Biochemical Pharmacology, Vol. 28, pp. 1323–1329.

0006-2952/79/0415–1323 $02.00/0

A STUDY OF THE EFFECT OF ANTICOAGULANTS ON [^{3}H]VITAMIN K_1 METABOLISM AND PROTHROMBIN COMPLEX ACTIVITY IN THE RABBIT

B. KEVIN PARK, JACQUELINE B. LECK, ADRIAN C. WILSON, MATTHEW J. SERLIN and ALASDAIR M. BRECKENRIDGE

Department of Pharmacology and Therapeutics, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

(*Received* 12 *July* 1978; *accepted* 24 *November* 1978)

Abstract—The effect of the anticoagulants warfarin, 2-chloro-3-phytyl-1,4-naphthoquinone (Cl-K), acenocoumarol, brodifacoum and difenacoum on [^{3}H]vitamin K_1 metabolism and prothrombin complex activity (P.C.A.) were studied in the rabbit. All five anticoagulants inhibited clotting factor synthesis and the rate of decline of P.C.A. was similar for each drug. Metabolic studies with [^{3}H]vitamin K_1 showed that warfarin, acenocoumarol, brodifacoum and difenacoum increased the plasma ratio of [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 but that Cl-K decreased the ratio when compared with controls. None of the anticoagulants produced any significant change in the rate of disappearance of [^{3}H]vitamin K_1 from plasma. The results show that the metabolism of vitamin K_1 and its relationship with P.C.A. are the same as in man. The effect of the anticoagulants on [^{3}H]vitamin K_1 metabolism are consistent with the concept that interruption of the vitamin K_1 epoxide cycle, at either the epoxidase step or the reductase step, will result in a reduction in vitamin K_1-dependent clotting factor synthesis.

INTRODUCTION

Vitamin K_1 is essential for normal blood coagulation because it is a cofactor for the postribosomal synthesis of clotting factors II (prothrombin), VII, IX and X [1]. The vitamin K_1 dependent step in clotting factor synthesis involves the γ-carboxylation of glutamic acid residues in clotting factor 'precursors' during which vitamin K_1 is converted into vitamin K_1 epoxide by vitamin K_1 epoxidase [2]. It is thought that for the continued syfithesis of clotting factors vitamin K_1 must be regenerated from the biologically inactive epoxide by vitamin K_1 epoxide reductase [3].

It would appear that interruption of the vitamin K_1 epoxide cycle, by inhibition of either the epoxidase or the epoxide reductase, will result in a reduction of clotting factor synthesis [4]. Thus it has been shown in both man and the rat that the coumarin anticoagulant warfarin inhibits the epoxide reductase, whereas studies with the rat indicate that 2-chloro-3-phytyl-1,4-naphthoquinone (Cl-K) prevents prothrombin synthesis by inhibiting vitamin K_1 epoxidase [4, 5].

There are inherent problems in studying the mechanism of action of anticoagulants in both man and the rat. In man there is a limitation on the dose of anticoagulant and radioactivity that can be administered safely. In the rat the liver must be removed for determination of [^{3}H]vitamin K_1 and its metabolites, and therefore only single point determinations can be made. We have therefore used the rabbit to investigate the mechanism of action of anticoagulants because the temporal relationship between changes in vitamin K_1 metabolism and changes in clotting factor activity may be measured in the same individual animal.

Vitamin K_1 metabolism and the effect of anticoagulants on this have not been studied previously in the rabbit. First we investigated the anticoagulants warfarin and Cl-K which have been studied in other species [4, 5] and having established the rabbit as a useful experimental model we investigated the anticoagulants acenocoumarol, brodifacoum and difenacoum whose mechanisms had not been studied previously *in vivo*.

MATERIALS AND METHODS

Male New Zealand White rabbits (2.5–3.0 kg) were used in these studies.

[1′,2′-$^3H_2$]Vitamin K_1 (S.A. 0.102 Ci/mmole) was a gift from Hoffman-La Roche, Basle. Unlabelled vitamin K_1 was obtained from Sigma and vitamin K_1 epoxide was synthesised by the method of Tishler *et al.* [6]. Racemic sodium warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] and racemic acenocoumarol were gifts from Ward Blenkinsop and from Geigy Pharmaceuticals. Difenacoum, brodifacoum and 2-chloro-3-phytyl-1,4-naphthoquinone (Cl-K) were gifts from Sorex Laboratories, Widnes. For intravenous injections sodium warfarin and sodium acenocoumarol were dissolved in 0.9% saline while [^{3}H]vitamin K_1 and 2-chloro-3-phytyl-1,4-naphthoquinone were dissolved in Tween 80 and diluted with 0.9% saline to make solutions containing 5% Tween. For intramuscular injections difenacoum and brodifacoum were dissolved in dimethylsulphoxide (2 mg/ml).

Scintillant (NE 260) was obtained from Nuclear Enterprises, Edinburgh, silica gel GF 254 chromatography plates from Kodak, and all other general reagents from B.D.H. All solvents were redistilled before use; chloroform was stabilized by the addition of methanol

(2%, v/v). Thromboplastin was obtained from the National (U.K.) Reference Laboratory for Anticoagulant Reagents and Control, Manchester.

General plan of study. Before each experiment the control prothrombin time for each animal was determined. Animals were dosed with anticoagulants either intravenously (warfarin, acenocoumarol, Cl-K) into the marginal ear vein or intramuscularly (difenacoum, brodifacoum) into the thigh. This injection was followed 1 hr later in the case of the animals dosed intravenously and 2 hr later in the case of the animals dosed intramuscularly by an intravenous injection of [^{3}H]vitamin K_1 (10 μCi). Blood samples (4 ml) were taken from the other marginal ear vein 1, 2, 3, 4, 5 and 6 hr after administration of [^{3}H]vitamin K_1 for determination of [^{3}H]vitamin K_1 and [^{3}H]vitamin K_1 epoxide concentrations. From 6 hr after injection of [^{3}H]vitamin K_1, blood samples (0.9 ml) were taken every four hr for prothrombin time measurements.

Prothrombin complex activity (P.C.A.). Prothrombin times were determined by the method of Quick [7]. Blood samples (0.9 ml) were collected into 3.8% trisodium citrate (0.1 ml) and centrifuged (8,000 *g* for 2 min). Thromboplastin (0.1 ml) was added to citrated plasma (0.1 ml) and incubated at 37° for 2 min, in duplicate. 0.025 M calcium chloride (0.1 ml) was added and the clotting times determined in a Schnitger and Gross coagulometer.

A standard curve of P.C.A. was obtained by determining prothrombin times of pooled normal citrated rabbit plasma diluted in adsorbed plasma (deficient in factors II, VII, IX and X) at concentrations of 1–100 per cent. The P.C.A. for each animal was expressed as a percentage of its own control taking 100 per cent as the beginning of each experiment.

Lipid extraction and chromatography of plasma lipids. Plasma lipid extracts were obtained using the method of Bligh and Dyer [8]. Methanol (1 ml) and chloroform (1 ml) were added to plasma (0.9 ml) and mechanically mixed for 10 min. The mixture was separated by centrifugation (1000 *g*; 20 min) and the organic phase removed. The aqueous phase was re-extracted with chloroform (1 ml). To the combined chloroform extracts was added carrier vitamin K_1 (100 μg) and carrier vitamin K_1 oxide (100 μg) and the solvent removed *in vacuo* at 35°. The lipid extracts were separated by reversed-phase partition t.l.c. [9] using the solvent system acetone–water (88:12, v/v). The areas corresponding to vitamin K_1 and vitamin K_1 oxide were visualised under u.v. light. The plates were

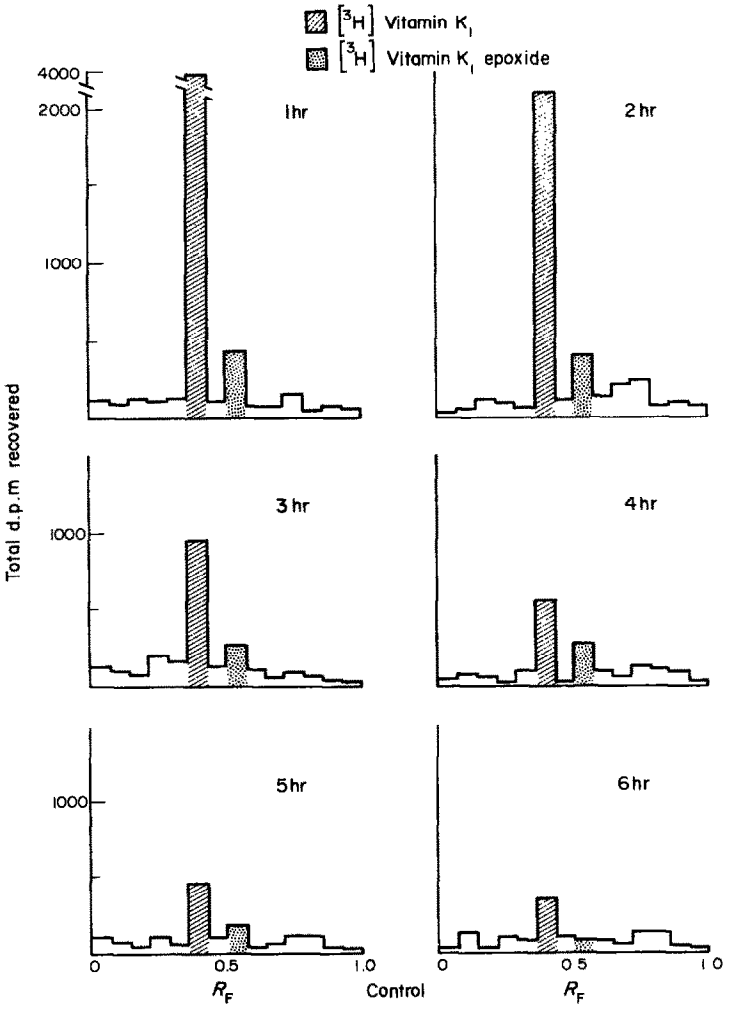

Fig. 1. Distribution on reversed-phase chromatograms of radioactive metabolites extracted from plasma taken from a rabbit at hourly intervals after injection of [^{3}H]vitamin K_1.

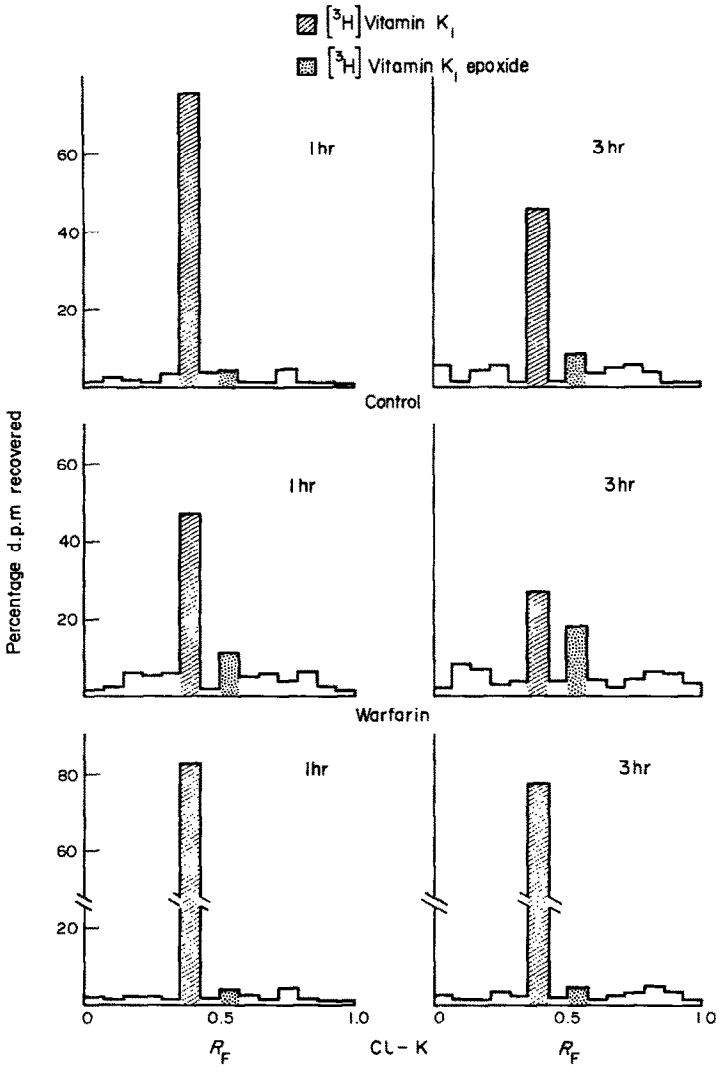

Fig. 2. Distribution on reversed-phase chromatograms of radioactive metabolites extracted from plasma taken from control, warfarin-treated and Cl-K treated rabbits 1 and 3 hr after injection of [^{3}H]vitamin K_1. The radioactivity in each zone of the chromatogram is expressed as a percentage of the total d.p.m. recovered from the chromatogram.

divided into 1 cm bands and the silica scraped into scintillation vials. Water (1 ml) was added and the vials vortexed (30 sec) before the addition of NE 260 scintillation fluid (4 ml) and then vortexed again. The radioactive content of the vials was measured using an Intertechnique SL 33 liquid scintillation counter.

RESULTS

Effect of anticoagulants on the metabolism of [^{3}H]*vitamin* K_1. The distribution on chromatograms of chloroform-extractable [^{3}H]vitamin K_1 metabolites from plasma taken from an untreated rabbit 1, 2, 3, 4, 5 and 6 hr after an intravenous dose of [^{3}H]vitamin K_1 (10 μCi) is shown in Fig. 1. The overall pattern of metabolites is similar to that obtained with humans [5]; only [^{3}H]vitamin K_1 and [^{3}H]vitamin K_1 epoxide were characterised. Figures 2 and 3 show the distribution on chromatograms of chloroform-extractable [^{3}H]vitamin K_1 metabolites from rabbits which had been treated with the various anticoagulants before dosing with [^{3}H]vitamin K_1. In each case the overall pattern of [^{3}H]vitamin K_1 metabolites was similar to that obtained with control rabbits but the proportion of [^{3}H]vitamin K_1 epoxide was increased after pretreatment with warfarin, ăcenocoumarol, brodifacoum and difenacoum and decreased after pretreatment with Cl-K. There appeared to be little difference in other more polar, minor metabolites which were not identified and require further study.

The change in [^{3}H]vitamin K_1 metabolism brought about by the anticoagulants investigated is illustrated in Figs. 4 and 5 which show the change in the [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 ratio with time.

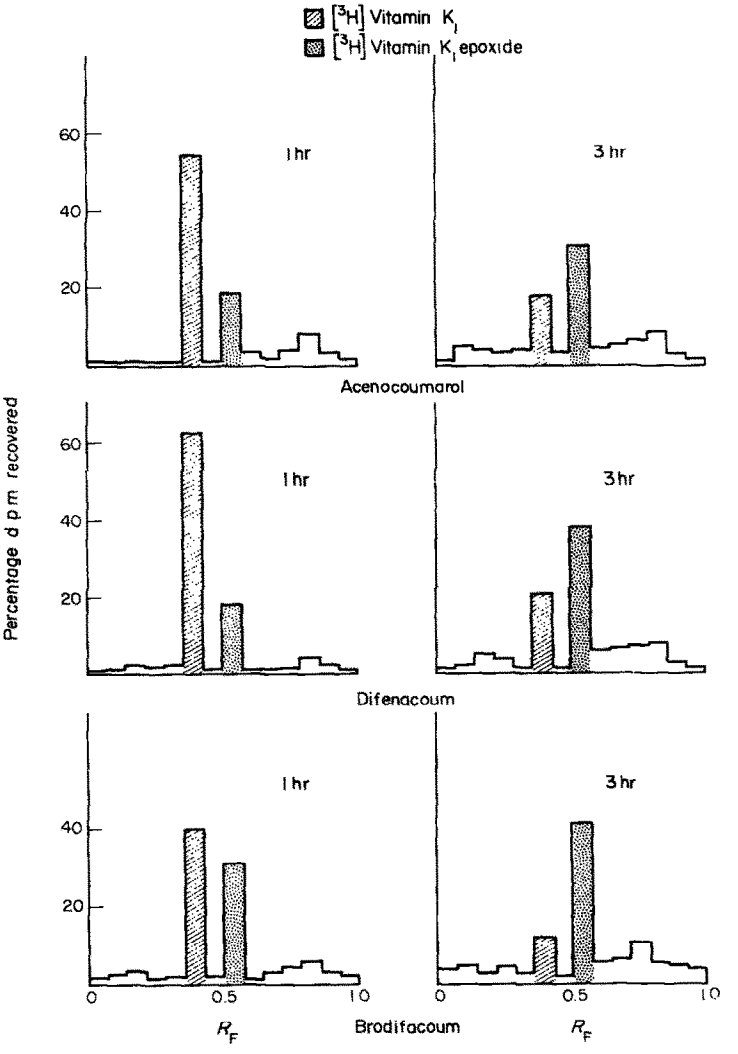

Fig. 3. Distribution on reversed-phase chromatograms of radioactive metabolites extracted from plasma taken from acenocoumarol, difenacoum and brodifacoum treated rabbits 1 and 3 hr after injection of [^{3}H]vitamin K_1. The radioactivity in each zone of the chromatogram is expressed as a percentage of the total d.p.m. recovered from the chromatogram.

Table 1. Effect of anticoagulants on the half-life and rate of disappearance of [^{3}H]vitamin K_1 from rabbit plasma

Drug	Dose (mg/kg)	n	$t_{\frac{1}{2}}$ Disappearance [^{3}H]-K_1 from plasma (hr)	Disappearance rate constant (k) of [^{3}H]-K_1 from plasma (hr^{-1})
None	- -	6	2.03	0.366 ± 0.107
Warfarin	2.5	4	1.76	0.421 ± 0.126
Cl-K	10	4	1.98	0.350 ± 0.018
Warfarin + Cl-K	2.5 + 10	4	2.37	0.347 ± 0.122
Difenacoum	1	4	1.61	0.429 ± 0.018
Brodifacoum	1	4	2.04	0.344 ± 0.046
Acenocoumarol	10	4	1.77	0.403 ± 0.086

Results are means ± S.D.
There was no significant difference in $t_{\frac{1}{2}}$ or k between the various groups.

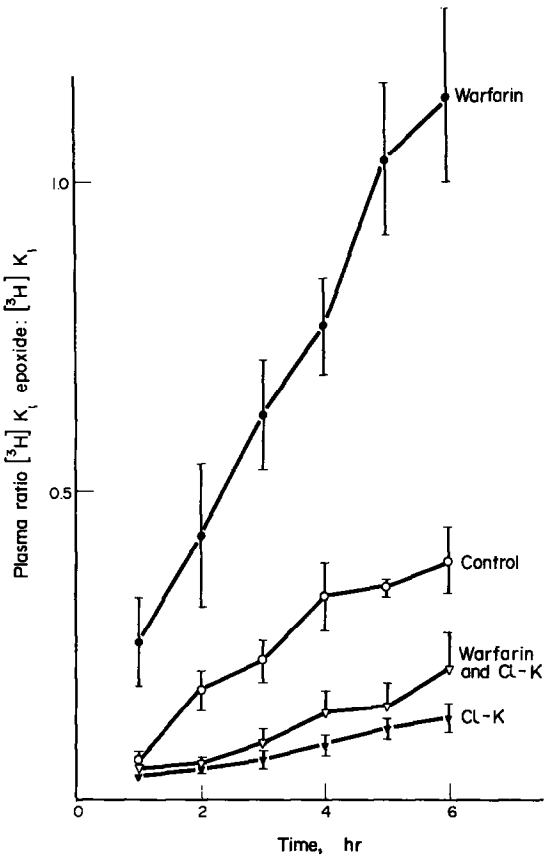

Fig. 4. The effect of warfarin (●), Cl-K (▼), warfarin and Cl-K (▽) on the plasma [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 ratio in rabbit plasma 1–6 hr after injection of [^{3}H]vitamin K_1, compared with controls (○). The results are expressed as the mean ± S.E.M.

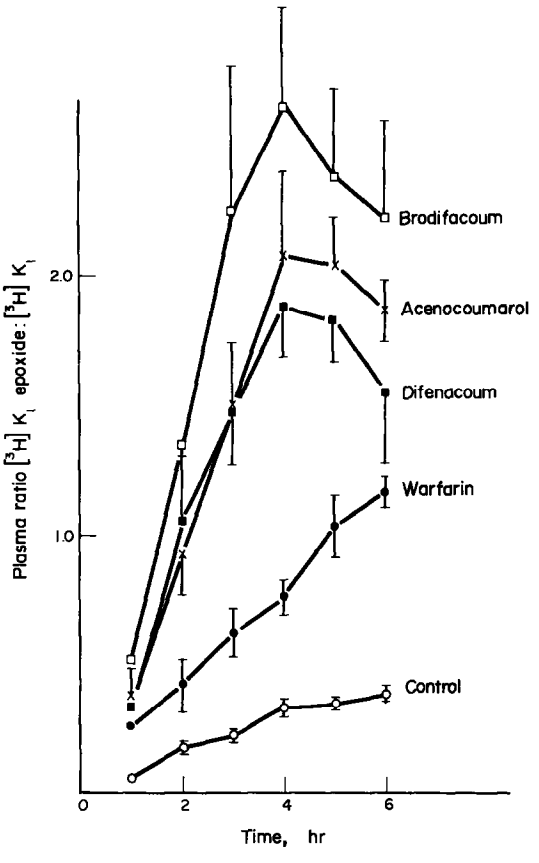

Fig. 5. The effect of warfarin (●), acenocoumarol (×), brodifacoum (□) and difenacoum (■) on the plasma [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 ratio in rabbit plasma 1–6 hr after injection of [^{3}H]vitamin K_1, compared with controls (○).

From Figure 4 it can be seen that warfarin produced an increase in the [^{3}H]vitamin K_1 oxide:[^{3}H]vitamin K_1 ratio when compared to controls whereas Cl-K produced a decrease. Administration of Cl-K with warfarin blocked the increase in the ratio which would have been seen otherwise; the mean ratios were close to those obtained with Cl-K alone. Figure 5 shows that acenocoumarol, brodifacoum and difenacoum all produced an increase in the [^{3}H]vitamin K_1 oxide:[^{3}H]vitamin K_1 ratio.

The rate of disappearance of [^{3}H]vitamin K_1 from plasma. The disappearance of [^{3}H]vitamin K_1 from plasma from normal rabbits was mono-exponential over the period studied (1–6 hr after dosing) with a mean half-life of 2.03 hr. The half-lives obtained from rabbits pretreated with various anticoagulants (Table 1) were similar, consistent with there being no change in the rate of uptake of [^{3}H]vitamin K_1 by the liver during this period [5].

Table 2. Effect of anticoagulants on the half-life and rate of degradation of prothrombin complex activity and minimum prothrombin complex activity in the rabbit

Drug	Dose (mg/kg)	n	$t_{\frac{1}{2}}$ degradation P.C.A. (hr)	Rate of degradation (k) of P.C.A. (hr^{-1})	Minimum P.C.A. (%)
None	—	6	—	—	100
Warfarin	2.5	4	6.13	0.114 ± 0.01	15.5 ± 6.3
Cl-K	10	4	6.05	0.117 ± 0.018	<2
Warfarin + Cl-K	2.5 + 10	4	6.20	0.112 ± 0.004	<2
Difenacoum	1	4	6.85	0.103 ± 0.015	<2
Brodifacoum	1	4	7.20	0.096 ± 0.005	<2
Acenocoumarol	10	4	6.33	0.109 ± 0.008	16.6 ± 8.0

Results are means + S.D.
There was no significant difference in $t_{\frac{1}{2}}$ and k between the various groups.

Effect of anticoagulants on prothrombin complex activity. The half-life of degradation of P.C.A. after pretreatment with the various anticoagulants are given in Table 2 together with the minimum prothrombin complex activity measured. The rate of degradation was the same after each drug pretreatment and confirmed that maximum inhibition of clotting factor synthesis had been achieved. The mean minimum P.C.A. of animals treated with acenocoumarol (10 mg/kg) and warfarin (2.5 mg/kg) but not [^{3}H]vitamin K_1 were 5.2 ± 3.0 and 10.7 ± 4.0 respectively compared with 16.6 ± 8 and 15.6 ± 6.3 with [^{3}H]vitamin K_1, indicating that the [^{3}H]vitamin K_1 administered, which has a mass of 43 μg, had a pharmacological effect.

DISCUSSION

The role of the vitamin K_1 epoxide cycle in the synthesis of clotting factors II, VII, IX and X has been studied in man [5] and in the rat [4] but has not been investigated previously in the rabbit.

The general pattern of metabolism of an intravenous dose of [^{3}H]vitamin K_1 in normal rabbits (Fig. 1) is similar to that observed in man by Shearer *et al.* [5]. Furthermore, the mean half-life of disappearance of [^{3}H]vitamin K_1 during 1–6 hr after administration was 2.03 hr (Table 1) which is similar to the range of half-lives (2–2.5 hr) found in man during the same period after an intravenous dose [10]; Shearer *et al.* [10], assumed that lipid-soluble radioactivity represented the injected [^{3}H]vitamin K_1. Little [^{3}H]vitamin K_1 epoxide was detected during this period in rabbit plasma and most of the lipid-soluble radioactivity was associated with carrier vitamin K_1, although there were small amounts of more polar metabolites that were not identified (Fig. 1).

Pretreatment of rabbits with warfarin reduced the P.C.A. to a mean of 15.6 per cent of normal and produced an accumulation of [^{3}H]vitamin K_1 oxide in plasma which significantly ($P < 0.05$) increased the [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 ratio when compared to controls (Fig. 4). There was no other change in the pattern of [^{3}H]vitamin K_1 metabolism (Fig. 2) and the rate of disappearance of [^{3}H]vitamin K_1 from plasma in warfarin treated rabbits was the same as for controls (Table 2). Therefore it would appear that warfarin produces its anticoagulant effect in the rabbit by inhibiting vitamin K_1 epoxide reductase as has been suggested in man [5] and the rat [4].

In vitro and *in vivo* studies with the rat have indicated that Cl-K may block prothrombin synthesis by inhibiting vitamin K_1 epoxidase [5]. At the doses used, Cl-K produced a greater reduction in P.C.A. than warfarin in the rabbit but the rates of degradation of P.C.A. were similar, indicating a common effect on clotting factor synthesis. In contrast to warfarin, Cl-K produced a significant decrease in the plasma ratio of [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 (Fig. 4) but did not change the rate of disappearance of [^{3}H]vitamin K_1 (Table 1) from plasma in keeping with the hypothesis that it is a direct antagonist of vitamin K_1 [4]. To test further the relationship of vitamin K_1 epoxidase and vitamin K_1 epoxide reductase in the vitamin K_1 epoxide cycle, Cl-K and warfarin were administered together. At a dose which reduced P.C.A. to 2 per cent, Cl-K prevented the accumulation of [^{3}H]vitamin K_1 epoxide in plasma that warfarin would otherwise have produced; the [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 ratios obtained were similar to those produced by Cl-K alone (Fig. 4). It is evident that inhibition of clotting factor synthesis in the rabbit will occur after interruption of the vitamin K_1 epoxide cycle at either the epoxidase or reductase and that the activities of these enzymes are reflected in the relative plasma concentrations of [^{3}H]vitamin K_1 and its metabolite [^{3}H]vitamin K_1 epoxide.

The present work, using the rabbit, has confirmed previous studies of the mechanism of the anticoagulant warfarin in both man and the rat [4, 5]. We have also found that Cl-K reduces the plasma [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 ratio in keeping with changes in ratios measured in rat liver [4]. Furthermore, we have shown that the rabbit is a useful experimental animal for studying anticoagulants because [^{3}H]vitamin K_1 metabolism, [^{3}H]vitamin K_1 pharmacokinetics and prothrombin complex activity can be measured in the same individual animal. The pharmacokinetics of the anticoagulant could also be studied in the same animal.

Acenocoumarol is a close structural analogue of warfarin (Fig. 6) and would be expected to act by the same mechanism, although the relative potencies in man of its stereoisomers are opposite to those of warfarin [11]. However, the plasma ratios of [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 in animals pretreated with acenocoumarol (Fig. 5) clearly show that the anticoagulant acts like warfarin by inhibiting vitamin K_1 epoxide reductase; the rate of disappearance of vitamin K_1 from plasma and rate of degradation of P.C.A. were similar to those obtained with warfarin (Tables 1 and 2).

The novel anticoagulants brodifacoum and difenacoum, although possessing the same 4-hydroxycoumarin ring system as warfarin (Fig. 6), are effective in warfarin-resistant rats [12] but their mechanism has not been elucidated. Both of the tetrahydronaphthyl anticoagulants were more potent than warfarin in the rabbit but the rates of degradation of P.C.A. obtained with all three drugs were the same, indicating a similar effect on clotting factor activity. Neither brodifacoum nor difenacoum affected the rate of disappearance of [^{3}H]vitamin K_1 from plasma but both produced an increase in the plasma [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 ratio, indicating that they also act by inhibiting vitamin K_1 epoxide.

There was no correlation between the increase in [^{3}H]vitamin K_1 epoxide:[^{3}H]vitamin K_1 ratios and anticoagulant potency in line with results obtained, using the rat, by other workers [13, 14]. It is thought that the vitamin K_1 dependent clotting factor precursors contain several glutamate residues which require γ-carboxylation for activation [15]. In cattle receiving large doses of dicoumarol, none of the glutamic acid residues are carboxylated [16] whereas in humans receiving only a therapeutic dose of warfarin, seven of the possible ten glutamic acid residues are carboxylated [17]. It may be that only a certain degree of inhibition of the vitamin K_1 epoxide cycle is necessary for blocking the synthesis of complete clotting factors. An alternative explanation is that the anticoagulant may have different effects on the individual vitamin K_1-dependent clotting factors.

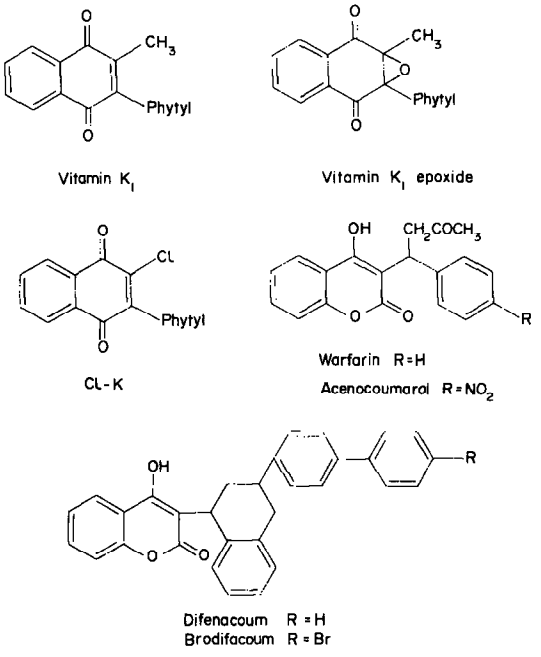

Fig. 6. Chemical structures.

In conclusion we have investigated the relationship between [^{3}H]vitamin K_1 metabolism and prothrombin complex activity in the rabbit with special reference to the effects of anticoagulants. We have found that the rabbit is a useful model for studying the mechanism of anticoagulants and have shown that acenocoumarol, brodifacoum and difenacoum exert their pharmacological effect by inhibiting vitamin K_1 epoxide reductase.

Acknowledgements—We would like to thank Dr. R. Long of Hoffman La Roche for a generous gift of tritiated vitamin K_1 and the Merseyside Regional Health Authority for financial support.

REFERENCES

1. J. W. Suttie, G. A. Grant, C. T. Emson and T. V. Shah, *Mayo Clin. Proc.* **49**, 933 (1974).
2. A. K. Willingham and J. T. Matschiner, *Biochem. J.* **140**, 435 (1974).
3. J. T. Matschiner, A. Zimmerman and R. G. Bell. *Thromb. Diath. haemorr.* Suppl. **57**, 45 (1974).
4. A. K. Willingham, R. E. Laliberte, R. G. Bell and J. T. Matschiner, *Biochem. Pharmac.* **25**, 1063 (1976).
5. M. J. Shearer, A. McBurney and P. Barkhan, *Br. J. Haemat.* **24**, 471 (1973).
6. M. Tishler, L. F. Fieser and N. L. Wendler, *J. Am. chem. Soc.* **62**, 2866 (1940).
7. A. J. Quick, in *Haemorrhagic Diseases*, (Ed. A. J. Quick). Lea and Febiger, Philadelphia p. 379 (1957).
8. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **8**, 911, (1959).
9. M. J. Shearer, A. McBurney, A. M. Breckenridge and P. Barkhan, *Clin. Sci.* **52**, 621 (1977).
10. M. J. Shearer, C. M. Mallinson, G. R. Webster and P. Barkhan, *Br. J. Haemat.* **22**, 579 (1972).
11. T. Meinertze, W. Kasper, C. Kahl and E. Jahnchen, *Br. J. clin. Pharm.* **5**, 187 (1978).
12. M. R. Hadler and R. S. Shadbolt, *Nature, Lond.* **253**, 275 (1975).
13. J. A. Sadowski and J. W. Suttie, *Biochemistry* **13**, 3696 (1974).
14. R. G. Bell, P. T. Caldwell and E. E. T. Holm, *Biochem. Pharmac.* **25**, 1067 (1976).
15. M. P. Esnouf, *Br. med. Bull.* **33**, 213 (1977).
16. J. Stenflo, *J. biol. Chem.* **248**, 6325 (1973).
17. M. P. Esnouf and C. V. Prowse, *Biochim. biophys. Acta.* **490**, 471 (1977).