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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

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Abstract—The effects of the anticoagulants warfarin and brodifacoum on [^{3}H]vitamin K_1 metabolism and clotting factor activity were studied in warfarin-susceptible and warfarin-resistant rats. Brodifacoum produced the same rate of degradation of prothrombin complex activity (PCA) as warfarin in the rat and significantly reduced the activity of clotting factors, II, VII, IX and X but did not affect factor V.

Metabolic studies using [^{3}H]vitamin K_1 showed that brodifacoum increased the hepatic concentration ratio of [^{3}H]vitamin K_1 epoxide: [^{3}H]vitamin K_1 in both warfarin-susceptible and warfarin-resistant rats at doses which produced anticoagulation. Brodifacoum produced a significant increase in the rate of excretion of [^{3}H]vitamin K_1 metabolites in bile but did not affect the urinary excretion of [^{3}H]vitamin K_1 metabolites. The results indicate that brodifacoum has the same mechanism of action as warfarin and support the concept that coumarin anticoagulants reduce vitamin K_1-dependent clotting factor synthesis by interrupting the vitamin K_1–epoxide cycle.

The novel anticoagulant brodifacoum possesses the same 4-hydroxycoumarin ring system as warfarin (Fig. 1) but is a more potent rodenticide and is effective in warfarin resistant rats [1]. It was therefore suggested that the novel side-chain in brodifacoum produces more effective binding to the warfarin receptor [1]. Warfarin and related 4-hydroxycoumarin anticoagulants produce their anticoagulant effect by interfering with the vitamin K_1-dependent γ-carboxylation of glutamic acid residues in clotting factor precursor proteins [2]. The warfarin receptor is thought to be associated with the enzyme vitamin K_1 epoxide reductase which is responsible for the regeneration of vitamin K_1 from its inactive epoxide in the physiologically important vitamin K_1-epoxide cycle [3].

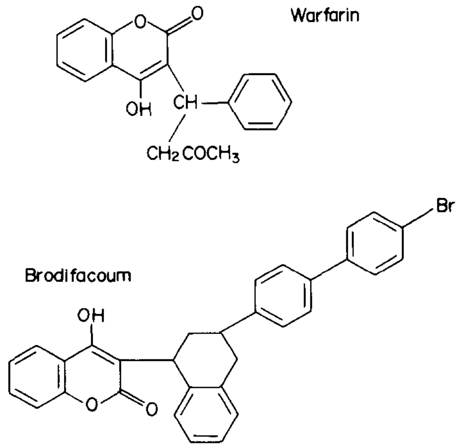

Fig. 1. Chemical structures of warfarin and brodifacoum.

In resistant animals warfarin is less able to inhibit the vitamin K_1 epoxide to vitamin K_1 conversion. It has been postulated that a mutation produces an epoxide reductase with a reduced binding affinity for both warfarin and vitamin K_1 epoxide [4, 5].

The mechanism of action of brodifacoum in the rat has not been determined but it was suggested that the novel 4′-bromo (1-1′ biphenyl) group may provide a point of attachment to a lipophilic site to which warfarin and other coumarin anticoagulants do not bind strongly [1]. We have compared the effect of brodifacoum with warfarin on the relationship between vitamin K_1 metabolism and clotting factor synthesis in both warfarin-susceptible and warfarin-resistant rats.

MATERIALS AND METHODS

Mature male rats of the Wistar strain (200–300 g) were obtained from Bantin and Kingman and male warfarin-resistant rats (250–300 g) of the Welsh Resistant strain from Sorex Laboratories, Widnes.

[5-$^3H_2$] vitamin K_1 (S.A. 21 Ci/mmole) was a gift from Hoffman La Roche, Basle. Unlabelled vitamin K_1 was obtained from Sigma, racemic sodium warfarin [3α-acetonylbenzyl)-4-hydroxycoumarin] from Ward Blenkinsop and brodifacoum 3-(3-4′-bromo(1-1′-biphenyl)]-4-yl-1,2,3,4-tetrahydro-1-naphthalenyl)-4-hydroxy-2*H*-1-benzopyran-2-one from Sorex Laboratories, Widnes. For intraperitoneal injections sodium warfarin was dissolved in 0.9% saline (1 ml/kg) and brodifacoum in dimethylsulphoxide (DMSO) (1 ml/kg). [^{3}H]vitamin K_1 was dissolved in Tween 80 and diluted with 0.9% saline to make solutions containing 5% Tween. It was necessary to chromatograph [^{3}H]vitamin K$_1$ in benzene:hexane (1:4 v/v) over silicic acid immediately before use.

Scintillant (NE 260) was obtained from Nuclear Enterprises, Edinburgh, silica gel GF 254 chromatography plates from Kodak, and all other general reagents from BDH, Poole, U.K. All solvents were redistilled before use; chloroform was stabilised by the addition of methanol (2% v/v). Thromboplastin was obtained from the National (U.K.) Reference Laboratory for Anticoagulant Reagents and Control, Manchester, and beagle dog factor VII deficient plasma was a gift from Dr. L. Poller. Factor II deficient substrate plasma and factor IX deficient substrate plasma were obtained from DADE American Hospital Suppliers. Factor X deficient substrate plasma and Russell's Viper Venom/Cephalin for use in the assay of factor X were obtained from Diagnostic Reagents Limited, Thame, Oxon, U.K. Platelet substitute with kaolin for use in the assay of factor IX was obtained from Diamed Diagnostics, Liverpool, U.K.

Measurement of prothrombin complex activity (PCA). For measurement of PCA, blood samples (0.9 ml) were collected from the tail artery into 3.8% trisodium citrate (0.1 ml) while the rat was under ether anaesthesia. Plasma was obtained using blank rat citrated plasma as previously described for rabbit plasma [7].

Measurement of individual clotting factor activity. Plasma samples for clotting factor assays were obtained as described for prothrombin measurements at 24 hr after administration of brodifacoum (1 mg/kg).

Factor V was assayed within 2 hr of collecting the plasma as this factor is known to be unstable. Factors II, VII, IX and X were assayed after completion of the experiment on frozen plasma aliquots. The aliquots were never refrozen and a freshly thawed aliquot was used for each assay. Assays for individual clotting factors [8] were carried out for all animals at the same time. A standard curve was obtained using serial dilutions of control pooled ($n = 4$) plasma. Clotting time was plotted against dilutions on double logarithmic graph paper [9]. The factor II assay was based upon the one-stage prothrombin time using DADE coagulation factor II deficient substrate plasma. The standard deviation of the assay when performed repeatedly on the same sample was ± 1.9%. Assay variation was determined according to Vainieri and Wingard (1977) [10]. The factor V assay was based upon the one-stage prothrombin time using human plasma made deficient in factor V by the method described by Quick (1960) [11]. The standard deviation of this assay was ± 1.4%. The factor VII assay was based upon the one-stage prothrombin time using beagle dog factor VII deficient plasma which has been shown to be comparable to using human factor VII deficient plasma [12]. The standard deviation of the assay was ± 2.3%. The factor IX assay was based on the one-stage Kaolin–Cephalin Clotting Time (KCCT) using human factor IX deficient substrate plasma. The standard deviation of the assay was ± 1.1%. The factor X assay was a one-stage method using DIAGEN factor X deficient substrate plasma and Russell's Viper Venom with cephalin as thromboplastin. The standard deviation of the assay was ± 3.6%.

Study of vitamin K$_1$ metabolism. For [^{3}H]vitamin K$_1$ metabolism studies the anticoagulant brodifacoum was injected i.p. in DMSO into test rats and DMSO alone was injected by the same route into control rats.

One hour later the animals were anaesthetised by urethane (14% solution: 1 ml/100 g) and the jugular vein was cannulated with pp50 tubing for an i.v. injection of [^{3}H]vitamin K$_1$ (5 μCi in 0.2 ml 5% Tween–saline) washed in with 0.9% saline (0.2 ml). Two hours after the administration of the [^{3}H]vitamin K$_1$ a blood sample (5 ml) was collected into heparin by cardiac puncture and the liver was dissected out, homogenized in 2 vol. KCl (1.15 M) and frozen (−30°) until assayed. Liver homogenate (1 ml) was extracted by mixing with acetone:ether (1:4 v/v; 2 ml) and mixing mechanically by tumbling for 10 min. The tubes were centrifuged (1000 ***g***; 20 min) and the top organic phase removed and the extraction procedure repeated on the remaining aqueous phase.

Carrier vitamin K$_1$ (100 μg) in chloroform (50 μl) and carrier vitamin K$_1$ epoxide (100 μg) in chloroform (50 μl) were added to the combined organic extracts and the solvent evaporated *in vacuo* at 35°. The sample was redissolved in ether (200 μl) and subjected to t.l.c. as described for rabbit plasma [7].

The efficiency of the extraction of [^{3}H]vitamin K$_1$ from liver was investigated by carrying out repeated extractions with acetone:ether (1:4, 2 ml) and measuring the radioactivity content in each extract after evaporation and addition of NE 260 (4 ml). An aliquot (1 ml) of the final aqueous phase was mixed directly with NE 260 (4 ml) vortexed and the radioactivity content measured.

Total radioactivity in the liver was calculated by adding NCS (200 μl) to liver homogenate (100 μl) and incubating at 50° for 4 hr. Glacial acetic acid (100 μl) was added to quench the reaction and NE 260 (12 ml) was added, the tubes were vortexed and the radioactive content measured.

In order to measure the rate of excretion of [^{3}H]vitamin K$_1$ metabolites in bile male Sprague–Dawley rats (200–300 g) were injected i.p. with brodifacoum in DMSO (1 mg/kg) in tests and DMSO alone in controls. They were anaesthetised 1 hr later with urethane (14% solution; 1 ml/100 g) and the jugular vein was cannulated with pp50 tubing for an injection of [^{3}H]vitamin K$_1$ (5 μCi). The common bile duct was also cannulated with pp50 tubing and bile was collected at 30 min intervals, after [^{3}H]vitamin K$_1$ administration, over 5 hr.

20 μl bile from each time interval was assayed for radioactive content and it was established that this amount of bile did not influence the efficiency of the scintillation counting. To investigate the nature of the excreted metabolites, pooled bile (0.2 ml) from test and control animals was extracted with ethyl acetate (1 ml) and the distribution of radioactivity determined. In other experiments bile (0.5 ml) was incubated with ketodase (12,500 Fishman units) in pH 5 acetate buffer (4.5 ml) at 40° for 24 hr after

Table 1. The effect of warfarin and brodifacoum on the half-life of degradation of PCA, the PCA at 24 hr and the minimum PCA observed in warfarin-sensitive and warfarin-resistant rats

Strain	Drug	Dose (mg/kg)	$t_{\frac{1}{2}}$ degradation PCA (hr)	PCA % 24 hr	PCA % minimum
WS	warfarin	0.1	5.12 ± 0.55*	55.2 ± 10.3	21.4 ± 7.1
WS	warfarin	1.0	3.91 ± 0.22	<2	<2
WS	brodifacoum	0.1	9.19 ± 4.16*	18.5 ± 8.7	18.5 ± 8.7
WS	brodifacoum	1.0	3.70 ± 1.11	<2	<2
WS	brodifacoum	10.0	4.08 ± 0.38	<2	<2
WR	warfarin	0.1	—	100	100
WR	warfarin	1.0	—	100	100
WR	brodifacoum	0.1	9.68 ± 2.95*	62.3 ± 6.9	30.3 ± 3.0
WR	brodifacoum	1.0	3.45 ± 0.53	<2	<2

Results are means ($n \geq 4$) ± SD.
WS = warfarin-sensitive Wistar rats.
WR = warfarin-resistant Wistar rats.
* Apparent half-life of degradation.

which the aglycone fraction was extracted with ethyl acetate and subjected to reversed-phase chromatography as described above.

For investigation of urinary metabolites of [^{3}H]vitamin K_1, male Sprague–Dawley rats (200–300 g) were injected i.p. with either brodifacoum (1 mg/kg) in DMSO for test animals or DMSO alone for control animals. One hour later both test and control animals were injected i.v. into the leg femoral vein with [^{3}H]vitamin K_1 (5 μCi) and placed in individual metabolism cages. Urine was collected every 12 hr for 60 hr.

The radioactive content of urine was calculated by adding NE 260 (4 ml) to urine (200 μl), vortexing and counting.

Whole urine (200 μl) was chromatographed on cellulose t.l.c. plates, the developing solvent being ethyl-acetate : ethanol : water : acetic acid (60 : 30 : 9 : 1, v/v). The pattern of metabolites was studied by cutting the plate into bands, each of which were scraped into scintillation vials. Water (1 ml) was added and the tubes were vortexed before adding NE260 (4 ml), vortexing again and counting.

Table 2. Clotting factor activity in rats 24 hr after brodifacoum treatment compared to control rats

Factor	Percentage of normal blood activity at 24 hr DMSO control	Brodifacoum
V	72.8 ± 12.4	79.6 ± 12.4
II	98.7 ± 37.0	15.7 ± 3.6*
VII	119 ± 29.7	<5
IX	109.5 ± 13.7	<12.5†
X	141.0 ± 38.7	<6†

Values are means of groups ($n = 4$) ± S.D. statistical significance from controls using non-paired Student's t-test. A standard curve was obtained using serial dilutions of pooled ($n = 4$) plasma from control rats.
* $P < 0.01$
† $P < 0.001$

RESULTS

Effect of anticoagulants on prothrombin complex activity and individual clotting factor activity

The half-lives of degradation of PCA in rats after pretreatment with either brodifacoum or warfarin are given in Table 1 together with the minimum PCA observed and the PCA at 24 hr. Both anticoagulants produced the same maximum rate of degradation of PCA in warfarin sensitive rats. In each case complete inhibition of clotting factor synthesis for 24 hr was obtained with 1 mg/kg. Intraperitoneal injection of either saline (1 ml/kg) or DMSO (1 ml/kg) did not significantly alter PCA. The mean control PCA of the warfarin-resistant rats used in this study was 27 per cent of the mean control PCA of the warfarin-susceptible Wistar rats. For comparative purposes the PCA for each individual rat was expressed as a per cent of its own control value.

Table 2 shows that the activities of the vitamin K_1-dependent clotting factors II, VII, IX and X are significantly decreased ($P < 0.01$ for factor II and $P < 0.001$ for factors VII, IX and X) 24 hrs after brodifacoum treatment. Factor V is not significantly different after brodifacoum treatment when compared to the controls.

The effect of anticoagulants on the metabolism of ^{3}[H]vitamin K_1

The double acetone : ether (1 : 4; v/v) extraction of [^{3}H]vitamin K_1 from liver homogenates was at least 95 per cent efficient. The main metabolites extracted were [^{3}H]vitamin K_1 and [^{3}H]vitamin K_1 epoxide. The more polar metabolites were not identified.

The rats were killed 2 hr after [^{3}H]vitamin K_1 administration and the liver [^{3}H]vitamin K_1 metabolites were expressed as a percentage of the dose of [^{3}H]vitamin K_1 administered (Table 3). Intraperitoneal injection of DMSO or saline did not significantly affect [^{3}H]vitamin K_1 metabolism. After warfarin administration the warfarin resistant rats showed no significant change in the [^{3}H]vitamin K_1 epoxide : [^{3}H[vitamin K_1 ratio while this ratio increased from 0.05 ± 0.01 to 0.36 ± 0.04 in war-

Table 3. [^{3}H]Vitamin K$_1$ epoxide:[^{3}H]vitamin K$_1$ ratios and the percentage of the radioactive dose found in liver [^{3}H]vitamin K$_1$ metabolites 2 hr after [^{3}H]vitamin K$_1$ administration in warfarin-susceptible and warfarin-resistant rats

Strain	Drug	Dose (mg/kg)	[^{3}H]vitamin K$_1$ epoxide: [^{3}H]vitamin K$_1$	% dose as [^{3}H]vitamin K$_1$	% dose as [^{3}H]vitamin K$_1$ epoxide	% dose as total hepatic radioactive metabolites
WS	—	—	0.05 ± 0.01	22.56 ± 4.04	0.89 ± 0.44	36.0 ± 9.3
WS	warfarin	1	0.36 ± 0.04‡	9.39 ± 1.18‡	3.36 ± 0.50‡	29.1 ± 5.2
WS	brodifacoum	0.1	0.12 ± 0.01‡	14.32 ± 2.19*	1.68 ± 0.35†	26.4 ± 7.4
WS	brodifacoum	1.0	0.25 ± 0.05‡	8.37 ± 3.75‡	2.03 ± 0.61†	27.8 ± 6.4
WS	brodifacoum	10.0	0.33 ± 0.15†	9.05 ± 3.39*	2.95 ± 1.72‡	32.4 ± 4.7
WR	—	—	0.24 ± 0.11	10.03 ± 2.84	2.12 ± 0.84	26.4 ± 3.1
WR	warfarin	1	0.21 ± 0.06	10.32 ± 3.33	2.08 ± 0.22	26.6 ± 3.2
WR	brodifacoum	1	0.91 ± 0.30†	5.55 ± 0.89‡	4.98 ± 1.50†	22.6 ± 3.1

Values are means of groups ($n \geq 4$) ± S.D.
WS = warfarin-sensitive Wistar rats.
WR = warfarin-resistant Wistar rats.
Statistical significance from appropriate controls using Student's *t*-test: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

farin-susceptible rats. It can also be seen that in warfarin-resistant rats warfarin caused no significant change in the percentage of [^{3}H]vitamin K$_1$ or [^{3}H]vitamin K$_1$ epoxide when compared to controls but in the warfarin-sensitive rats the [^{3}H]vitamin K$_1$ concentration was decreased and the [^{3}H]vitamin K$_1$ epoxide concentration increased when compared to controls.

In both warfarin-susceptible and warfarin-resistant rats an increase in the [^{3}H]vitamin K$_1$ epoxide: [^{3}H]vitamin K$_1$ ratio occurs on brodifacoum administration. Furthermore, it can be seen that brodifacoum administration causes a significant change in the percentage of the injected dose occurring as [^{3}H]vitamin K$_1$ and [^{3}H]vitamin K$_1$ epoxide, the former being a decrease and the latter an increase in both warfarin-sensitive and warfarin-resistant rats.

Rate of excretion of [^{3}H]vitamin K_1 metabolites in bile

The excretion of [^{3}H]vitamin K$_1$ metabolites in bile was found to be significantly increased by treatment with 1 mg/kg brodifacoum (Fig. 3). However, there was no significant increase in bile flow with 1 mg/kg brodifacoum. The radiolabelled metabolites present in bile were water-soluble but after treatment with β-glucuronidase 48 per cent of the radioactivity could be extracted with ethyl acetate. Vitamin K$_1$ itself could not be detected. All the metabolite(s) present in the aglycone fraction were more polar than vitamin K$_1$ and vitamin K$_1$ epoxide.

Effect of brodifacoum on urinary excretion of [^{3}H]vitamin K_1 metabolites

Figure 2 represents the total cumulative radioac-

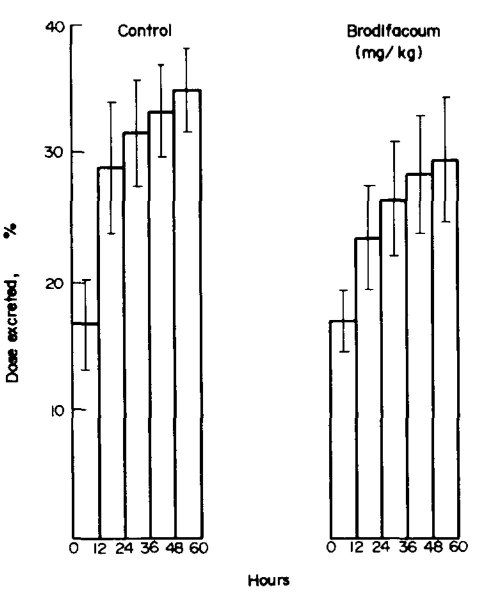

Fig. 2. Cummulative excretion of radioactivity in urine after administration of [^{3}H]vitamin K$_1$ in control rats and rats pretreated with brodifacoum (1 mg/kg). Values are means ($n = 5$) ± S.D.

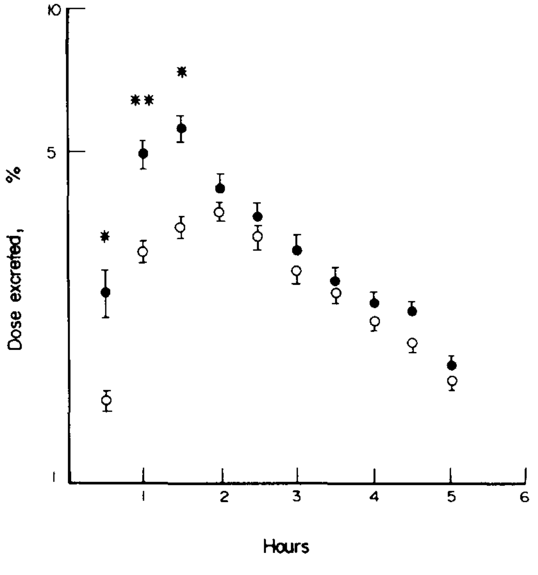

Fig. 3. Excretion of radioactivity in bile after administration of [^{3}H]vitamin K$_1$ in control rats (○) and rats pretreated with brodifacoum (●). Values are means ($n = 4$) ± S.D. Statistical significance between the groups using Student's *t*-test, *$P < 0.01$ **$P < 0.05$.

tivity excreted in urine in 12 hr periods up to 60 hr. At 60 hr the PCA was less than 5 per cent in brodifacoum treated rats indicating that clotting factor synthesis was maximally inhibited throughout the collection period. Brodifacoum had no significant effect on the total radioactivity excreted.

The pattern of metabolites in urine was investigated on radiochromatograms and found to be complex. It was therefore difficult to detect any differences between test and control animals.

DISCUSSION

The anticoagulant action of brodifacoum was established in normal Wistar rats by measuring prothrombin complex activity in tail artery plasma. Brodifacoum (1 mg/kg) produced the same maximum rate of degradation of PCA as warfarin indicating a similar effect on clotting factor activity. However, brodifacoum proved equally effective in warfarin-susceptible and warfarin-resistant rats and produced the same maximum rate of degradation of PCA in both strains as shown in Table 1. The control PCA of the resistant rats used in this study was 27 per cent of the normal controls and was not affected by warfarin (1 mg/kg) in keeping with results obtained using other resistant strains [13].

Furthermore it was found that brodifacoum like warfarin [10] depressed the activity of all the individual vitamin K_1-dependent clotting factors II, VII, IX and X but did not significantly alter factor V which is vitamin K_1 independent (Table 2). The activities of the individual clotting factors at 24 hr are consistent with their rates of degradation [10].

Doses of brodifacoum which inhibit clotting factor synthesis also produced significant changes in the metabolism and disposition of [^{3}H]vitamin K_1 and its metabolites. There was a significant increase in [^{3}H]vitamin K_1 epoxide concentrations and a significant decrease in [^{3}H]vitamin K_1 concentrations. These changes are similar to those produced by warfarin in our experimental system (Table 3) indicating that brodifacoum also interrupts the vitamin K_1-epoxide cycle at the epoxide reductase in the rat.

Having established that brodifacoum produced changes in the metabolism of [^{3}H]vitamin K_1 we investigated the rate of excretion of [^{3}H]vitamin K_1 metabolites; [^{3}H]vitamin K_1 itself is not excreted unchanged. Caldwell *et al.* [14] found that warfarin did not significantly alter the urinary excretion of [^{3}H]vitamin K_1 metabolites in the rat and we were unable to show any change with brodifacoum (Fig. 2). However, only 30 per cent of the injected dose appeared in urine after 60 hr indicating that this may not be the major route of excretion of vitamin K_1 metabolites in the rat. We have therefore investigated the biliary excretion of [^{3}H]vitamin K_1 metabolites and found that 30 per cent of the dose was excreted within 5 hr. Furthermore the half-life of [^{3}H]vitamin K_1 metabolite concentration in bile is similar to the elimination half-life in plasma of [^{3}H]vitamin K_1 [15]. Our preliminary investigations indicate the [^{3}H]vitamin K_1 is metabolised to more polar products which then form glucuronides before excretion in the bile. Administration of brodifacoum produced a significant increase in the biliary excretion of [^{3}H]vitamin K_1 metabolites (Fig. 3) without altering bile flow. The increased rate of excretion is consistent with an increased hepatic turnover of [^{3}H]vitamin K_1 mediated via the 2,3-epoxide and its further metabolites. Warfarin produces both quantitative and qualitative changes in the urinary excretion of [^{3}H]vitamin K_1 in man where the molecular weight threshold for biliary excretion is greater than in the rat [16].

The [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 ratio in warfarin resistant rats was 5-fold greater than in warfarin-susceptible rats but was not affected by warfarin. On the basis of similar observations Bell and Caldwell [4] have suggested that warfarin-resistant rats possess an epoxide reductase with a reduced affinity for both vitamin K_1 epoxide and warfarin. Brodifacoum produced an increase in the hepatic [^{3}H]vitamin K_1 epoxide concentrations and a decrease in [^{3}H]vitamin K_1 concentrations in warfarin resistant rats suggesting that it is able to inhibit the epoxide reductase enzyme in warfarin resistant as well as warfarin susceptible rats at doses which inhibit clotting factor synthesis.

In conclusion we have shown that brodifacoum and warfarin have the same effect on the relationship between clotting factor synthesis and vitamin K_1 metabolism in the rat in keeping with our previous work in the rabbit [7]. These results support the concept that 4-hydroxycoumarins inhibit clotting factor synthesis by interrupting the vitamin K_1-epoxide cycle at the epoxide reductase.

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