

Plasma protein binding is a major determinant in the pharmacokinetic parameters of warfarin in both the rat and human. Following an i.v. injection to rats, warfarin concentration in plasma declines in three phases. However, no apparent correlation is found between the free fraction of warfarin and the intercompartmental distribution rate constants /88/, although strong correlations occur between the free fraction and other pharmacokinetic parameters which will be discussed later. The duration of anticoagulant effects correlates reasonably well with the interval in which the drug is retained in the plasma /89/ and in the liver /90/. An excellent correlation also exists for the strength of binding to albumin and to the intracellular receptor site for anticoagulant activity /80,90/.

3.3 In vivo metabolism

Liver metabolism of compounds occurs with two main consequences. Either the substrate is pharmacologically activated to give a more reactive (and potentially toxic) species, or it becomes inactivated to a less pharmacologically effective product. In the case of warfarin, metabolism results in pharmacological inactivation, as judged by the decreased anticoagulant activity of its metabolites. The metabolism of warfarin has been extensively studied and many metabolites have been identified including (Fig. 3). The main metabolic routes are:

i) *keto reduction* to warfarin alcohols, each enantiomer yielding two diastereoisomeric products;

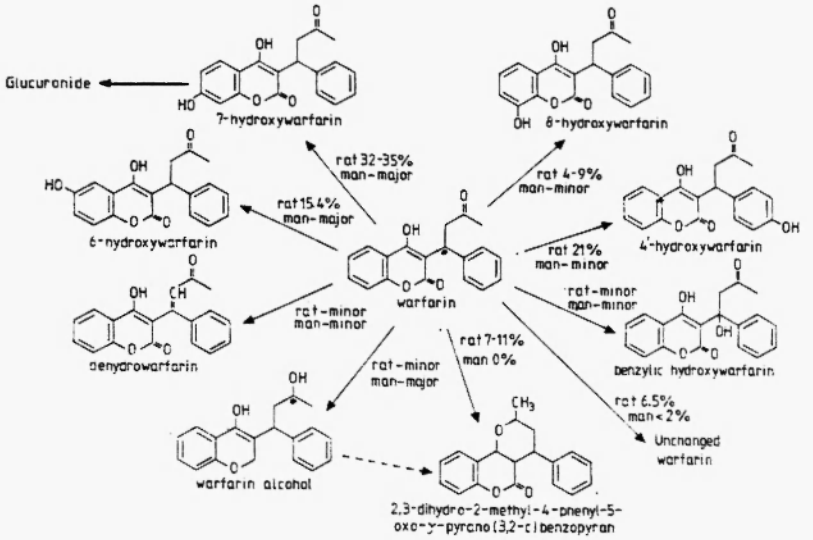

Fig. 3: Metabolic profile of warfarin in the rat and man
→, dehydration and ring closure
*, chiral centre

Indicated numbers are the percentage of dose excreted.

ii) *dehydration of warfarin alcohol* resulting in ring closure, yielding a benzopyrano derivative;
iii) *hydroxylation reactions* yielding the regioisomeric products 6-, 7-, 8-, 4'- and 9-hydroxy(benzylic) warfarin;
iv) *dehydration of 9-hydroxywarfarin* to yield 9, 10-dehydrowarfarin and
v) various *conjugation reactions* /91/.

Substantial metabolic differences occur between animal species, strains and organs, and substantial interspecies differences in warfarin metabolism have been well documented between rat, man, guinea-pig, dog and mouse /92-98/.

The first report describing the metabolic fate of warfarin appeared in 1962 /92/. Since then further studies have demonstrated the formation of 6-hydroxy, 7-hydroxy, 8-hydroxy and 4'-hydroxy warfarin, 2,3-dihydro-2-methyl-4-phenyl-5-S-oxo-γ-pyrano (3,2-c)benzopyran, and a glucuronide conjugate of 7-hydroxywarfarin in the rat (Fig. 3), in addition to unchanged warfarin /93/. These results are supported by

other workers /94,95/. Studies *in vitro* by Ikeda *et al.* /96/ have shown that the production of 6-, 7-, and 8-hydroxy warfarins is associated with the microsomal fraction of the liver and requires molecular oxygen and a source of NADPH. Thus the properties of the enzyme system fulfill the criteria for a mixed function oxidase (MFO) in the requirements mentioned, in addition to the fact that carbon monoxide inhibits hydroxylation /97/. There is a divergence of evidence concerning keto reduction to produce warfarin alcohols in the rat. Townsend *et al.* /97/ did not show the presence of the alcohols using t.l.c., but warfarin alcohol was found to have the same chromatographic properties as an unknown metabolite described by Ikeda *et al.* /96/. However, later studies /91/ indicated that the warfarin alcohols are indeed formed in the rat.

In addition to the involvement of the MFO system giving rise to hydroxylated metabolites, ketone reduction of warfarin occurs, most likely catalysed by the enzyme warfarin reductase. The enzyme is NADPH-dependent, is present in the soluble fraction of both human and rat liver and kidney /98/, and appears similar to the ketone reductases reported by Liebman /99/. Alcohol dehydrogenase is not considered to be involved in the reaction /98/.

The metabolism of racemic warfarin in man results in the formation of seven fluorescent compounds. Four of these were identified by Lewis and Trager /100/ as 6- and 7-hydroxywarfarin and two diasteromeric alcohols. In man, warfarin appears to undergo similar metabolism to the rat, and although 8- and 9-hydroxywarfarin have not been identified, these may be minor metabolites. The pyranocoumarin found in the rat is not present in man. This metabolite is thought to be formed from warfarin alcohols (present in man) with dehydration and ring closure /101/ (Fig. 3).

The biotransformation of various congeners of warfarin in the rat have also been studied, e.g., dicoumarol and phenprocoumon. Dicoumarol is metabolised by mixed function oxidases to hydroxylated products, mainly in the 7 position /102/. Phenprocoumon (3-(α-ethylbenzyl)-4-hydroxycoumarin) is metabolised mainly to 4′-, 6-, 7-, and 8 hydroxyphenprocoumon by the MFO enzymes. These metabolites have been identified using t.l.c. and mass spectrometry /103/. A sensitive acid labile conjugate of phenprocoumaron itself is formed by metabolism, and is postulated to be the enol ether 4-O-glucuronide of the drug /103/. In the metabolism of warfarin, approximately twice as much

7-hydroxy compared to 6-hydroxy warfarin is found, and for phenprocoumon, the reverse is true, more 6-hydroxy metabolite being produced. The relative amounts of 8- and 4'-hydroxy metabolites produced are the same for warfarin and phenprocoumon /103/. Given the similarities between warfarin and phenprocoumon, it seems reasonable to speculate that the same enzyme system is responsible for the formation of 6-, 7-, and 8-hydroxy metabolites of both phenprocoumon and warfarin, i.e., the cytochrome P-450-dependent MFO system. The differences in metabolites produced from the above two substrates suggest possible differences in the modes of binding of the substrates to the enzyme, leading to different products, or the possibility that the two substrates are metabolised by different isoenzymes of cytochrome P-450, the terminal haemoprotein component of the MFO system.

Acenocoumarol metabolism has been studied in man /62/. It is metabolised to 7-hydroxy (14-22% of the administered dose), 6-hydroxy (7-18%) acenocoumarols, an amino metabolite (8-12%) an acetamido metabolite (11-19%) as well as two acenocoumarol alcohols (7-13%). Thus the primary paths of biotransformation are oxidation and reduction at different positions in the molecule. Nitro reduction results in the amino metabolite, the major portion of which is further metabolised to the corresponding N-acetyl-derivative. Reduction of the ketone group to yield two diastereoisomeric alcohols corresponds to the formation of alcohols of warfarin in man and rat. Oxidation of the coumarin nucleus occurs giving predominantly 6- and 7-hydroxy metabolites of acenocoumarol /100,101,104,105/.

Similarly, in the guinea-pig warfarin is biotransformed to hydroxy-metabolites /71/, although these have not been as extensively studied as in man and rat. However, the biotransformation pathways seem very similar in man, rat and guinea-pig /71/. Man, rat, dog and rhesus monkey have been used comparatively to study interspecies variations in biotransformation of warfarin and dicoumarol /106/. The observations are consistent with the idea that the two anticoagulants are subject to the same major biotransformation pathways /106/. The half-life of warfarin increases in the order rat < monkey < dog < man, and reflects the rate of biotransformation of warfarin. In the rabbit, marked differences in the metabolism of anticoagulants have been reported /104/. For example, ethylbiscoumacetate is metabolised by hydroxylation in man but by de-esterification in the rabbit. Also coumarin (which

has no anticoagulant activity) is transformed to 7-hydroxycoumarin in man but to 7-hydroxycoumarin in rabbit, and hydroxy derivatives and carbon dioxide in the rat /100/.

Warfarin exists as two distinct enantiomers - R(+) and S(-) warfarin, the therapeutic agent being a racemate containing equal quantities of the two enantiomers. In the rat and in man, S(-) warfarin is the more potent anticoagulant than R(+) warfarin /68/. However, while S(-) enantiomer is cleared more rapidly than the R(+) enantiomer in man /101/, the reverse is the case in the rat /108/. The two enantiomers also differ in their metabolic routes, S(-) warfarin being primarily oxidised to 7-hydroxywarfarin and reduced to S,S warfarin alcohol /101/. 6-Hydroxywarfarin is produced equally on metabolism of both enantiomers. Thus when warfarin is administered to man, the differences in pharmacodynamics and pharmacokinetics of the two enantiomers should be taken into account.

In summary, interspecies differences in metabolism do occur, namely in the rate of metabolism, relative amounts of the metabolites formed and more rarely in the routes of biotransformation of warfarin and congeners.

3.4 In vitro metabolism

As has been stated above, warfarin is metabolised *in vivo* in the rat to 6-, 7- and 8-hydroxywarfarin. In addition, various *in vitro* systems have been used to investigate metabolism, including enzymatic and non-enzymatic methods. For example, enzymatic hydrolysis of warfarin has been studied using liver homogenates and microsomes. These studies have demonstrated enzymic conversion of warfarin to 6-, 7- and 8-hydroxy metabolites with no evidence of 5-hydroxywarfarin. Other methods have shown equal amounts of 6-, 7- and 8-hydroxywarfarin are formed /109,110/. Also comparisons of microsomal cytochrome P-450 and purified cytochrome P-450-catalysed warfarin metabolism must be made with caution due to the potential for different rate-controlling steps in the two systems. It can therefore be seen that although quantitatively similar, microsomal metabolic models may produce quantitative differences in metabolites compared with *in vivo* findings, and therefore cannot be used to accurately predict the *in vivo* response.

An important consideration when administering oral anticoagulants is whether the metabolic products of biotransformation have anticoagu-

lant activity. The 6-, 7- and 8-hydroxywarfarins have no anticoagulant activity /58/ and of the hydroxywarfarins, only 4′-hydroxywarfarin shows significant anticoagulant activity /94/. In addition, the four warfarin alcohols all show weak anticoagulant activity. Since warfarin is a racemic mixture of R(+) and S(-) warfarin, on ketone reduction a second chiral centre is generated producing four different configurations of warfarin alcohol: (S,S), (S,R), (R,S) and (R,R) /111/. These metabolites have anticoagulant activity in man and monkey, and it appears that the (R,S) enantiomer is the most active, whilst (R,R) is the least active. Anticoagulant activity is only 5-10% of the activity of racemic warfarin, implying that the metabolites of warfarin are not important in the pharmacological action of the drug /112/.

The metabolism of R(+) and S(-) warfarin by rabbit liver microsomes differs from that of rat microsomes with respect to the overall rates of metabolism, the relative rates of formation of the different metabolites and the effects of inducing agents on the metabolism. Fasco *et al.* /113/ used purified and partially purified, reconstituted rabbit liver microsomal cytochrome P-450 (P-450LM) to investigate the stereoselective metabolism of warfarin. Based on metabolite formation, two groups of cytochrome P-450 (LM_2 and LM_4) were found to have markedly different activities. *P-450LM*$_4$ produced metabolites which had the phenyl portion of the coumarin moiety hydroxylated in the 6, 7 and 8 positions, and had greater activity with R(+) warfarin. In contrast, the *P-450LM*$_2$ isoenzyme could react with the phenyl substituent of warfarin in the R(+) configuration.

It has been proposed that in the rat, metabolism of warfarin to hydroxylated derivatives occurs via arene oxides /111/, a mechanism that has been shown to occur in other compounds metabolised by cytochrome P-450 /114/. If the same mechanism occurs in the rabbit, results show that cytochrome P-450LM_2 only catalyses 6,7 arene oxide formation, whereas cytochrome P-450LM_4 gives both 6,7 and 7,8 arene oxides. Phenobarbitone (PB)-induced microsomes show the same metabolic profile as the purified cytochrome P-450LM_2, whereas β-naphthoflavone (BNF)-induced microsomes and P-450LM_4 are less similar. This suggests that other forms apart from P-450LM_4 contribute to metabolism in BNF-induced microsomes. The regio- and stereoselectivities of the cytochrome P-450LM enzymes were essentially maintained on purification from microsomal membranes /113/.

Similarly, in the rat, different cytochrome P-450 forms have been

found to be responsible for the formation of different metabolites of warfarin. Studies by Pohl *et al.*/109/ using rat liver microsomes showed the formation of 7- and 8-hydroxy warfarin was stereoselective for the R(+) enantiomer. The converse stereoselectivity was shown for 4′-hydroxylation. The data suggested at least three distinct enzymatic processes were operating, which may or may not have been distinct haemoproteins. Fasco *et al.* /110/ showed that metabolic patterns of warfarin enantiomers varied following induction of cytochrome P-450 by phenobarbitone (PB) and cytochrome P-448 by 3-methylcholanthrene (MC). This study concluded that R(+) and S(-) warfarin bind to two separate forms of cytochrome P-450 and two separate forms of cytochrome P-448, both of which are different from uninduced (constitutive) cytochrome P-450. The interactions of R(+) and S(-) warfarin with these different forms of cytochrome P-450 resulted in the formation of a variety of monohydroxylated metabolites. Further work using either NADPH- or cumene hydroperoxide-supported microsomal hydroxylation of warfarin, showed fairly uniform results with control, PB, MC and 3-β-hydroxy-20-oxo-pregn-5-ene-16-α -carbonitrile (PCN) induced microsomes. This suggested that for each metabolite of warfarin the cytochrome P-450 responsible for its production is the same, irrespective of the mode of induction of the microsomes. The exception to this was 6-hydroxylation activity, where different cytochrome P-450's are responsible for its formation in differently-induced microsomes. The cytochrome P-450's for the formation of 7- and 8-hydroxywarfarin are different from those catalyzing the other metabolites, and the cytochrome P-450's catalyzing 7- and 8-hydroxylation are different from each other.

To try and elucidate which cytochrome P-450's are responsible for the production of which hydroxylated metabolite, purified forms of rat liver cytochrome P-450 (P-450-UT) have been used. Marked differences are shown between uninduced, PB-induced and MC-induced cytochrome P-450 forms /115,116/. The PB-induced enzyme appears particularly active in 7-hydroxylating R(+) warfarin, whereas the MC-induced cytochrome P-450 is more active in the 8-hydroxylation of R(+) warfarin. Untreated liver microsomes contain P-450 (UT-A) which appears highly selective for the 6,7- and 4′ positions, for which the stereoselectivity is low, and for the 9 (benzyl) position with high stereoselectivity. Cytochrome P-450 (UT-C) is also present but is less active than the (UT-A) form. Thus the structural selectivity of

hydroxylation of warfarin combines product regioselectivity in that two warfarin enantiomers show differences in the various routes of hydroxylation, and substrate stereoselectivity in that different cytochrome P-450's preferentially hydroxylate R(+) or S(-) warfarin to a greater extent than the other enantiomer.

A wide variety of extrahepatic rat tissues contain cytochrome P-450 similar to major forms of hepatic microsomal P-450. The cytochrome P-450 of MC-induced liver, lung and kidney microsomes is similar, but different from PB-induced or control microsomes /115/. PB-induction is effectively different in liver, lung and kidney, thereby giving differences in warfarin metabolism. However, in PB-induced kidneys, a warfarin metabolite pattern is found which is similar to that from MC-induced liver microsomes. Compared to liver microsomes, warfarin is a poor substrate for cytochrome P-450 of control rat kidney and lung microsomes /115/.

Within the liver mass, different cytochrome P-450's show different distribution patterns. Using antibodies to two purified fractions of cytochrome P-450B (PB-B or MC-B), immunostaining occurred in parenchymal cells throughout the liver lobule /117/. Centrilobular hepatocytes were found to contain a greater concentration of cytochrome P-450 than did cells in the peripheral region of the lobule. Therefore, the structural selectivities of warfarin metabolism may be affected by species, strain, tissue and localization within a given tissue.

The above differences in warfarin metabolism are thought to be due to cytochrome P-450 multiplicity, and characterisation of the numerous cytochrome P-450 forms and their functional selectivities may allow quantitative analysis of the differences in warfarin metabolism.

As well as interspecies variation and differential organ metabolism, as described above, differences also occur within a given species. This includes sex differences, strain differences, differences in stereoselectivity of metabolism and differential organ metabolism. For example, sex differences in pentobarbital sensitivity have been shown in mice /118/, male mice sleeping longer than females. Interstrain variation in hexobarbitone sleeping time in mice is caused by variation in the rate of hexobarbitone metabolism. The sleeping time is inversely proportional to liver hexobarbitone hydroxylase activity. Correlation of hexobarbitone sleeping time and zoxazolamine paralysis times in these strains may be due to genetic variation of a component of the microsomal system /119/, and could be involved in several, if not all, hydroxylations.

Accordingly, sex differences in warfarin metabolism by the mixed function oxidase system may occur by a similar mechanism. In fact, it has been shown that in rats, marked sex differences do occur in that female rats metabolise warfarin more slowly than do male rats /118/. This also corresponds to female rats sleeping longer than males after pentobarbitone administration. The average half-life of warfarin is 18 hours in male rats and 28 hours in female rats /48/, possibly illustrating sex differences in metabolism. The anticoagulant potency of S(-) warfarin is 6.6 times greater than that of R(+) isomer in male rats. This difference is partly explained by a difference in the plasma half-life of the two enantiomers and partly by a difference in the inhibition of prothrombin complex synthesis, R(+) warfarin being 1.9 times less potent than S(-) warfarin /108/. There are therefore sex and enantiomeric differences in metabolism of warfarin in the rat, S(-) warfarin being more slowly metabolised and having greater anticoagulant activity /108/. However, in man, although the S(-) isomer of warfarin is 3.8 times more potent as an anticoagulant than R(+) warfarin, it is metabolised at a greater rate as reflected by its shorter half-life compared to R(+) warfarin /107/. Therefore the metabolic rate cannot be responsible for the different potencies of the enantiomers in their anticoagulant action. It is concluded (since GIT absorption, plasma protein binding, volume of distribution, metabolic rate, and excretion are not responsible) that differences in the biological activity of the enantiomorphs of warfarin are due to differences in intrinsic activity, as a result of permeability to, or affinity for a receptor /69/.

Interindividual variation in drug metabolism also occurs on administration of the oral anticoagulants. Dicoumarol is an example of this. Thus, large differences in the metabolism of dicoumarol occur between individuals /120/. Variation arises from genetic rather than environmental factors, the rate of dicoumarol decay being controlled by the operation of recessive genes. However, the biochemical site of expression of these genes is not known. The half-life of dicoumarol ranges from 7-74 hours in man, with the half-life being dose-dependent /123/, implying that excretion does not obey first order kinetics. It is postulated that natural selection in certain environmental circumstances may have favoured individuals with high levels of drug-metabolising enzymes /121/. Genetic control, resulting in inter-individual variation is shown for basal and phenobarbitone induced levels of a mixed function oxidase which hydroxylates coumarins /121/. Male mice of one of four strains showed

3-4 times the 7-hydroxylase activity of the other three, and in phenobarbitone-induced mice this figure became 4-6 times higher. The total levels of cytochrome P-450 were equal in all the strains of mice, but it is possible that one particular isoenzyme of cytochrome P-450 may have been preferentially induced or synthesised followed phenobaritone pretreatment, thus rationalising the observed increase in 7-hydroxylase activity. In support of this conclusion, multiplicity of cytochrome P-450 forms has been shown in various species including rat /122,123/ rabbit /124/ mouse /125/ and human /126/.

3.5 Excretion

Excretion of drugs may occur via the urine, bile, faeces and/or respiratory gases. Warfarin and related anticoagulants undergo relatively extensive metabolism, thereby becoming more polar and more susceptible to excretion, particularly in the urine.

In the rat, over a 96 hour period after oral administration, warfarin and its metabolites were found in both bile (39.4%) and urine (18%), and the same figures after i.v. dosage were bile (47.5%) and urine (21.3%) /127/. These results were derived from rats fitted with biliary fistulas, to ensure that enterohepatic circulation did not occur. Enterohepatic circulation entails the passage of warfarin or warfarin metabolites from the liver to the bile and then into the gut, from which drugs may be reabsorbed and recirculated to be excreted in part by the kidney. Thus, elevated levels of metabolite excretion via the urine may be measured, representing in part warfarin and metabolites excreted initially into the bile. By fitting biliary fistulas, transport of warfarin and metabolites to the gut cannot occur /127/. This process can be seen to occur in the rat to a small extent since the excretion of warfarin after oral and i.v. dosing in normal rats (no biliary fistula) is as follows: after oral dosing 43% found in urine, 41% in urine after i.v. dosing (compared to the 18.0 and 21.3% above). In faeces 35% of the dose is excreted. Urinary excretion represents 6-, 7- (or glucuronide), 8-, 4′-hydroxywarfarins and 2,3-dihydro-2-methyl-4-phenyl-5-oxo-α-pyrano(3,2-c) benzopyran. These metabolites were also found in the faeces by Barker *et al.* /93/ but were not seen in the study by Losito Rousseau /127/. After the administration of [4-^{14}C]-warfarin, no radiolabel was detected in the respiratory gases, indicating that decarboxylation does not take place /128/.

In man, the same 7 metabolites of warfarin are excreted in urine as in the rat /100/ and less than 2% of the administered dose appears as unchanged warfarin. In man, the main metabolites excreted in the urine are 6-, and 7-hydroxy warfarin and two diastereoisomeric warfarin alcohols, but the rate of warfarin elimination differs considerably between individuals, with half lives varying between 20 and 50 hours. Acenocoumarol, in contrast, has a half-life of elimination of 8.5 hours in man with the major part of the dose being excreted in the first 3 days /62/. Between 59 and 73% of acenocoumarol is excreted via the kidney into the urine, thus constituting the major route of excretion.

The guinea-pig, like man, excretes warfarin and its metabolites predominantly in urine (70% in 48 hours), after oral dosing. About 43% of warfarin is excreted into the bile in the 12 hours after injection /74/. Considering the molecular weight threshold for biliary excretion in the guinea-pig (400±50), it is surprising that warfarin with a molecular weight of 308 and hydroxy-warfarin metabolites with molecular weights of 324, are excreted into the bile /71/, suggesting that factors other than molecular weight may affect biliary excretion. A majority of warfarin and its metabolites excreted in the bile, undergo enterohepatic circulation and are disposed of in the kidney in this species.

In the rabbit, unlike the rat and guinea-pig, the bile is a minor excretory route for warfarin, and only polar and bulky conjugates of the drug are eliminated by this pathway. The main excretory route in the rabbit is via the urine, 90% of the warfarin dose being recovered within 4 days after oral administration /129/. The major urinary metabolites are the warfarin alcohols, 4′-6- and 7-hydroxywarfarin, and also present was unchanged warfarin /129/. Biliary metabolites in the rabbit show that the majority are glucuronide conjugates (70%) or highly polar metabolites (10%). The biliary metabolites in rat and guinea-pig, in contrast, are mainly unconjugated warfarin and hydroxylated warfarin metabolites. Species differences in routes of excretion are probably due to differences in threshold molecular weight for biliary excretion /129/.

From the above discussion it is quite clear that the routes of excretion of warfarin and its metabolites substantially differ in various species including rat, man, guinea-pig and rabbit.

The excretion of the enantiomers of warfarin has been studied primarily in man, but also in the rat. In the rat, S(-) warfarin is more potent in producing an anticoagulant effect than R(+) warfarin. The

S(-) isomer is also eliminated less quickly, thus partly explaining its greater potency in the rat /130/. In man, although S(-) warfarin is the more potent isomer, it is eliminated more rapidly than R(+) warfarin, and both warfarin and phenprocoumon are excreted mainly in the urine. S(-) warfarin potency is 5-8 times as potent as the R(+) isomer, and S(-) phenprocoumon is 3 times more potent than the R(+) isomer. In both cases the least potent isomer is excreted more rapidly. However, the *in vitro* potency ratio of the drug enantiomers is not explained by the moderate difference in gross excretion data /131/. There is no evidence to suggest that the enantiomers of warfarin are excreted via different routes, although their excretion rates do differ.

There is a positive correlation between the elimination rate constant and the Vd_{app} of warfarin in rats also affecting the excretion of the drug and its metabolites /132/. The effect of plasma protein binding on warfarin elimination can be predicted on the basis of a linear relationship between the total plasma clearance of warfarin and the free fraction of this drug in the serum /133/. Intersubject variation in elimination of warfarin may be explained by differences in plasma protein binding, since differences in metabolism are not responsible /134/.

IV. MECHANISMS OF WARFARIN-RESISTANCE

4.1 Vitamin K-dependent resistance

Warfarin-resistance has been reported in both man and rodents, the resistance being vitamin K-dependent. Various types of resistance have been documented in man including dietary resistance caused by an excess intake of dietary vitamin K_1 which is reversible upon discontinuation of the excess intake /135/. Acquired resistance is shown by an initial response to warfarin followed by a decrease in this response. Finally, hereditary resistance is documented where no initial response is seen to warfarin. This condition is transmitted as an autosomal dominant trait and doses in excess of twenty times normal have been required before effects are seen in rats /136/. In this review, the latter type, hereditary resistance, will be discussed in detail.

Understanding resistance in both man and rodents is important for effective anticoagulant therapy, and control of resistance in rodents using rodenticides effective in such strains. Once the biochemical site of

action of the warfarin resistance gene has been fully elucidated, development of new rodenticides, effective in resistant rats, should be considerably easier.

Recently, in man, cases of warfarin resistance due to unrecognised vitamin K_1 supplementation in liquid nutrient products has been described /135,137/. The increased intake of vitamin K antagonises the action of the anticoagulant, and clotting factor synthesis may still occur. Although the daily requirement is vitamin K_1 is not well established, an estimated minimum requirement of 0.03 μg/kg body weight in man /138/. The normal dietary intake of vitamin K_1 ranges from 300-500 μg/day, and dietary supplements can raise this figure to approximately 1.5 mg/day /138/. In addition to liquid nutrient products, excess dietary vitamin K_1 may be obtained, for example, from vegetable-rich, weight-reducing diets, and cases have been documented where a change from this type of diet restores normal anticoagulation on subsequent warfarin administration /139,140/.

Resistance to oral anticoagulants may occur due to increased metabolic rate, and subsequent increased elimination of the drug from the body. This is usually due to microsomal enzyme inducing agents such as barbiturates being administered at the same time as warfarin /141,142/. In this case, the dose of warfarin may be increased, or if possible, the inducing agent withdrawn, in order to obtain normal responses to warfarin.

Acquired warfarin-resistance occurs when a patient gradually becomes resistant to oral anticoagulants, and in these cases, the half-life of elimination and absorption of warfarin appear to be normal /143/. In addition, high vitamin K_1 intake or interference with coumarin metabolism by other drugs do not appear to be responsible for resistance. Acquired resistance may also be transient. Such a case has been reported /144/ wherein a patient developed resistance to the warfarin congeners, phenprocoumon and acenocoumarol. Large doses appeared to give satisfactory anticoagulation but after 8 months of these large doses, the patient suddenly developed haemorrhagic diathesis. No explanation for this resistance can be given, other than perhaps development of a transient receptor defect for either vitamin K_1 or coumarin anticoagulants. Acquired resistance to anticoagulants is a rare phenomenon compared to hereditary resistance, the latter being first reported in man in 1964 /145/, the patient requiring 25-30 times the usual dose of warfarin to remain anticoagulated. Increased warfarin

metabolism was not the cause, since plasma warfarin levels were also elevated. The patient was also very sensitive to the antidotal action of vitamin K_1. Studies of the rest of the family showed the resistance to be an heritable autosomal dominant trait. Further studies in this kindred, and others discovered more recently /146,147,136/, have suggested that this form of resistance is due to a genetic mutation in the warfarin 'receptor', which may be the enzyme vitamin K_1-epoxide reductase (Fig. 1). The mutation is thought to cause the 'receptor' to have a moderately decreased affinity for vitamin K_1, whilst the affinity for warfarin is markedly decreased. Although any of the following mechanisms might account for the resistance including decreased absorption, increased destruction or excretion, altered volume of distribution of the anticoagulant, increased concentration of vitamin K_1, increased production or biological half-life of vitamin K_1-dependent clotting factors, presence of an alternative pathway for clotting factor synthesis, bypassing vitamin K_1, or the presence of a hepatic receptor site or protein with altered affinity for, or permeability to vitamin K_1 or anticoagulant drugs, all but the last mechanism have been ruled out in the kindreds studied.

Anticoagulants were first used as rodenticidal agents in the 1950's /148/ and warfarin was used extensively to control both rats and mice. Inevitably, rodent populations evolved with genetically determined resistance to its effects. Resistance in wild rats Rattus norvegicus was found in populations from Scotland in 1958 /149/ and the English-Welsh border regions in 1959 /150/. Since then resistant rats have also been reported in Denmark /151/ and the USA /152/. In 1969, Greaves and Aryer /153/ found linkages between genes for coat colour and resistance to warfarin in the Norway rat *R. norvegicus*. The Welsh resistance gene is classed Rw^2, shows a single autosomal dominant effect, and is present on chromosome 7 /153/. Resistant rats have an abnormally high requirement for vitamin K_1 /154/, essential for maintenance of vitamin K-dependent clotting factor synthesis. It has been suggested that a protein involved in clotting factor synthesis, which interacts with both warfarin and vitamin K_1 is altered in resistant rats so that its affinity for both compounds is decreased, but that for warfarin is decreased more than that for vitamin K_1 /154/. Whereas resistance is manifested as a dominant effect /153/, a monogenic recessive inheritance of a haemorrhagic trait has been found. The gene involved is linked with the *p locus*, and may well be identical to or

allelic for Rw^2. An interesting factor is that a single biochemical mechanism has been proposed which connects the dominance of Rw^2 with respect to resistance to warfarin, with a recessive effect in respect of vitamin K_1 requirement. It would be expected that a gene as rare as Rw^2 would normally be recessive and deleterious in its expression /153/. It seems that resistance to warfarin and an increased vitamin K_1 requirement are pleiotropic effects of the same allele (Rw^2).

The Scottish resistance gene is not the same as the Welsh gene Rw^2 as the two strains have different vitamin K_1 requirements (Welsh resistant rats have a greater requirement than Scottish resistant rats). Demonstration of segregation with respect to resistance of warfarin, vitamin K_1 requirement or site of action is needed to test the hypothesis that Welsh and Scottish genes are allelic with one another /155/. An interesting study /156/ showed that on withdrawal of anticoagulant administration in a natural population containing resistant individuals, the change in the frequency of resistance reflects the relative fitness of the three possible genotypes under natural conditions. In this case, the decline in frequency of resistance is consistent with a hypothesis of reduced fitness of both Rw^2 Rw^2 and Rw^1 Rw^2 genotypes relative to Rw^1 Rw^1 under natural conditions. Estimates for fitness of genotypes under natural conditions gave values of: Rw^2 Rw^2 (0.46), Rw^1 Rw^2 (0.77) and Rw^1 Rw^1 (1.00) /156/, indicating that Rw^1 Rw^1 is the normal allele selected in the absence of anticoagulant administration.

Determination of warfarin-resistance genotype is based on changes in blood coagulation activity brought about by the administration of warfarin and vitamin K_1-epoxide and by feeding a vitamin K_1-deficient diet for 4 days according to the method of Martin *et al.* /157/. This method has proved effective in identifying the genotype of wild rats carrying the warfarin resistance gene Rw^2.

Notwithstanding the identification of the major gene controlling warfarin resistance in the rat, the biochemical manifestation of the resistance gene could be any one of various mechanisms as discussed previously for heritable resistance in man. It has been shown that increased metabolism of warfarin does not account for resistance in the rat /158,97/. The half-life of enantiomers of warfarin in male rats was found to be almost identifical in resistant and susceptible rats, although both male strains eliminate R(+) and S(-) warfarin faster than their respective females /158/. Comparing female rats, the half-lives of respective isomers in resistant rats is about two-thirds that of

susceptible rats, due to an increased elimination rate in resistant females. Also, no difference in metabolites excreted in urine or faeces occurs in resistant rats, compared to normal susceptible animals /97/. These pieces of evidence support the idea that a faster rate of warfarin metabolism and excretion is not responsible for warfarin resistance, although in females, the elimination rate of warfarin is increased, it is unlikely that the small change would contribute significantly to warfarin resistance. The fact that the Rw^2 gene confers resistance to both orally administered and intravenous warfarin suggests that a defect in absorption of warfarin is not responsible for resistance.

Sex differences in metabolism in warfarin-resistant rats do occur, since the female eliminates warfarin at a slower rate than the male. In the susceptible rat, the male rat is less sensitive than the female, and females are less likely to become vitamin K_1 deficient with increasing age /159/. In both resistant and non-resistant rats, the half-life of S(-) warfarin is greater than that for R(+) warfarin. This applies for both sexes of resistant and susceptible rats indicating no great sex difference with respect to resistance. In both resistant and non-resistant strains, the half-life of warfarin enantiomers is longer in females than in males.

To achieve PCA values comparable to normal susceptible rats, heterozygous resistant rats need twice as much vitamin K_1 and homozygous resistant rats twenty times as much vitamin /160/. This could be due to an increased destruction of vitamin K_1 in the tissues of warfarin resistant rats or an altered protein (necessary for clotting factor synthesis) with which vitamin K_1 acts, having a slightly decreased affinity for the vitamin, and also a greatly reduced affinity for warfarin, so causing resistance /154/. The former mechanism has been ruled out by Thierry *et al.* /155/. The similarity in both whole body and subcellular distribution of vitamin K_1 in normal and resistant rats make it improbable that distribution of the vitamin has anything to do with its increased requirement in resistant rats. Hence, the second mechanism appears to be a more likely explanation for resistance.

Thus, having established that the metabolism of warfarin, or alteration in the destruction of vitamin K_1 do not account for resistance, research was directed to find the binding protein which was thought to have altered affinity for vitamin K_1 and warfarin so conferring resistance. The site of resistance was postulated to be somewhere between the point of transfer of warfarin from plasma albumin and excretion from the liver. As has been discussed previously,

the anticoagulant effect of warfarin is due to an antagonistic effect on the role of vitamin K in post-translational carboxylation of glutamyl residues in blood clotting factors II, VII, IX and X. The γ-glutamyl carboxylase enzyme appears to require the reduced (hydroquinone) form of vitamin K_1 as cofactor. Administration of warfarin to rats results in an increase in the ratio of vitamin K_1-2,3-epoxide to vitamin K_1 /159/. This effect is more pronounced in warfarin-susceptible than in resistant rats, suggesting that resistance may be due to an alteration at some point in the vitamin K cycle. The site of warfarin inhibition in the susceptible animal has not been entirely elucidated, although the enzyme vitamin K_1-epoxide reductase is thought to be the main site of inhibition /16/. A warfarin binding protein has been shown in ribosomes in liver cells /154/, and the ribosomes of susceptible rats bind 2 or 3 times as much warfarin as those from resistant rats. This warfarin binding site is localized in microsomal membranes /154/. Although decreased binding in warfarin-resistant rats was shown, it is not known if the binding protein is absent or just exhibits a decreased affinity /33/. Considering this evidence, and the lack of accumulation of vitamin K_1-2,3-epoxide in resistant rats treated with warfarin, the vitamin K_1-epoxide reductase enzyme appears a likely candidate for the altered protein in resistance /162/. Administration of vitamin K_1-2,3-epoxide to resistant rats can stimulate prothrombin synthesis in the presence of warfarin, suggesting that the epoxide reductase enzyme is less sensitive to warfarin inhibition in resistant rats /163,163/. Further evidence for the decreased sensitivity of the epoxide reductase enzyme in resistance is given by Leck and Park /165/ using brodifacoum (Fig. 2), an anticoagulant which is effective in warfarin-resistant rats /166/. This study demonstrated that brodifacoum increases the hepatic ratio of vitamin K_1-epoxide to Vitamin K_1 in both resistant and susceptible animals, although warfarin does not show this effect in the resistant rat. A new metabolite of vitamin K_1 (2(3),-hydroxy-2,3-dihydro-2-methyl,3-phytyl-1,4-naphthoquinone) (hydroxyvitamin K_1) has apparently been identified as a product of vitamin K_1-epoxide metabolism in microsomes from warfarin resistant /167/, but not those derived from normal rats, and is thought to be mechanistically related to warfarin resistance. The slight changes in the structure, decreasing the affinity of vitamin K-epoxide reductase for this metabolite, or altering the positions of the proton-donating groups at the active site, could also lower the affinity for warfarin. The decreased affinity of the enzyme

vitamin K-epoxide reductase for warfarin may be due to changes in the tautomeric equilibrium between keto and enol forms of the bound drug /167/. However, further research has shown that hydroxyvitamin K_1 is formed in greater quantities after long incubation times using solubilized microsomes, and as such may be due to artifactual vitamin K_1-epoxide reduction /168/.

The enzyme vitamin K-epoxide reductase requires dithiothreitol for *in vitro* activity, as does vitamin K_1-epoxide-dependent protein γ-glutamyl carboxylation. Both reactions are warfarin-sensitive in susceptible rats, and it has been postulated that the dithiothreitol-dependent reductions of both vitamin K_1 and vitamin K_1-epoxide are a property of the same enzyme /40/. Subsequent studies have not clearly shown whether both reductive steps are catalysed by the same site on a single enzyme, by separate sites on a single enzyme, or indeed by two different enzymes. Purification of the enzyme(s) which are altered in warfarin resistance may aid elucidation of this problem. A protein which binds warfarin with a lower affinity in resistant rats has been located in microsomes and subsequently purified by Searcey *et al.* /169/. Until this protein is sequenced and its enzyme activity determined the above question will remain unanswered. As has been stated previously, the Scottish and Welsh resistant rats do not have identical resistance genes, although these are allelic /155/. Studies of the vitamin K cycle enzymes have been undertaken using Welsh resistant and Scottish resistant rats /170/ (HW and HS respectively) and compared to control susceptible rats (Tolworth Albino strain - TAS). Wild resistant rats, *R. norvegicus* were inbred with *TAS rats* to produce the two strains HS and HW, homozygous for either the Scottish (HS) or Welsh (HW) resistance genes, but closely related to the laboratory susceptible TAS rats /157/. In both resistant strains, the vitamin K_1 reductase activities are less sensitive to warfarin /170/. However, susceptible and HS rats are sensitive to co-administration of warfarin and vitamin K_1. The HW strain is also resistant to warfarin's anticoagulant effect under these conditions. Therefore in the HW strain, it is postulated that the major anticoagulant effect of warfarin is not that observed on vitamin K_1 reductase, although this enzyme is less susceptible to warfarin inhibition in this resistant strain. The expression of the Rw gene only appears to affect the vitamin K-epoxide reductase enzyme /170/, although the vitamin K-epoxide reductase enzyme has not been investigated in this strain.

Since most studies only compare one resistant strain with a susceptible one, the above results may present an over-simplified picture. Thus, both the epoxide reductase and vitamin K_1 reductase enzymes may be involved in resistance, depending on the strain of resistant rat studied.

Studies on the vitamin K_1-dependent γ-glutamyl carboxylase enzyme have been performed, and it has been reported that γ-glutamyl carboxylase is inhibited by warfarin *in vitro* /171/. The activity of the enzyme is induced by development of a hypoprothrombinaemic state due to vitamin K deficiency, or treatment with anticoagulants, and there is an increased level of γ-glutamyl carboxylase in resistant rats /172/. It is possible that this increase in activity maintains sufficient vitamin K-dependent clotting factors in the face of a marginal supply of the vitamin. Millimolar concentrations of warfarin are needed to inhibit the carboxylase enzyme, but it is unlikely that altered γ-glutamyl carboxylase is a result of expression of the resistance gene in the rat.

In summary, the warfarin-resistance gene is thought to manifest itself in an altered warfarin binding protein which has a slightly decreased affinity for vitamin K_1 and a greatly reduced affinity for warfarin. However, the nature of this binding protein has not been characterised. The alteration of affinities and properties of enzymes in the vitamin K cycle have been discussed, providing various possibilities for an explanation of warfarin-resistance. However, the biochemical expression of the warfarin-resistant gene in the rat essentially remains uncharacterised.

4.2 Vitamin K_1-independent resistance

Warfarin was first used as a rodenticide in the 1950's and infestations of the house mouse became increasingly difficult to control, even after continuous warfarin treatment /173/. Although warfarin resistant rats have been shown to possess an increased dietary requirement for vitamin K_1, there are not reports of such a requirement in the warfarin-resistant house mouse *Mus musculus*. Therefore, in mice resistance would appear to be vitamin K_1-independent.

Infestations of the house mouse are generally more difficult to control with warfarin than rats due to warfarin being comparatively less toxic to mice and the mouse being a more sporadic and diffuse feeder and therefore may ingest sublethal doses /174/. Bait shyness is not

encountered in the mouse, which may be a problem at times in control of rat infestations. Early studies /174,175/ suggested that more than one gene may be involved in anticoagulant resistance in the house mouse. Investigations undertaken by Wallace and MacSwiney /176/ have shown the existence of a major gene controlling warfarin resistance in the house mouse. The spread of a certain 'cream' mutant in a wild population of house mouse was noted. The hypothesis that the gene responsible for this colour (extreme chinchilla c^e) has spread because of linkage with a major gene for warfarin-resistance was tested by linkage backcrosses. The results show that a major gene does exist, that it is very closely linked with frizzy, fr, on chromosome 7, which in turn is linked with extreme chinchilla, c^e /176/. The gene is fully dominant in females at 4 months old and its partial dominance in males is under the control of modifiers. The position of the gene, War, may be analogous with the position of the resistance gene Rw in the rat. The presence of the single major gene, War, rules out the possibility of acquired warfarin resistance following several sublethal doses of the anticoagulant.

The physiological mechanism(s) involved in warfarin resistance in mice is (are) unknown. This resistance, however, may be due to abnormal supplies of vitamin K_1, poor absorption of warfarin from the gastrointestinal tract, rapid excretion or metabolic transformation of warfarin, or altered enzyme activity resulting in a general decrease in sensitivity to warfarin. Toxicity studies have indicated that some mice, particularly females, have a low response to large doses of warfarin and there is evidence of induced tolerance on repeated anticoagulant administration /177/. The same investigation showed that some resistance mice are able to live for several weeks with practically incoagulable blood without any sign of haemorrhages. This physiological state has been reported as occurring spontaneously in other small rodents, where these animals do not bleed /178,179/. This is compared to normal susceptible mice which have incoagulable plasma after 3 days, whereas the PCA in resistant mice is either not decreased or has returned to normal before 21 days continuous feeding /177/.

It has also been reported /166/ that administration of sulphaquinoxaline (which decreases the bacterial flora in the gut of mice and therefore diminishes vitamin K_2 synthesis) does not render either resistant or normal mice more susceptible to anticoagulant action. The vitamin K requirement of mice, especially those which are resistant to warfarin is not as large as that seen in the rat.

A sex variation in response to warfarin is documented in adult mice, females being more resistant to anticoagulant action than males. Male mice become more susceptible to warfarin poisoning with maturity but females do not show the same trend. Therefore, both sex and age have effects on warfarin resistance in the house mouse /51/. Resistant mice have also been shown to exhibit sex differences, females again being more resistant than males to the anticoagulant action of warfarin. Studies on mice given warfarin by injection, and in feed, indicate that in females, the level of resistance to warfarin may be assessed on the basis of their response to a single injected dose of warfarin /177/.

Few studies have been undertaken to investigate the biochemical manifestation(s) of the warfarin resistance gene of mice. However, evidence so far uncovered suggests that a more complex resistance mechanism is operating in the house mouse as compared to the rat.

Wood and Conney /121/ suggested that genetic variation in coumarin hydroxylase activity in the mouse was a biochemical consequence of resistance. Of the four strains of mice studied one showed a 3-4 times higher coumarin hydroxylase activity than the others. In addition this strain was less sensitive to the effects of warfarin. Whether this is related to the formation of 7-hydroxywarfarin has been further investigated. Sixteen strains of mice were used to try and correlate coumarin 7-hydroxylase activity with warfarin resistance in mice, as it seemed plausible that the two events may be related due to the fact that warfarin is inactivated by hydroxylation. However, no correlation between coumarin-hydroxylating ability and warfarin resistance was found /180/. Correlation of hexobarbitone sleeping time and zoxazolamine paralysis times with warfarin resistancse in several mouse strains, could be due to genetic variation of a component of the microsomal system which is involved in several hydroxylations /119/. However, this component is clearly not coumarin 7-hydroxylase.

The evidence from the studies suggest that the biochemical expression of the warfarin resistance gene in the mouse may have an effect on absorption, distribution or metabolism of the anticoagulant. Further investigation is needed to elucidate the physiological site of warfarin resistance in the house mouse.

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REFERENCES

1. O'Leary, T.J. and Abbrecht, P.H. Predicting oral anticoagulant response using a pharmacodynamic model *Annal. Biomed. Eng.* 1981; **9**: 199-216.
2. Heimark, L.D. and Trager, W.F. Stereoselecstive metabolism of conformational analogues of warfarin by β-naphthoflavone-inducible-cytochrome P-450. *J. Med. Chem.* 1985; 28:503-505.
3. Thierry, M.J., Hermondson, M.A. and Suttie, J.W. Vitamain K and warfarin distribution and metabolism in the warfarin-resistant rat. *Am. J. Physiol.* 1970; 219:854-859.
4. Hewick, D.S. The plasma half-lives of the enantiomers of warfarin in warfarin-resistant and warfarin-susceptible rats. *J. Pharm. Pharmac.* 1972; 24: 661-662.
5. Bell, R.G. and Caldwell, P.T. Mechanism of warfarin resistance: warfarin and the metabolism of vitamin K. *Biochemistry* 1973; 12: 1759-1762.
6. Stenflo, J. Vitamin K, prothrombin and γ-carboxyglutamic acid. *New Engl. J. Med.* 1977; 296: 624-625.
7. Nelsestuen, G.L., Zytokovitz, T.M. and Howard, J.B. The mode of action of vitamin K. Identification of γ-carboxyglutamic acid as a component of prothrombin. *J. Biol. Chem.* 1974; 249: 6347-6350.
8. Jackson, C.M. and Suttie, J.W. Recent developments in understanding the mechanism of vitamin K and vitamin K-antagonist drug action and the consequences of vitamin K in blood coagulation. *Prog. Haematol.* 1977; 10: 333-359.
9. Hauschka, P.V., Friedman, P.A., Traverso, H.P. and Callop, P.M. Vitamin K dependent γ-carboxyglutamic acid formation by kidney microsomes *in vitro*. *Biochem. Biophys. Res. Commun.* 1976; 71: 1207-1213.
10. Buchtal, S.D. and Bell, R.G. Vitamin K dependent carboxylation in spleen and kidney. In: Suttie, J.W., ed., Vitamin K metabolism and vitamin K-dependent proteins. Baltimore: University Park Press, 1979; 299-302.
11. Bell, R.G. Vitamin K-dependent carboxylation in lung microsomes. In vitamin K metabolism and vitamin K-dependent proteins. In: Suttie, J.W., ed., Vitamin K metabolism and vitamin K-dependent proteins. Baltimore: University Park Press, 1979; 286-293.
12. Traverso, H.P., Haschka, P.V. and Gallop, P.M. Vitamin K-dependent carboxylation in microsomal preparations derived from cultural kidney cells, chick embryo fibroblasts and pancreas. In: Suttie, J.W., ed., Vitamin

K metabolism and vitamin K-dependent proteins. Baltimore: University Park Press, 1979; 311-314.

13. Friedman, P.A., Hauschka, P.V., Shia, M.A. and Wallace, J.K. Characteristics of the vitamin K-dependent carboxylating system in human placenta. *Biochem. Biophys. Acta* 1979; 583: 261-265.
14. Hauschka, P.V., Lian, J.B. and Gallop, P.M. Direct identification of the calcium-binding amino-acid γ-carboxyglutamate in mineralised tissue. *Proc. Nat. Acad. Sci. (USA)* 1975; 72: 3925-3929.
15. Hauschka, P.V. Osteocalcin in developing bone systems. In: Suttie, J.W., ed., Vitamin K metabolism and vitamin K-dependent proteins. Baltimore: University Park Press, 1979; 227-236.
16. Matschiner, J.T., Zimmerman, A. and Bell, R.G. The influence of warfarin on vitamin K epoxide reductase. *Thomb. Diat. Haemorrh. (suppl)* 1974; 57: 45-52.
17. Suttie, J.W., Geweke, L.O., Martin, S.L. and Willingham, A.K. Vitamin K epoxidase: Dependence of epoxidase activity on substrates of the vitamin K-dependent carboxylation reactions. *FEBS Lett.* 1980; 109: 267-270.
18. Patterson, J.L., Preusch, P.C. and Suttie, J.W. Purification and stereospecificity of the microsomal vitamin K 2,3-epoxide reductase. *Fed. Proc.* 1981; 40: (abs) 1584.
19. Campbell, H.A. and Link, K.P. Studies on the haemorrhagic sweet clover disease IV. Isolation and crystallization of the haemorrhagic agent. *J. Biol. Chem.* 1941; 138:21-29.
20. Dam, H. The antihaemorrhagic vitamin of the chick. *Biochem. J.* 1935; 29: 1273-1285.
21. Doisy, E.A. Jr. Nutritional hypoprothrombinaemia and metabolism of vitamin K. *Fed. Proc.* 1961; 989-991.
22. Quick, A.J. One-stage prothrombin time in the control of anticoagulant therapy. In: Nichol, E.S., et al., eds., Anticoagulant therapy in ischemic heart disease. New York: Grune and Stratton, 1969; 20: 381-388.
23. Arora, R.B. and Mathur, C.N. Relationship between structure and anticoagulant activity of coumarin derivatives. *Br. J. Pharmacol.* 1963; 20: 29-35.
24. O'Reilly, R.A. and Aggeler, P.M. Studies on coumarin anticoagulant drugs: Initiation of warfarin therapy without a loading dose. *Circulation* 1968; 38: 169-173.
25. Vainieri, H. and Wingard, L.B. Jr. Effects of warfarin on the kinetics of vitamin K-dependent clotting factors in rats. *J. Pharmacol. Exp. Ther.* 1977; 201: 507-517.
26. Lowenthal, J and Birnbaum, H. Vitamin K and coumarin anticoagulants. Dependence of anticoagulant effect on inhibition of vitamin K transport. *Science* 1969; 181-183.
27. Goynes, L.M. and Bridges, R.A. Studies on the mode of action of dicoumarol. *Biochem. Pharmacol.* 1965; 14: 579-587.
28. Hemker, H.C., Veltkamp, J.J., Hensen, A. and Loeliger, E.A. Nature of prothrombin biosynthesis: Preprothrombinaemia in vitamin K deficiency. *Nature* 1963; 200: 589-590.

29. Vermeer, C., Soute, B.A.M., Govers-Riemslag, J. and Hemker, H.C. *In vitro* prothrombin synthesis from a precursor protein. I. Development of a bovine liver cell-free system. *Biochem. Biophys. Acta* 1976; 44: 926-929.
30. Vermeer, C., Soute, B.A.M. and Hemker, H.C. *In vitro* prothrombin synthesis from a purified precursor protein. II. Partial purification of bovine carboxylase. *Biochem. Biophys. Acta* 1978; 523: 494-505.
31. Friedman, P.A., Rosenburg, R.D., Hauschka, P.V. and Fitz-James, A. A spectrum of partially carboxylated prothrombins in the plasma of coumarin-treated patients. *Biochem. Biophys. Acta* 1977; 494: 271-276.
32. Levy, R.J. and Lian, J.B. γ-carboxyglutamate excretion and warfarin therapy. *Clin. Pharmacol. Ther.* 1972; 25:562-570.
33. Lorusso, D.J. and Suttie, J.W. Warfarin binding to microsomes isolated from normal and warfarin-resistant rat liver. *Mol. Pharmacol.* 1972; 8: 197-203.
34. McBurney, A., Schearer, M.J. and Barkhan, P. Preparative isolation and characterisation of the urinary aglycones of Vitamin K_1 (phylloquinone) in man. *Biochem. Med.* 1980; 24: 250-267.
35. Caldwell, P.T., Ren, P. and Bell, R.G. Warfarin and the metabolism of vitamin K_1. *Biochem. Pharmacol.* 1974; 23: 3353-3362.
36. McBurney, A., Schearer, M.J. and Barkhan, P. Changes in the urinary metabolites of phylloquinone (vitamin K_1) in man following therapeutic anticoagulation with warfarin. *Biochem. Pharmacol.* 1978; 27: 273-278.
37. Ren, P., Stark, P.Y., Johnson, R.L. and Bell, R.G. Warfarin and the metabolism of vitamin K_1. *Biochem. Pharmacol.* 1974; 23: 3355-3357.
38. Wingard, L.B. Jr. and Levy, G. Kinetics of the oral anticoagulant effect of dicoumarol in rats. *J. Pharmacol. Exp. Ther.* 1973; 184: 253-260.
39. Willingham, A.K. and Matschiner, J.T. Changes in phylloquinone epoxidase activity related to prothrombin synthesis and microsomal clotting activity in the rat. *Biochem. J.* 1974; 140: 435-441.
40. Whitlon, D.S., Sadowski, J.A. and Suttie, J.W. Mechanism of coumarin action: Significance of vitamin K epoxide reductase inhibition. *Biochemistry* 1978; 17: 1371-1377.
41. Fasco, M.J., Hildebrandt, E.F. and Suttie, J.W. Evidence that wwarfarin anticoagulant action involves two distinct reductase activities. *J. Biol. Chem.* 1982; 257: 11210-11212.
42. Hildebrandt, E.F. and Suttie, J.W. Mechanism of coumarin action: Sensitivity of vitamin K-metabolising enzymes of normal and warfarin-resistant rat liver. *Biochemistry* 1982; 21: 2406-2411.
43. Lowenthal, J. and MacFarlane, J.A. The nature of the antagonism between vitamin K and indirect anticoagulants. *J. Pharmacol. Exp. Ther.* 1964; 143: 273-277.
44. Olson, R.E. New concepts relating to the model of action of vitamin K. *Vitam. Horm.* 1974; 32: 483-511.
45. Silverman, R.B. Model studies for a molecular mechanism of action of oral anticoagulants. *Am. J. Chem. Soc.* 1981; 103: 3910-3915.
46. Silverman, R.B. A model for a molecular mechanism of anticoagulant

activity of 3-substituted 40hydroxycoumarins. *J. Am. chem. Soc.* 1980; 102: 5421-5423.

47. Weiner, M., Brodie, B.B. and Burns, J.J. In: Koller, T. and Merz, W.R., eds., Thrombosis and embolism. Proceedings of 1st International Conference. Benno, Schwabe and co., 1954; 181-193.
48. Pyörälä, K. Sex differences in the clotting factor response to warfarin and in the rate of warfarin metabolism in the rat. *Ann. Med. Exp. Fenn.* 1968; 46: 23-24.
49. Hall, D.E. blood coagulation and its disorders in the dog. London: Bailliere Tindal, 1972; 54-55.
50. Metta, V.C. and Johnson, B.C. Effect of feeding vitamin K-deficient diets to female rats. *J. Nutr.* 1960; 72: 455-458.
51. Rowe, F.P. and Redfern, R. The effect of sex and age on the response to warfarin in a non-inbred strain of mice. *J. Hyg Cambr.* 1967; 65: 55-60.
52. Shah, D.V. and Suttie, J.W. Vitamin K requirement and warfarin tolerance in the hamster (38987). *Proc. Soc. Exp. Biol. Med.* 1975; 150: 126-130.
53. Bell, R.G. Metabolism of vitamin K and prothrombin synthesis: Anticoagulants and the vitamin K-epoxide cycle. *Fed. Proc.* 1978; 37: 2599-2604.
54. Shearer, M.J., McBurney, A. and Barkhan, P. Effect of warfarin anticoagulation of vitamin K_1 metabolism in man. *Br. J. Haemat.* 1973; 24: 471-479.
55. Park, B.K., Leck, J.B., Wilson, A.C., Serlin, M.J. and Breckenridge, A.M. A study of the effect of anticoagulants on ^3H vitamin K_1 metabolism and prothrombin complex activity in the rabbit. *Biochem. Pharmacol.* 1979; 28: 1323-1329.
56. Harris, P.A. and Riegelman, S. Influence of the route of administration on the area under the plasma concentration-time curve. *J. Pharm. Sci.* 1969; 58: 71-75.
57. Julkunen, R.J.K., Pyörälä, K., Airaksinen, M.M. and Slurala, M. The Effect of pH on the absorption of warfarin from rat small intestine in vitro. Ann. Med. Exp. Biol. Fenn. 1971; 49: 38-44.
58. Coon, W.W. and Willis, P.W. Some aspects of the pharmacology of oral anticoagulants. *Clin. Pharmacol. Ther.* 1970; 11: 312-336.
59. Schanker, L.S., Tocco, D.J., Brodie, B.B. and Hogben, C.A.M. Absorption of drugs from the rat small intestine. *J. Pharmacol. Exp. Ther.* 1958; 123: 81-88.
60. Kakemi, K., Arita, T., Hori, R., *et al.* Absorption and excretion of drugs. An aspect of the mechanism of drug absorption from the intestinal tract in rats. *Chem. Pharm. Bull.* 1969; 17: 255-261.
61. Breckenridge, A.M. and Orme, M. Kinetics of warfarin absorption in man. *Clin. Pharmacol. Ther.* 1973; 14: 955-961.
62. Diertle, W., Faigle, J.W., Montigel, C., Sulc, M. and Theobald, W. Biotransformation and pharmacokinetics of acenocoumarol (Sintrom) in man. *Eur. J. Clin. Pharmacol.* 1977; 11: 367-375.
63. O'Reilly, R.A., Aggeler, P.M., and Leong, L.S. Studies on coumarin anticoagulant drugs: Pharmacodynamics of warfarin in man. *J. Clin. Invest.* 1963; 42: 1542-1551.

64. Park, B.K. and Leck, J.B. On the mechanism of salicylate-induced hypoprothrombinaemia. *J. Pharm. Pharmacol.* 1980; 33: 25-28.
65. Gosselin, R.E., Smith, R.P. and Hodge, H.C. Clinical toxicology of commercial products. Baltimore: Williams and Wilkins, 1984; 395-397.
66. Fristedt, B. and Sterner, N. Warfarin intoxication from percutaneous absorption. *Arch. Environ. Health* 1965; 11: 205-208.
67. Martin-Bouyer, G., Linh, P.D., Tuan, L.C., *et al.* Epidemic of haemorrhagic disease in Vietnamese infants caused by warfarin-contaminated talcs. *Lancet* 1983; 1: 230-231.
68. Lewis, R.J., Trager, W.F., Chan, K.K., *et al.* Warfarin. Stereochemical aspects of its action and the interaction with phenylbutazone. *J. Clin Invest.* 1974; 53: 1607-1617.
69. O'Reilly, R.A. Studies on the topicalenantiomorphs of warfarin in man. *Clin. Pharmacol. Ther.* 1974; 16: 348-354.
70. Anderson, G.F. The distribution of warfarin (coumadin) in the rat. *Thromb. Diath. Haemorrh.* 1967; 18: 754-758.
71. Wong, L.T., Solomonaj, G. and Thomas, B.H. Fate of ^{14}C-warfarin in guinea-pigs: Effects of a lconcomitant single dose of salicylate. *J. Pharm. Pharmac.* 1978; 30: 240-243.
72. Jaques, L.B. Anticoagulant therapy and pharmacological principles. Springfield, lll: Thomas C, 1965; 105-109.
73. Takada, K. and Levy, G. Comparative pharmacokinetics of coumarin anticoagulants XLIII: Concentration-dependent hepatic uptake of warfarin in rats. *J. Pharm. Sci.* 1979; 63: 1569-1572.
74. Takada, K. and Levy, G. Comparative pharmacokinetics of coumarin anticoagulants XLIV: Dose-dependent pharmacokinetics in the rat. *J. Pharm. Sci.* 1980; 69: 9-14.
75. Laliberte, R., Chakrabarti, S. and Brodeur, J. The influence of fasting on the disposition of warfarin in rats. *J. Pharmacol. Exp. Ther.* 1977; 200: 44-51.
76. Covell, D.G. and Abbrecht, P.H. Changes in warfarin pharmacology during chronic administration. *Fed. Proc.* 1980; 39: 746.
77. O'Reilly, R.A. and Kowitz, P.E. Studies on the coumarin anticoagulant drugs: Interaction of human plasma albumin and warfarin sodium. *J. Clin. Invest.* 1967; 46: 829-837.
78. O'Reilly, R.A. Interaction of several coumarin compounds with human and canine plasma albumin. *Mol. Pharmacol.* 1970; 7: 209-218.
79. Koch-Weser, J. and Seller, E.M. Binding of drugs to serum albumin. *New Engl. J. Med.* 1967; 294: 311 and 526.
80. O'Reilly, R.A. The binding of sodium warfarin to plasma albumin and its displacement by phenylbutazone. *Ann. NY Acad. Sci.* 1973; 226: 293-308.
81. Overman, R.S., Stahmann, M.A., Sullivan, W.R., Heubner, C.F., Campbell, H.A. and Link, K.P. Studies on the haemorrhagic sweet clover disease VII: The effect of 3-3 methylene bis-(4-hydroxycoumarin) on the prothrombin time of the plasma of various animals. *J. Biol. Chem.* 1942; 142: 941-943.
82. Hagen, E.C. and rodomski, J.L. The toxicity of 30(acetonylbenzyl)-4-

hydroxycoumarin (warfarin) to laboratory animals. *J. Am. Pharm. Ass.* 1953; 42: 379-382.

83. Deckert, F.W. Coumarin anticoagulants: A review of some current research areas. *5th Med. J.* 1974; 67: 1191-1202.
84. Seller, E.M., Lang-Sellers, M.L. and Koch-Weser, J. Comparative warfarin binding to albumin from various species. *Biochem. Pharmacol.* 1977; 26: 2445-2447.
85. Chignell, C.F. Optical studies of drug-protein complexes. The interaction of warfarin and dicoumarol with human serum albumin. *Mol. Pharmacol.* 1970; 6: 1-12.
86. Sellers, E.M. and Koch-Weser, J. Interaction of warfarin stereoisomers with human albumin. *Pharmac. Res. Commun.* 1975; 7: 331-339.
87. Wosilait, W.D., Ryan, M.P. and Byington, K.H. Competition between serum albumin and the soluble fraction of liver for binding of wawrfarin and other drugs. *Res. Commun. Chem. Pathol. Pharmacol.* 1981; 32: 113-121.
88. Yacobi, A. and Levy G. Comparative pharmacokinetics of coumarin anticoagulants. XXI: Effect of plasma protein binding on the distribution kinetics of warfarin. *J. Pharm. Sci.* 1977; 66: 567-572.
89. Jaques, L.B. Froese, E.L., O'Toole, R. and Spinks, J.W.T. Relationship between duration of hypoprothombinaemia with dicoumarol and the level of the drug in the liver. *Arch. Int. Pharmacol.* 1957; 111: 478-489.
90. Routledge, P.A., Chaptman, P.H., Davies, D.M., Rawlins, M.D. Pharmacokinetics and pharmacodynamics of warfarin at steady state. *Br. J. Clin. Pharmacol.* 1979; 8: 243-247.
91. Testa, B. Regio- and stereoselectivity in drug metabolism: two example. *Pharm. Int.* 1981; 2:34-37.
92. O'Reilly, R.A., Aggeler, P.M., Hoag, M.S. and Leong, L. Studies on the coumarin anticoagulant drugs: The assay of warfarin and its biological application. *Thomb. Diath. Haemorrh.* 1962; 8: 82-95.
93. Barker, W.M., Hermondsen, M.A. and Link, K.P. The metabolism of 4-^{14}C warfarin sodium by the rat. *J. Pharmacol. Exp. ther.* 1970; 171: 307-313.
94. Kaminsky, L.S. Dannan, G.A. and Guengerich, F.P. Composition of cytochrome P-450 isozymes from hepatic microsomes of C57BL/6 and DBA/2 mice, assessed by warfarin metabolism, immunoinhibition, and immunoelectrophoresis and anti-rat cytochrome P-450. *Eur. J. Biochem.* 1984; 141: 141-148.
95. Ikeda, M., Ullrich, V. and Staudinger, H. Metabolism *in vitro* of warfarin by enzymic and non-enzymic systems. *Biochem. Pharmacol.* 1968; 17: 1663-1669.
96. Ikeda, M., Conney, A.H. and Burns, J.J. Stimulatory effects of phenobarbital and insecticides on warfarin metabolism in rats. *J. Pharm. Exp. Ther.* 1968; 162: 338-343.
97. Townsend, M.G., Odam, E.M. and Page, J.M.J. Studies of the microsomal drug metabolism system in warfarin-resistant and susceptible rats. *Biochem. Pharmacol.* 1975; 24: 729-735.

98. Hewick, D.S. and Moreland, T.A. An NADPH-dependent warfarin reductase in human liver and kidney soluble fraction. *Br. J. Pharmacol.* 1975; 53: 441.
99. Liebman, K.C. Reduction of ketones in liver cytosol. *Xenobiotica* 1971; 1: 97-104.
100. Lewis, R.J. and Trager, W.F. Warfarin metabolism in man: Identification of metabolites in urine. *J. Clin. Invest.* 1970; 49: 907-913.
101. Lewis, R.J. and Trager, W.F. The metabolic fate of warfarin: Studies on metabolites in the plasma. *Ann. NY Acad. Sci.* 1971; 179: 205-212.
102. Christensen, F. Studies on the fate of dicoumarl-^{14}C in the rat. *Acta Pharmacol. toxicol.* 1956; 22: 141-151.
103. Haddock, R.E., Trager, W.F. and Pohl, L.R. Biotransformation of phenprocoumon in the rat. *J. Med. Chem.* 1975; 18: 519-523.
104. Burns, J.J., Weiner, M., Simson, G. and Brodie, B.B. The biotransformation of ethyl biscoumacetate (Tromexan) in man, rabbit and dog. *J. Pharmacol. Exp. Ther.* 1953; 108: 33-41.
105. Shilling, W.H., Crampton, R.F., Longland, R.C. Metabolism of coumarin in man. *Nature* 1969; 221: 664-665.
106. Nugashima, R. and Levy, G. Comparative pharmacokinetics of coumarin anticoagulants: Kinetics of warfarin elimination in the rat, dog, and rhesus monkey compared to man. *J. Pharm. Sci.* 1969; 58: 845-849.
107. Hewick, D.S., and McEwen, J. Plasma half-lives, plasma metabolites and anti-coagulant efficacies of the enantiomers of warfarin in man. *J. Pharm. Pharmac.* 1973; 25: 458-459.
108. Breckenridge, A.M. and Orme, M. The plasma half-lives and the pharmacological effect of the enantiomers of warfarin in therat. *Life Sci.* 1972; 11: 337-345.
109. Pohl, L.R., Nelson, S.D., Porter, W.R., *et al.* Warfarin: stereochemical aspects of its metabolism by rat liver microsomes. *Biochem. Pharmacol.* 1976; 25: 2153-2162.
110. Fasco, M.J., Piper, L.J. and Kaminsky, L.S. Differences in the interactions of R and S warfarin with hepatic microsomal cytochrome P-450. In: Ullrich, V., Roobis, 1., Hildebrandt, A., Estabrook, R.W., Conney, A.H., eds., Microsomes and drug oxidations. Pergamon Press, 1976; 136-143.
111. Chan, K.J., Lewis, R.J. and Trager, W.F. Absolute configurations of the four warfarin alcohols. *J. Med. Chem.* 1972; 15: 1265-1270.
112. Lewis, R.J., Trager, W.F., Robinson, A.J. and Chan, K.J. Warfarin metabolites: The anticoagulant activity and pharmacology of warfarin alcohols. *J. Lab. Clin. Med.* 1973; 81: 925-931.
113. Fasco, M.J., Vatsis, K.P., Kaminsky, L.S. and Coon, M.J. Regio- and stereoselective hydroxylation of R and S warfarin by different forms of purified cytochrome P-450 from rabbit liver. *J. Biol. Chem.* 1978; 253: 7813-7820.
114. Jerina, D.M. and Daly, J.W. Arene oxides. A new aspect of drug metabolism. *Science* 1974; 185: 573-582.
115. Kaminsky, L.S., Fasco, M.J. and Guengerich, F.P. Comparison of different

forms of liver, kidney and lung microsomal cytochrome P-450 by immunological inhibition of regio- and stereoselective metabolism of warfarin. *J. Biol. Chem.* 1979; 254: 9657-9662.

116. Kaminsky, L.S. Fasco, M.J., and Guengerich, F.P. Comparison of different forms of purified cytochrome P-450 from rat liver by immunological inhibition of regio- and stereoselective metabolism of warfarin. *J. Biol. Chem.* 1980; 255: 85-91.
117. Baron, J., Redick, J.A. and Guengerich, F.P. Immunohistochemical localizations of cytochromes P-450 in the rat liver. *Life Sci.* 1978; 23: 2627-2631.
118. Westfall, B.A., Boules, B.M., Shields, J.L. and Garb, S. Sex differences in pentobarbital sensitivity in mice. *Proc. Soc. Exp. Biol. Med.* 1964; 115: 509-510.
119. Lush, I.E. A survey of the response of different strains of mice to substances metabaolised by microsomal oxidation: hexabarbitone, zoxazolamine and warfarin. *Chem. Biol. Interact.* 1976; 12: 363-373.
120. Vessell, E.S. and Page, J.G. Genetic control of dicoumarol levels in man. *J. Clin. Invest.* 1968; 47: 2657-2663.
121. Wood, A.W. and Conney, A.H. Genetic variation in coumarin hydroxylase activity in the mouse ('Mus musculus'). *Science* 1974; 185: 612-613.
122. Sato, R. and Omura, T. A short history of cytochrome P-450. In Sato, R., Omura, T., eds., Cytochrome P-450. New York: Kodansha Ltd., Academic Press.
123. Pickett, C., Jeter, R., Moris, J. and Lu, A.Y.H. electroimmunochemical quanitation of cytochrome P-450, cytochrome P-448 and epoxide hydrolase in rat liver microsomes. *J. Biol. Chem.* 1981; 256: 8815-8820.
124. Johnson, E.F. Contrasting properties of cytochrome P-450 isozynes: Characterisation identification and significance of multiple forms of rabbit microsomal cytochrome P-450. In: Gustafsson, J.A., Caristedt-Duke, J., Mode, A. and Rafter, J., eds., Biochemistry, Biophysics and Regulation of Cytochrome P-450. Elsevier/North Holland Biomedical Press, 1980; 65-72.
125. Haugen, M., Coon, M.J. and Nebert, D.W. Induction of multiple forms of mouse liver cytochrome P-450. *J. Biol. Chem.* 1976; 251: 1817-1827.
126. Beaune, P., Dansette, P., Flinois, J., Columelli, S., Mansuy, D. and Leroux, J.P. Partial purification of human liver cytochrome P-450. *Biochem. Biophys. Res. Commun.* 1979; 88: 826-832.
127. Losito, R. and Rousseau, A.M. ^{14}C-warfarin excretion in the rat. *Thomb Diath. Haemorrh.* 1972; 27: 300-308.
128. Link, K.P., Berg, D. and Barker, W.M. Partial fate of warfarin in the rat. *Science* 1965; 150: 378.
129. Wong, L.T., and Solomonraj, G. Biliary and urinary excretion of ^{14}C warfarin in rabbits. *Xenobiotica* 1980; 10: 210-210.
130. Breckenridge, A.M., Orme, M., Wesseling, H., Lewis, R.J., and Gibbons, R. Pharmacokinetics and pharmacodynamics of the enantiomers of warfarin in man. *Clin. Pharmacol. Ther.* 1973; 15: 424-430.
131. Goding, L.A. and West, B.D. Synthesis and relative urinary excretion rates

of the enantiomers of warfarin-4^{14}C and phenprocoumon-2-^{14}C. *J. Med. Chem.* 1969; 12: 517-518.

132. Yacobi, A., Wingard, L.B. and Levy, G. Comparative pharmacokinetics of coumarin anticoagulants X: relationship between distribution, elimination and anticoagulant action of warfarin. *J. Pharm. Sci.* 1974; 63: 868-872.
133. Levy, G. and Yacobi, A. Effect of plasma protein binding on the elimination of warfarin. *J. Pharm. Sci.* 1974; 63: 803-806.
134. Yacobi, A. and Levy, G. Comparative pharmacokinetics of coumarin anticoagulants XIV: relationship between protein binding, distribution and elimination kinetics. *J. Pharm. Sci.* 1975; 64: 1660-1664.
135. Lee, M., Schwartz, R.N. and Sharifi, R. Warfarin resistance and vitamin K. *Ann. Intern. Med.* 1981; 94: 140-141.
136. Holt, R.J. and Freytes, C.O. Familial warfarin resistance. *Drug. Int. Clin. Pharmacol.* 1983; 17: 281-281.
137. Zallman, J.A., Lee, D.P. and Jeffrey, P.L. Liquid nutrition as a cause of warfarin resistance. *Am. J. Hosp. Pharm.* 1981; 38: 1174.
138. Lader, E., Yang, L. and Clarke, A. Warfarin dosage and vitamin K in Osmolite. *Ann. Intern. Med.* 93: 373.
139. Qureshi, G.D., Reinders, T.P., Swift, J.J. and Slate, M.B. Acquired resistance and weight-reducing diet. *Arch. Int. Med.* 1981; 141: 507-509.
140. Kempin, S.J. Warfarin resistance caused by broccoli. *New Engl. J. Med.* 1983; 308: 1229-1230.
141. Corn, M. Effect of phenobarbital and glutethimide on the biological half-life of warfarin. *Thomb. Diath. Haemorrh.* 1966; 16: 602-612.
142. Hanse, J.M., Stersboek-Nielson, K., Kristensen, M., *et al.* Effect of diphenylhydantoin on the metabolism of dicoumarol in man. *Acta Medica Scandinavica* 1971b; 189: 15-19.
143. Barnett, D.B. and Hancock, B.W. Anticoagulant resistance: an unusual case. *Br. J. Med.* 1975; 1: 608-609.
144. DeWolff, F.A. and Ten Cate J.W. Transient acquired resistance to the coumarin anticoagulants phenprocoumon and acenocoumarol. *Scand. J. Haematol.* 1979; 23: 437-441.
145. O'Reilly, R.A., Aggeler, P.M., Hoag, M.S., Leong, L.S. and Kropatkin, M. Hereditary transmission of exceptional resistance to coumarin anticoagulant drugs. *New Engl. J. Med.* 1964; 271: 809-813.
146. O'Reilly, R.A. The second reported kindred with hereditary resistance to oralanticoagulant drugs. *New Engl. J. Med.* 1970; 282: 1448-1451.
147. Marcus, P. and Kantor, T.G. Exceptional coumarin resistance in a black male. *Clin. Pharmacol. Ther.* 1973; 14: 141.
148. Greaves, J.H. Resistance to anticoagulants in rodents. *Pestic. Sci.* 1971; 2: 276-279.
149. Boyle, C.M. Case of apparent resistance of *Rattus norvegicus* Berkenhout to anticoagulant poisoning. *Nature* 1980; 188: 517.
150. Drummong, D.C. and Bentley, E.W. The resistance of rodents to warfarin in England and Wales. Rep. Int. Conf. on Rodents and Rodenticides. E.P.P.O., Paris, 1965; 57-67.

151. Lund, M. Resistance to warfarin in the common rat. *Nature* 1964; 203: 774.
152. Jackson, W.B., and Kaukeinen, D. Resistance of wild Norway rats in North Carolina to warfarin rodenticide. *Science* 1972; 176: 1343-1344.
153. Greaves, J.H. and Aryes, P. Linkages between genes for coat colour and resistance to warfarin in *Rattus norvegicus. Nature* 1969; 224: 284-285.
154. Hermondson, M.A., Suttie, J.W. and Link, K.P. Warfarin resistance and vitamin K requirements in the warfarin-resistant rat. *Am. J. Physiol.* 1969; 217: 1316-1319.
155. Greaves, J.H. and Aryes, P. Warfarin resistance and vitamin K-requirement in the rat. *Lab. Animals* 1973; 7: 141-148.
156. Partridge, G.G. Relative fitness of genotypes in a population of *Rattus norvegicus* polymorphicfor warfarin resistance. *Heredity* 1979; 43: 239-246.
157. Martin, A.D., Redfern, R., Gill, J.E. and Huson, L.W. Warfarin resistance genotype determination in the Norway rat *Rattus norvegicus. Lab Animals* 1979; 13: 209-214.
158. Hewick, D.S. The plasma half-lives of the enantiomers of warfarin in warfarin-resistant and warfarin-susceptible rats. *J. Pharm. Pharmac.* 1972; 24: 661-662.
159. Bell, R.G. and Caldwell, P.T. Mechanisms of warfarin resistance: warfarin and the metabolism of vitamin K. *Biochemistry* 1973; 12: 1759-1762.
160. Hermondson, M.A., Suttie, J.W. and Link, K.P. Warfarin resistance in the rat. *Fed. Proc.* 1968; 28: 386.
161. Thierry, M.S., Hermondson, M.A., and Suttie, J.W. Vitamin K and warfarin distribution and metabolism in the warfarin-resistant rat. *Am. J. Physiol.* 1970; 219: 854-859.
162. Bell, R.G. and Matschiner, J.T. Warfarin and the inhibition of vitamin K activity by an oxide metabolite. *Nature* 1972; 237: 32-33.
163. Ren, P., Lalibert, R.E. and Bell, R.G. Effects of warfarin, phenylilndane-dione, tetrochloropyridinol, and chloro-K on prothrombin synthesis and vitamin K_1 metabolism in normal and warfarin-resistant rats. *Mol. Pharmacol.* 1974; 10: 373-380.
164. Elliot, G.R., Odan, E.M. and Townsend, M.G. An assay procedure for lthe vitamin K_r2,3 epoxide reducing system of rat liver involving HPLC. *Biochem. Soc. Trans.* 1976; 4: 615-617.
165. Leck, J.B. and Park, B.K. A comparative study of the effects of warfarin and brodifacoum on the relationship between vitamin K_1 metabolism and clotting factor activity in warfarin-susceptible and warfarin-resistant rats. *Biochem. Pharmacol.* 1981; 30: 123-127.
166. Rowe, F.P. and Redfern, R. Laboratory studies in the toxicity of anticoagulant rodenticides to wild house mice *(Mus musculus L.). Ann. Appl. Biol.* 1968; 61: 322-326.
167. Fasco, M.J., Preusch, P.C., Hildebrandt, E. and Suttie, J.W. Formation of hydroxy-vitamin K by vitamin K-epoxide reductase of warfarin-resistant rats. *J. Biol. Chem.* 1983; 258: 4370-4380.
168. Hildebrandt, E.S., Preusch, P.C., Patterson, J.L. and Suttie, J.W. Solubiliza-

tion and characterization of vitamin K epoxide reductase from normal and warfarin-resistant rat liver microsomes. *Arch. Biochem. Biophys.* 1984; 228: 480-492.

169. Searcey, M.T., Graves, C.B. and Olson, R.E. Isolation of a warfarin-binding protein from liver endoplasmic reticulum of Sprague-Dawley and warfarin-resistant rats. *J. Biol. Chem.* 1977; 252: 6260-6267.
170. MacNicoll, A.D., Nadian, A.K. and Townsend, M.G. Inhibition by warfarin of liver microsomal vitamin K-reductase in warfarin-resistant and susceptible rats. *Biochem. Pharmacol.* 1984; 33: 1331-1336.
171. Sadowski, J.A., Easmon, C.T. and Suttie, J.W. Vitamin K-dependent carboxylase-requirements of the liver microsomal enzyme system. *J. Biol. Chem.* 1976; 251: 2770-2776.
172. Shah, D.V. and Suttie, J.W. Vitamin K-dependent carboxylase: Liver activity in various species. *Proc. Soc. Exp. Biol. Med.* 1979; 161: 498-501.
173. Dodswirth, E. Mice are spreading despite such poisons as warfarin. *Municipal Engineering London* 1961; 3746: 1668-1670.
174. Rowe, F.P. and Redfern, R. Toxicity tests on suspected warfarin-resistant house mice *(Mus musculus L.). J. Hyg. Cambr.* 1965; 63: 417-425.
175. Roll, R. Uber die wirking eines Cumarinpräparates (Warfarin) auf Häusmause *(Mus musculus L.). Z. Angew. Zool.* 1966; 53: 311-314.
176. Wallace, M.E. and MacSwiney, F.J. A major gene controlling warfarin resistance in the house mouse. *J. Hyg. Cambr.* 1976; 76: 173-181.
177. Rowe, F.P. and Redfern, R. The effect of warfarin on plasma clotting time in wild house mice *(Mus musculus L.). J. Hyg. Cambr.* 1968; 66: 159-174.
178. Lechler, E. and Penick, G.D. Blood clotting defect in hibernating ground squirrel *(Citeuustridecemlineatus). Am. J. Physiol.* 1963; 205: 985-988.
179. Denyes, A. and Carter, J.D. Clotting time of cold-exposed and hibernating hamsters. *Nature* 1961; 190: 450-451.
180. Lush, I.E. and Arnold, C.J. High coumarin 7-hydroxylase does not protect mice against warfarin. *Heredity* 1975; 35: 279-281.

ABBREVIATIONS

GLU	glutamic acid
GLA	γ-carboxyglutamic acid
PIVKA	protein induced by vitamin K antagonism
PCA	prothrombin clotting activity
DTT	dithiothreitol
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form
LD_{50}	lowest dose required to kill 50% of animals dosed
Vd	volume of distribution
VOL_{app}	apparent volume of distribution
CL_{INT}	intrinsic clearance
CL_T	total clearance
HSA	human serum albumin
MFO	mixed function oxidase
HW	homozygous Welsh resistant rats
HS	homozygous Scottish resistant rats