*Biochemical Pharmacology*, Vol. 31, No. 22, pp. 3635–3639, 1982.
Printed in Great Britain.

0006-2952/82/223635-05 $03.00/0


# A COMPARISON OF VITAMIN K ANTAGONISM BY WARFARIN, DIFENACOUM AND BRODIFACOUM IN THE RABBIT

B. KEVIN PARK* and JACQUELINE B. LECK

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

(*Received* 25 *January* 1982; *accepted* 17 *May* 1982)

**Abstract**—The pharmacological response to vitamin $K_1$ (Konakion®) in anticoagulated (prothrombin complex activity < 30%) New Zealand white rabbits was determined by measuring prothrombin complex activity (P.C.A.) in peripheral plasma. In animals pretreated with either brodifacoum (1 mg/kg or 10 mg/kg) or difenacoum (0.85 mg/kg or 8.5 mg/kg) P.C.A. reached a maximum 4 hr after administration of vitamin $K_1$ (0.5 mg/kg) and declined at a rate indicating complete inhibition of clotting factor synthesis. A different response to vitamin $K_1$ (0.5 mg/kg) was observed in rabbits pretreated with warfarin (63 mg/kg); after an initial rise P.C.A. appeared to plateau for 11 hr and then fall at a rate which indicated incomplete inhibition of clotting factor synthesis. The response to several doses of vitamin $K_1$ (0.5, 1, 2.5 and 5.0 mg/kg) was investigated in the same group of brodifacoum (1 mg/kg) anticoagulated animals. There was a linear relationship between the duration of clotting factor synthesis and the logarithm of the dose of the vitamin K; the pharmacological half-life of vitamin $K_1$ was only 1.7 ± 0.1 hr. The duration of action of brodifacoum and difenacoum was much longer than that of warfarin. Six weeks after administration of brodifacoum (1 mg/kg) animals were still anticoagulated (P.C.A. < 30%). In conclusion, we have found that brodifacoum and difenacoum are both more potent and persistent antagonists of vitamin $K_1$ than warfarin *in vivo*. In cases of poisoning with these compounds it will be necessary to give repeated and frequent doses of vitamin K to maintain clotting factor synthesis.

Difenacoum and brodifacoum are two novel 4-hydroxycoumarin anticoagulants which have been developed as rodenticides [1]. Coumarin anticoagulants, such as warfarin, inhibit the vitamin K-dependent step in the synthesis of clotting factors II, VII, IX and X, which involves the γ-carboxylation of glutamic acid residues in clotting factor precursors [2]. During γ-carboxylation vitamin $K_1$ hydroquinone ($KH_2$) is converted into vitamin $K_1$ 2,3-epoxide which must be reduced back to the vitamin, by vitamin K epoxide reductase, for clotting factor synthesis to continue. It is thought that coumarin anticoagulants interrupt the physiologically important vitamin $K_1$ epoxide cycle by inhibiting vitamin $K_1$ epoxide reductase [3]. The cyclic interconversion of vitamin $K_1$ and its 2,3-epoxide is referred to as the vitamin K-epoxide cycle.

In keeping with this hypothesis, it has been shown that difenacoum and brodifacoum produce an accumulation of [$^3$H]vitamin $K_1$ epoxide, after administration of [$^3$H]vitamin $K_1$ in rats and rabbits and also reduce the activity of clotting factors II, VII, IX and X, but do not affect factor V [4, 5].

However, it has been shown that difenacoum and brodifacoum are more potent anticoagulants than warfarin and that they are equally effective in warfarin-susceptible and warfarin-resistant rats [1, 4, 5]. To investigate further the mechanism of action of these novel anticoagulants, we have compared their ability to antagonise vitamin $K_1$ *in vivo* with that of warfarin in the rabbit. The rabbit was used in these studies because a large number of blood samples, for the determination of P.C.A., may be easily obtained from the same animal [4]. The duration of action of vitamin K, when administered in pharmacological doses is discussed in relation to its metabolism.

* Author to whom correspondence should be addressed.

## MATERIALS AND METHODS

Male New Zealand White rabbits (2.5–3.0 kg) were used in these studies. Vitamin $K_1$ (Konakion®) was obtained from Hoffmann-La Roche, Welwyn Garden City, racemic sodium warfarin from Ward Blenkinsop while difenacoum and brodifacoum were gifts from Sorex Laboratories, Widnes. For intraperitoneal injections sodium warfarin was dissolved in 0.9% saline while difenacoum and brodifacoum were dissolved in dimethylsulphoxide [4]. Animals had free access to food and water. The rabbits were maintained on Diet R14 Labshore Animal Foods, Poole, U.K., average daily intake of vitamin $K_1$ was *ca.* 60 μg/kg.

*Prothrombin complex activity (P.C.A.).* Prothrombin complex activity was determined as previously described [4]. Blood samples (0.9 ml) were collected into 3.8% trisodium citrate (0.1 ml) in polypropylene tubes and centrifuged (8000 *g* for 2 min) immediately. 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 pro-

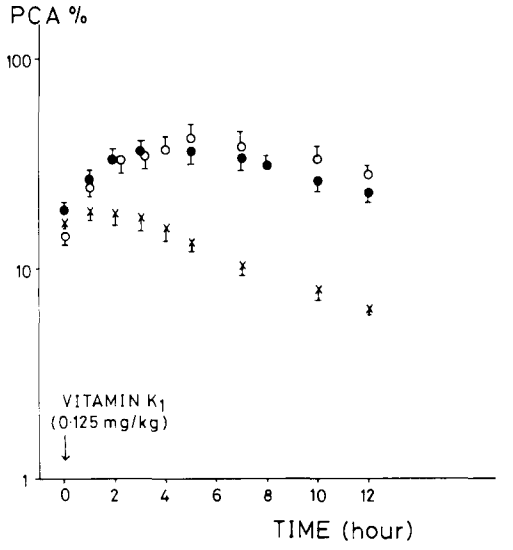


Fig. 1. The effect of intravenous vitamin $K_1$ (0.125 mg/kg) on prothrombin complex activity in rabbits anticoagulated with warfarin (63 mg/kg), ○ warfarin (189 mg/kg) ● and brodifacoum (1 mg/kg) ×. The anticoagulants were given 18 hr before vitamin $K_1$. Results are presented as means ($n = 4$) ± S.E.M.

thrombin times of pooled normal citrated rabbit plasma diluted in absorbed 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.

*General plan of study.* The antagonism of vitamin $K_1$ by coumarin anticoagulants was studied *in vivo*, by measuring P.C.A., at regular intervals, after an intravenous dose of vitamin $K_1$ (0.125, 0.5, 1.0, 2.5 or 5 mg/kg) which was given 18 hr after the coumarin anticoagulant. Thus, P.C.A. was less than 30% of control when vitamin $K_1$ was given.

The duration of action of a single dose of difenacoum (0.85 mg/kg and 8.5 mg/kg) and a single dose of brodifacoum (1 mg/kg) was investigated by measuring P.C.A. at regular intervals. In the early part of the study subcutaneous administration of vitamin K (4 mg/kg: alternate days) was necessary to prevent death from hemorrhage. During this period P.C.A. was measured immediately before each vitamin $K_1$ injection.

*Statistical analysis.* For comparative purposes 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. The data was analysed using the Student's *t*-test. Results are presented as the means ± S.E.M.

## RESULTS

The effect of a single intravenous dose of vitamin $K_1$ (0.125 mg/kg) on P.C.A. in rabbits anticoagulated with warfarin, 63 mg/kg and 189 mg/kg, is shown in Fig. 1. The results were identical for each group indicating that maximum antagonism of vitamin $K_1$ by warfarin had been achieved. The response to the same dose of the vitamin in rabbits anticoagulated with brodifacoum (1 mg/kg) is shown for comparison. The antagonism of vitamin $K_1$ produced by brodifacoum is greater than that obtained by warfarin because P.C.A. was significantly less ($P < 0.01$) in the brodifacoum group from 2 hr after the vitamin K injection onwards.

From Fig. 2 it can be seen that both difenacoum (0.85 mg/kg) and brodifacoum (1 mg/kg) produced a greater antagonism of vitamin $K_1$ at a dose of 0.5 mg/kg, than warfarin (63 mg/kg); P.C.A. was significantly less ($P < 0.01$) in the brodifacoum group and in the difenacoum group from 5 hr after the vitamin $K_1$ injection onwards. For all three anticoagulants, the initial (0–1 hr) rate of clotting factor synthesis was between three and four times normal

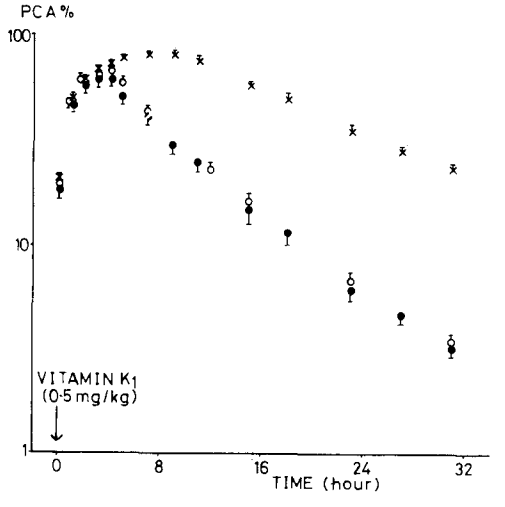


Fig. 2. The effect of intravenous vitamin $K_1$ (0.5 mg/kg) on prothrombin complex activity in rabbits anticoagulated with warfarin (63 mg/kg) × brodifacoum (1 mg/kg) ○ and difenacoum (0.85 mg/kg) ●. The anticoagulants were given 18 hr before vitamin $K_1$. Results are presented as means ($n = 4$) ± S.E.M.

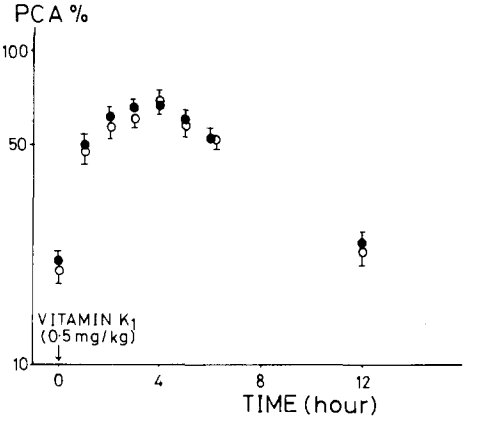


Fig. 3. The effect of intravenous vitamin $K_1$ (0.5 mg/kg) on prothrombin complex activity in rabbits anticoagulated with difenacoum (0.85 mg/kg) ○ and difenacoum (8.5 mg/kg) ●. The anticoagulant was given 18 hr before vitamin $K_1$. Results are means ($n = 4$) ± S.E.M.

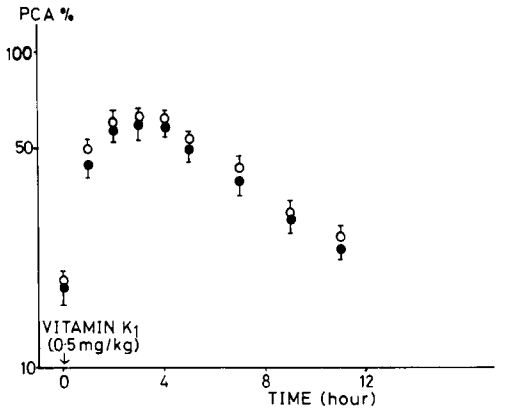


Fig. 4. The effect of intravenous vitamin $K_1$ (0.5 mg/kg) on prothrombin complex activity in rabbits anticoagulated with brodifacoum (1 mg/kg) ○ and brodifacoum (10 mg/kg) ●. The anticoagulant was given 18 hr before vitamin $K_1$. Results are presented as means ($n = 4$) ± S.E.M.

when calculated according to the method of Nagashima *et al.* [6]. The mean half-lives of degradation of P.C.A. in the difenacoum group and in the brodifacoum were 6.4 ± 0.4 hr and 6.0 ± 0.3 hr, respectively. This is the maximum rate of degradation of P.C.A. in the rabbit [4] and indicates complete inhibition of clotting factor synthesis. The mean apparent half-life of degradation of P.C.A. for the warfarin group was 12.1 ± 1.7 hr which indicates partial inhibition of clotting factor synthesis. The antagonism of vitamin $K_1$ produced by difenacoum and brodifacoum in this experiment was maximal, because identical results were obtained when the dose of the novel anticoagulants was increased ten-fold (Figs. 3 and 4).

The duration of the pharmacological action of difenacoum (0.85 and 8.5 mg/kg) in the rabbit is illustrated in Fig. 5. For the first 21 days of the experiment vitamin $K_1$ (4 mg/kg) was administered subcutaneously every other day, immediately before vitamin $K_1$ administration P.C.A. was measured. P.C.A. rose gradually in each group during vitamin $K_1$ administration. In the group which received the higher dose, P.C.A. never rose above 25% of normal and on withdrawing vitamin $K_1$ P.C.A. fell to less than 2% and three of the four animals died within 4 days. In the group given 0.85 mg/kg P.C.A. rose to 66% of normal during vitamin $K_1$ administration, but fell to 33% when vitamin $K_1$ was withdrawn; thereafter P.C.A. increased gradually over a period of several weeks.

In a similar experiment it was found that when vitamin $K_1$ was withdrawn from rabbits six weeks after administration of a single dose of brodifacoum (1 mg/kg) P.C.A. fell to 32.8 ± 4.3% within 24 hr and to 18.0 ± 2.4% within 72 hr.

The effect of several doses of vitamin $K_1$ (0.5, 1.0, 2.5 and 5.0 mg/kg) on P.C.A. in one rabbit is shown in Fig. 6. It can be seen that the duration of action of vitamin $K_1$, which we have arbitrarily assigned as the time to reach maximum P.C.A., is dependent on dose. There was a linear relationship between the

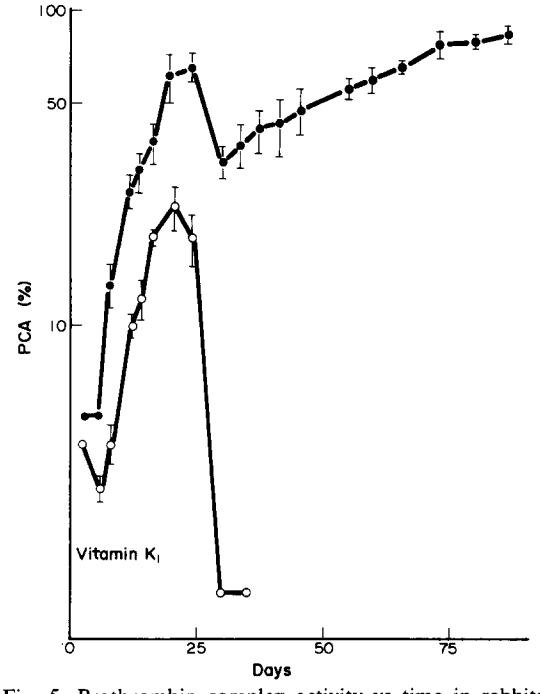


Fig. 5. Prothrombin complex activity vs time in rabbits after a single dose of difenacoum (0.85 mg/kg) ● and difenacoum (8.5 mg/kg) ○. Vitamin $K_1$ (4 mg/kg) was given subcutaneously on alternative days for the first 21 days. Results are presented as means ($n = 4$) ± S.E.M.

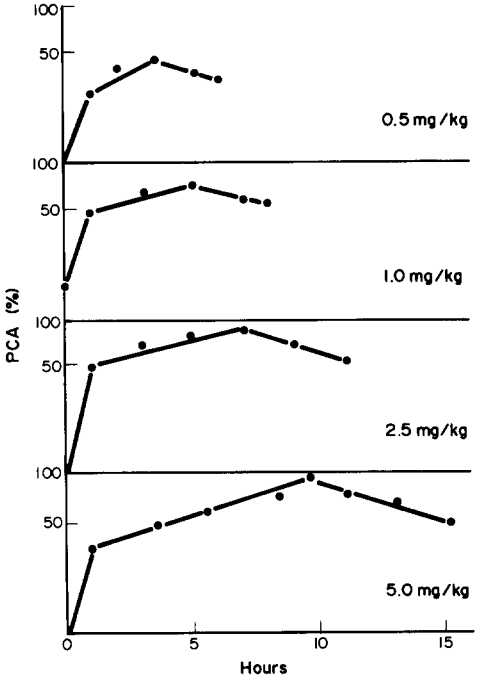


Fig. 6. The effect of intravenous vitamin $K_1$ (0.5 mg/kg, 1.0 mg/kg, 2.5 mg/kg and 5.0 mg/kg) on prothrombin complex activity in a rabbit anticoagulated with brodifacoum (1 mg/kg).

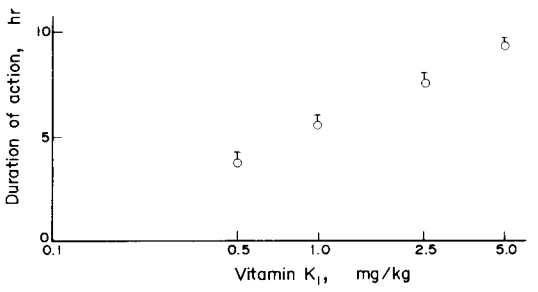


Fig. 7. The relationship between the duration of action of vitamin $K_1$ and the logarithm of the dose of vitamin $K_1$. Results are presented as means ($n = 4$) ± S.E.M.

duration of action of vitamin $K_1$ and the logarithm of the dose (Fig. 7). The pharmacological half-life of vitamin K, defined as the increase in duration of action obtained by doubling the dose of the vitamin, was $1.71 \pm 0.1$ hr ($n = 4$). The pharmacological response to vitamin K (0.5 mg/kg) was identical one and eight days after administration of brodifacoum (1 mg/kg).

## DISCUSSION

The therapeutic ratio of coumarin anticoagulants is small and patients who are unduly sensitive may become anticoagulated to a dangerous degree. However, surprisingly little is known about the duration of action of vitamin $K_1$ during chronic anticoagulation with 4-hydroxycoumarin anticoagulants. In man, it is generally agreed that repeated and frequent administration of vitamin $K_1$ may be necessary to restore clotting factor synthesis; but more precise details are lacking [7, 8].

Lowenthal and MacFarlane [9] investigated the antagonism of vitamin $K_1$ by warfarin in the rat and found that the antagonism is neither competitive nor non-competitive and that warfarin cannot completely inhibit the action of vitamin $K_1$. On the basis of metabolic studies, Matschiner and co-workers [10] have suggested that the principal mechanism of action of coumarin anticoagulants is inhibition of the regeneration of vitamin $K_1$ from its metabolite vitamin $K_1$ epoxide. In keeping with this hypothesis we have found that warfarin, difenacoum and brodifacoum all produce an accumulation of [$^3$H]vitamin $K_1$ epoxide in rabbit plasma after administration of [$^3$H]vitamin $K_1$ [4].

In the rabbit, the maximum antagonism of vitamin $K_1$ by warfarin was produced by a dose of 63 mg/kg: increasing the dose of warfarin did not reduce further the effect of vitamin $K_1$. The response to vitamin $K_1$ (0.5 mg/kg) was characterised by an initial fast rise in P.C.A. during which the rate of clotting factor synthesis was greater than normal: this is probably due to rapid γ-carboxylation of accumulated clotting factor precursors [11]. Thereafter P.C.A. appeared to plateau and then fall at a rate which indicated that clotting factor synthesis was only partially inhibited [4, 6]. These findings are consistent with the hypothesis that coumarin anticoagulants act by inhibiting the enzyme vitamin $K_1$ epoxide reductase but do not directly inhibit the vitamin K-dependent γ-carboxylation of glutamic acid residues in clotting factor precursors [3].

Brodifacoum and difenacoum both produced a greater maximum antagonism of vitamin $K_1$ than warfarin *in vivo*, despite the fact that the latter was administered in a dose of 100 times greater, on a molar basis. After administration of vitamin $K_1$ (0.5 mg/kg) to animals anticoagulated with difenacoum or brodifacoum, P.C.A. rose for between 3 and 5 hr and then fell at a rate which indicated complete inhibition of clotting factor synthesis.

There are at least two possible explanations for the greater maximum pharmacological activity of brodifacoum and difenacoum. One is that brodifacoum and difenacoum may have a greater affinity for the enzyme vitamin $K_1$ epoxide reductase. This implies that warfarin cannot produce complete inhibition of vitamin $K_1$ epoxide reductase *in vivo*. A second explanation is that the 4-hydroxycoumarin anticoagulants may interrupt the vitamin K-epoxide cycle at more than one site *in vivo* as has been suggested by *in vitro* work [12].

The duration of anticoagulation produced by brodifacoum and difenacoum is much longer than that of warfarin [4, 13] in the rabbit. After a single dose (0.85 mg/kg) of difenacoum, repeated vitamin $K_1$ administration was necessary, even for partial clotting factor synthesis, over a period of several weeks. During this period the response of P.C.A. to vitamin $K_1$ increased (Fig. 5), presumably reflecting the slow clearance of difenacoum from its site of action.

The pharmacological action of brodifacoum was even more persistent. Rabbits required vitamin K for up to six weeks after administration of brodifacoum (1 mg/kg) when the experiment was terminated. Furthermore it was found that the response to vitamin $K_1$ was identical one day and eight days after administration of brodifacoum (1 mg/kg).

The persistent and consistent antagonism of vitamin K by brodifacoum made possible an investigation of the response to several doses of vitamin $K_1$ in the same group of animals. The mean pharmacological half-life of vitamin K, which we have arbitrarily defined as the increase in duration of clotting factor synthesis obtained by doubling the dose of vitamin K, was found to be only 1.71 hr. On the basis of these experiments, the daily dose of vitamin $K_1$ required to maintain 100% clotting factor synthesis would be approximately 640 mg/kg. It has been estimated that the daily dietary requirement for vitamin K, in both man and the rat, is of the order of 1 μg/kg [14, 15].

The pharmacological half-life of vitamin $K_1$, estimated in this study, is similar to the terminal plasma half-life of tritiated vitamin $K_1$ in the rabbit [16], in man [17, 18], and in the rat [19] and is also similar to the half-life of tritiated vitamin $K_1$ metabolites in the rat [5]. Therefore, there is a close relationship between the pharmacological half-life of vitamin $K_1$ in animals given brodifacoum (in which vitamin K epoxide reductase is inhibited), and the normal metabolic clearance of vitamin $K_1$ which is not affected by anticoagulants [16–19].

The major route of metabolism of vitamin K involves ω-oxidation and β-oxidation in the mito-

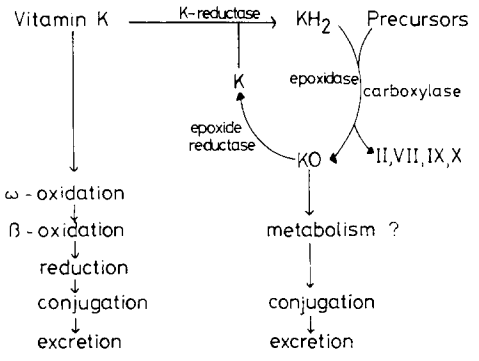


Fig. 8. The relationship between the metabolism and the physiological action of vitamin $K_1$.

chondria followed by glucuronidation in the endoplasmic reticulum [20, 21]. Only when coumarin anticoagulants are administered does metabolism via the epoxide become apparent. Therefore, the vitamin K-epoxide cycle may serve to maintain the small pool of vitamin $K_1$ associated with clotting factor synthesis and prevent the rapid metabolism by mitochondrial enzymes that would otherwise occur (Fig. 8). Once the endogenous pool is depleted, by inhibition of vitamin $K_1$ epoxide reductase, relatively high and frequent doses of vitamin $K_1$ are required to maintain the concentration of vitamin $K_1$ necessary for clotting factor synthesis because of competition from the enzymes that metabolise vitamin $K_1$ rapidly by $\omega$-oxidation and $\beta$-oxidation. Thus although dietary requirements of vitamin $K_1$ are low, the liver has the ability, somewhat paradoxically, to metabolise vitamin $K_1$ rapidly.

In conclusion, we have found that brodifacoum and difenacoum are more persistent and more potent antagonists of vitamin $K_1$ than warfarin. There are, therefore, essential pharmacodynamic and pharmacokinetic differences between the novel anticoagulants and warfarin which explain why these compounds are more effective rodenticides. These studies are of both theoretical and practical interest as a case of poisoning with one of these compounds, difenacoum, has already been reported [22].

*Acknowledgements*—We should like to thank the Merseyside Regional Health Authority for financial support. We are grateful to Professor A. M. Breckenridge for his advice and encouragement throughout this study. We should also like to thank Miss S. Newby, Mr. P. Roberts and Mr. D. Trafford for expert technical assistance and Mrs. C. Clarke for typing the manuscript.

## REFERENCES

1. M. R. Hadler and R. S. Shadbolt, *Nature, Lond.* **253**, 275 (1975).
2. C. M. Jackson and J. W. Suttie, *Prog. Haemat.* **10**, 333 (1977).
3. R. G. Bell, *Fedn Proc. Fedn Am. Socs exp. Biol.* **37**, 2599 (1978).
4. B. K. Park, J. B. Leck, A. C. Wilson, M. J. Serlin and A. M. Breckenridge, *Biochem. Pharmac.* **28**, 1323 (1979).
5. J. B. Leck and B. K. Park, *Biochem. Pharmac.* **30**, 123 (1981).
6. R. Nagashima, R. A. O'Reilly and G. Levy, *Clin. Pharmac. Ther.* **10**, 22 (1969).
7. T. D. Bjornsson and T. F. Blaschke, *Lancet* **ii**, 846 (1978).
8. M. J. Shearer and P. Barkham, *Lancet* **i**, 266 (1979).
9. J. Lowenthal and J. A. MacFarlane *J. Pharm. exp. Ther.* **143**, 273 (1964).
10. J. T. Matschiner, A. Zimmerman and R. G. Bell, *Thromb. Diath. Haemorrh.* **57**, suppl. 45 (1974).
11. J. W. Suttie, *Archs Biochem.* **141**, 571 (1970).
12. C. W. Siegfried, *Biochem. biophys. Res. Comm.* **83**, 1488 (1978).
13. B. K. Park, J. B. Leck and A. Breckenridge, *Biochem. Pharmac.* **29**, 1601 (1980).
14. P. G. Frick, G. Riedler and H. Brogli, *J. appl. Physiol* **23**, 387 (1967).
15. G. G. Partridge, *Comp. Biochem. Physiol.* **66A**, 87 (1980).
16. B. K. Park, J. B. Leck, A. C. Wilson and A. M. Breckenridge, in *Vitamin K Metabolism and Vitamin K Dependent Proteins* (Ed. J. W. Suttie), pp. 348–353. University Park Press, Baltimore (1980).
17. M. J. Shearer, A. McBurney and P. Barkham, *Vitamin and Hormones*, **32**, 513 (1974).
18. T. D. Bjornsson, P. J. Meffin, S. E. Swezey and T. F. Blaschke, *J. Pharmac. exp. Ther.* **210**, 322 (1979).
19. W. Schmidt, D. Beerman, F. Oesch and E. Jahnchen, *J. Pharm. Pharmac.* **31**, 490 (1979).
20. M. Watanabe, M. Toyoda, I. Imada and H. Morimoto, *Chem. Pharm. Bull.* **22**, 176 (1974).
21. A. McBurney, M. J. Shearer and P. Barkham, *Biochem. Med.* **24**, 250 (1980).
22. A. M. Barlow, A. L. Gay and B. K. Park, *Br. med. J.* **285**, 541 (1982).