Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90,* 147.
Hatlen, L. E., Amaldi, F., and Attardi, G. (1969), *Biochemistry 8,* 4989.
Kashel, M., and Kalbacher, B. (1970), *J. Biol. Chem. 245,* 2309.
Marzluff, W. F., Jr., Murphy, E. C., Jr., and Huang, R. C. C. (1973), *Biochemistry 12,* 3440.
Miller, O. L., Jr., and Beatty, B. R. (1969), *Science 164,* 965.
Perry, R. P. (1962), *Proc. Nat. Acad. Sci. U. S. 48,* 2179.
Price, R., and Penman, S. (1972a), *J. Virol. 9,* 621.
Price, R., and Penman, S. (1972b), *J. Mol. Biol. 70,* 435.
Reeder, R. H., and Roeder, R. G. (1972), *J. Mol. Biol. 67,* 433.
Roeder, R. G. (1974), *J. Biol. Chem. 249,* 249.
Roeder, R. G., Reeder, R. H., and Brown, D. D. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 727.
Smellie, E. J., and Burdon, R. H. (1970), *Biochim. Biophys. Acta 213,* 248.
Suzuki, Y., Gage, L. P., and Brown, D. D. (1972), *J. Mol. Biol. 70,* 637.
Wallace, R. D., and Kates, J. (1972), *J. Virol. 9,* 627.
Williamson, R., and Brownlee, G. G. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3,* 306.
Zylber, E., and Penman, S. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 2861.

Mechanism of Action of Coumarins. Significance of Vitamin K Epoxide†

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ABSTRACT: The 2,3-epoxide of phylloquinone is a normal metabolite of the vitamin, and it has been demonstrated that coumarin anticoagulants inhibit an enzyme system that converts this metabolite to the vitamin. It has been postulated that this epoxide is a competitive inhibitor of vitamin K at the metabolic site where it is involved in prothrombin synthesis, and that coumarins act as anticoagulants because they increase the tissue levels of this epoxide. This study demonstrates that there is little correlation between the effectiveness of four different coumarins as anticoagulants, and their effect on phylloquinone epoxide metabolism. It also demonstrates that administration of phylloquine epoxide to vitamin K deficient, hypoprothrombinemic rats causes a significant initiation of prothrombin synthesis within 15 min. During this 15-min period, the ratio of the epoxide to the vitamin in the liver is considerably in excess of that which has previously been postulated to be inhibitory. These data would appear to rule out the hypothesis that vitamin K epoxide is an inhibitor of vitamin K action and that warfarin functions as an anticoagulant by increasing the tissue ratio of vitamin K epoxide to vitamin K.

Dicoumarol was identified by Link (Campbell and Link, 1941) as the active hemorrhagic agent in spoiled sweet clover, and since that time a large number of coumarins, including 3-(α-acetonylbenzyl)-4-hydroxycoumarin (warfarin), have been used as rodenticides and therapeutic anticoagulants. The mechanism by which the coumarin anticoagulants antagonize the action of vitamin K and depress prothrombin synthesis has not been unambiguously defined (Suttie, 1975) although it has often been assumed that they compete with vitamin K for a receptor protein or proteins at the site where vitamin K exerts its biological activity. It has also been suggested (Olson, 1970) that the coumarins interact with an alternate site on such a receptor protein, or (Lowenthal and Birnbaum, 1969) that these anticoagulants block the transport of the vitamin to its physiologically active site. More recently, Matschiner *et al.* (1970) have identified the 2,3-epoxide of phylloquinone as a natural metabolite of vitamin K and have shown (Bell and Matschiner, 1970) that warfarin administration increases the tissue levels of this metabolite. It has been postulated (Bell and Matschiner, 1972; Bell *et al.*, 1972) that warfarin exerts its anticoagulant effect through an inhibition of the enzyme which converts the epoxide to the vitamin. The epoxide of vitamin K is assumed to be the true inhibitor of the action of the vitamin, and the increase in the liver ratio of the epoxide to the vitamin is postulated as the cause of the inhibition of vitamin K dependent clotting factor synthesis. Although it has been shown that warfarin treatment will inhibit the reductase (Matschiner *et al.*, 1974) and increase the liver ratio of vitamin K epoxide to vitamin K[1] definitive proof that this increase is responsible for the antagonism of vitamin K action is lacking. All of the data that have been used to support the correlation of high oxide:K_1 ratios with antagonism of the vitamin have been obtained under conditions where the animals have also received warfarin. The studies reported here indicate that vitamin K epoxide is not an inhibitor of vitamin K action, and suggest that the effect of warfarin on prothrombin production is not due to the increased tissue ratio of vitamin K epoxide to vitamin K which it causes.

Materials and Methods

Male, 190–210-g Holtzman strain rats were used throughout the study. Vitamin K deficiency was produced by feeding a vitamin K deficient diet (Mameesh and Johnson, 1959) for 8 days in cages that prevented coprophagy. The coumarin derivatives used were injected intraperitoneally in saline as the sodium salt. Plasma prothrombin concentrations were measured by the two-stage method of Ware and Seegers as modified by

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[1] The tissue ratio of phylloquinone 2,3-epoxide to phylloquinone will be referred to as the oxide:K_1 ratio.

TABLE I: Effect of Coumarins on Plasma Prothrombin Concentration.[a]

Coumarin Injected	Plasma Prothrombin (% of control)	
	5.5 hr	10.5 hr
I	60 ± 1	28 ± 2
II	57 ± 5	32 ± 3
III	59 ± 3	29 ± 1
IV	72 ± 3	71 ± 11

[a] Rats were injected with 13 μmol/kg body weight of anticoagulants I–IV (see Figure 1) at 0 time. Blood samples were drawn by cardiac puncture at 5.5 and 10.5 hr. Control prothrombin concentrations were 220 units/ml. Values are mean ± SE for 3–4 rats per group.

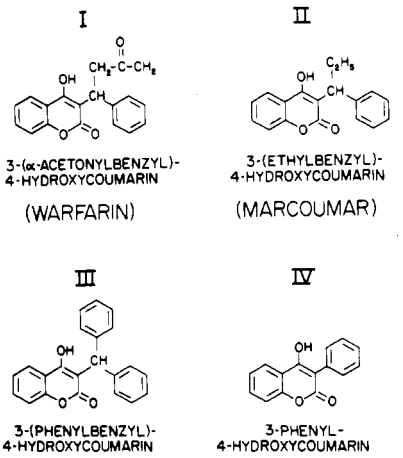

FIGURE 1: The four coumarins studied.

Shapiro and Waugh (1966). Liver microsomal extracts were prepared and assayed for prothrombin as previously described (Shah and Suttie, 1972). Tritiated and unlabeled vitamin K_1 epoxide were prepared and purified according to Matschiner *et al.* (1970). They were dissolved in Tween-80 and diluted with 0.9% sodium chloride to give solutions containing from 5 to 10% Tween, and 0.2 ml was injected intracardially. Vitamin K epoxide and vitamin K were assayed in hexane extracts of liver as described by Matschiner *et al.* (1970). Radioactivity was measured in a liquid scintillation spectrometer using external standardization. The radioactive vitamin used, 5,6,7,8-[^{3}H]phylloquinone,was a gift from Dr. John Matschiner, University of Nebraska and the coumarin derivatives used were a gift from Dr. K. P. Link, University of Wisconsin.

Results

If coumarins act by influencing the concentration of vitamin K epoxide in the liver, the relative anticoagulant effectiveness of various coumarins would be expected to parallel their effect on vitamin K metabolism. Four different coumarins including warfarin (Figure 1) were given to rats at a dose level which was equimolar to that of 5 mg/kg body weight warfarin.

The data in Table I indicate that warfarin (I) and compounds II and III were equally effective as anticoagulants. The half-life of prothrombin has been estimated at from 6 to 8 hr, and these data would indicate that prothrombin synthesis had been effectively halted when these three anticoagulants were used. In contrast to the effectiveness of these compounds, compound IV lowered plasma prothrombin only slightly at 5.5 hr without any further decrease at 10.5 hr, indicating that it caused an incomplete inhibition of prothrombin synthesis.

The effect of the administration of these coumarins on the metabolism of vitamin K and vitamin K oxide is illustrated in Table II. When the rats were given a tracer dose (0.042 μg/rat) of vitamin K 30 min after coumarin injection, there was no effect on plasma prothrombin concentrations 5 hr later. This amount of radioactive vitamin did, however, allow a determination of the effects of these anticoagulants on vitamin K metabolism. Warfarin injection increased the oxide:K_1 ratio to 1.37 compared to 0.08 for control animals. The other three coumarins had an intermediate effect. Compounds II and III, although their anticoagulant effect was equal to that of warfarin, increased the ratios of oxide to K_1 much less than warfarin. Administration of compound IV caused an oxide:K_1 ratio that was indistinguishable from that of the controls, and which was much lower than that which had previously been postulated (Bell *et al.*, 1972) to be required for inhibition of prothrombin synthesis. This compound was, however, an active anticoagulant even though it was not as effective on an equimolar basis as warfarin and compounds II and III.

TABLE II: Effect of Coumarins on Metabolism of Vitamin K_1 and Vitamin K_1 Epoxide.[a]

Coumarin Injected	[^{3}H]Vitamin K				[^{3}H]Vitamin K Epoxide			
	% Dose in Liver	% Liver ^{3}H as		Oxide: K_1	% Dose in Liver	% Liver ^{3}H as		Oxide: K_1
		Oxide	Vitamin			Oxide	Vitamin	
Control	24.5 ± 0.3	3.2 ± 0.6	40.7 ± 2.0	0.08 ± 0.02	34.3 ± 0.4	30.6 ± 1.0	20.8 ± 0.7	1.47 ± 0.01
I	34.5 ± 2.0	14.2 ± 1.8	10.5 ± 0.3	1.37 ± 0.21	41.9 ± 3.0	30.5 ± 0.7	0.53 ± 0.9	64.8 ± 14.6
II	36.5 ± 3.6	9.8 ± 2.3	16.4 ± 0.8	0.59 ± 0.10	42.6 ± 2.7	33.4 ± 3.2	1.8 ± 0.3	18.3 ± 2.1
III	27.5 ± 0.2	7.0 ± 0.4	15.8 ± 1.3	0.45 ± 0.02	35.3 ± 2.5	33.5 ± 3.8	2.1 ± 0.2	16.2 ± 2.3
IV	22.4 ± 1.0	3.5 ± 0.4	32.1 ± 1.8	0.11 ± 0.02	33.7 ± 2.7	31.3 ± 1.9	10.1 ± 1.6	3.22 ± 0.39

[a] Three rats per treatment were injected with the various coumarins as indicated in Table I; 30 min after this, they were injected intracardially with [^{3}H]vitamin K_1 (0.042 μg, 1.31 μCi) or [^{3}H]vitamin K_1 epoxide (200 μg, 1.31 μCi). Rats were killed after 5 hr and the % of the ^{3}H injected which was localized in the liver, and the % of the liver ^{3}H which was present as vitamin K and vitamin K epoxide was determined. The tracer dose of vitamin K had no effect on prothrombin levels after 5 hr and the 200-μg dose of vitamin K epoxide had an effect only in the animals given coumarin IV. In this case, the prothrombin levels at 5.5 hr were 101% of controls (compare to 71.6% in Table I). Control animals received radioactive vitamins but no anticoagulant. Values mean ± SE.

TABLE III: The Effect of Vitamin K_1 and Vitamin K_1 Epoxide on Microsomal Prothrombin Concentrations.[a]

Pre-treatment	Treatment	Time (min)	Liver Prothrombin (units/g of liver)	Oxide:K_1
Deficient (8)	None		<3	
Deficient (3)	K_1	10	11.2 ± 0.4	
Deficient (7)	K_1	15	6.8 ± 0.9	0.28 ± 0.04
Deficient (7)	K_1 Epoxide	10	8.3 ± 0.7	
Deficient (8)	K_1 Epoxide	15	11.5 ± 1.6	2.31 ± 0.66
Warfarin (4)	None		<3	
Warfarin (3)	K_1	15	10.2 ± 0.3	1.12 ± 0.03
Warfarin (3)	K_1 Epoxide	15	<3	>20

[a] Rats were made hypoprothrombinemic (plasma prothrombin <20% of controls) by an injection of 5 mg/kg of warfarin intraperitoneally 24 hr before the experiments, or by placing them on a vitamin K deficient diet for 8 days. The rats were injected intracardially with 1 mg of [^{3}H]vitamin or epoxide (2 μCi/mg) and they were killed at the times indicated. Liver prothrombin concentration was determined on all animals and oxide:K_1 ratios on the animals killed at 15 min. Values are mean ± SE for the number of rats in parentheses.

Table II also shows the effect of the anticoagulants on the metabolism of 200 μg of tritiated vitamin K oxide. The oxide was not effective in overcoming the inhibition caused by compounds I (warfarin), II, or III, but did reverse the partial inhibition caused by compound IV; 5 hr after injection of the oxide, the control animals showed an oxide:K_1 ratio as high as those previously reported to be inhibitory to prothrombin synthesis yet the circulating level of prothrombin was not diminished. Likewise, the partial inhibition of prothrombin synthesis caused by compound IV was reversed even though the oxide:K_1 ratio was higher than would be expected if the oxide were an inhibitor of vitamin K action. Warfarin, compound II, and compound III strongly inhibited the conversion of oxide to vitamin, but compound IV had only a slight effect on this conversion. Up to 10% of the total liver tritium could be identified as vitamin K_1 when compound IV was used, whereas with the more effective anticoagulants, less than 2% of the liver tritium was in the form of the vitamin. The percentage of liver tritium identifiable as the oxide was virtually the same for all cases. The results from this experiment were inconsistent with the hypothesis that the oxide is an inhibitor of vitamin K, since prothrombin synthesis was observed when the oxide:K_1 ratio was as high or higher than those ratios previously reported to be inhibitory to vitamin K action on prothrombin synthesis.

When the vitamin or the oxide was administered to the animals in the presence of any of the anticoagulants, there was a dramatic increase in the amount of tritium present in the liver that could not be identifiable as either the vitamin or the oxide by the methods employed. The difference seems to lie in the amount of material which could not be extracted from the liver by hexane, since less total tritium was extracted from the livers of those animals given anticoagulant than from those given just the vitamin or the oxide. The recovery of the tritium as the vitamin or the oxide from the silicic acid column was comparable irrespective of the treatment, and accounted for almost 100% of the hexane extractable radioactivity. Presumably, the coumarins enhance the conversion of vitamin K and its oxide to more polar forms which accumulated in the presence of warfarin and were not extractable with hexane.

Previous attempts to demonstrate that high oxide:K_1 ratios are not in themselves inhibitory have been unsuccessful because of the rapid conversion of oxide to vitamin. If the oxide is injected into vitamin K deficient hypoprothrombinemic animals, the oxide:K_1 ratio reaches control levels substantially before prothrombin begins to appear in the plasma. A response in microsomal prothrombin concentrations can, however, be detected much before the appearance of plasma prothrombin (Shah and Suttie, 1972) and the data in Table III indicate that both the vitamin and the oxide can initiate a rapid increase in microsomal prothrombin. When 1 mg of vitamin K was injected intracardially to vitamin K deficient rats, the peak of microsomal prothrombin was seen at 10 min, and by 15 min this had begun to fall as prothrombin moved from the liver to the plasma. The oxide:K_1 ratio at 15 min was 0.28. When vitamin K_1 oxide was injected, the peak microsomal prothrombin concentrations were seen at 15 rather than 10 min after injection. The maximum response was, however, equal to that elicited by the vitamin, even though the oxide:ratio at 15 min was 2.3.

The effect of warfarin on the initiation of microsomal prothrombin synthesis by vitamin K and the oxide was also examined. Table III indicates that 1 mg of vitamin K was capable of overcoming the warfarin inhibition of prothrombin synthesis in 15 min, whereas 1 mg of oxide was completely ineffective. The data indicate that the conversion of oxide to the vitamin was completely blocked in the warfarin-treated animals, and that no vitamin K could be identified in the liver when vitamin K oxide was administered to warfarin-treated rats. It was also noted that the oxide:K_1 ratio in vitamin K deficient animals given a large dose of vitamin K was greater (0.28) than that found (Table II) in control animals given a tracer dose of the vitamin (0.08). This might indicate that the enzyme(s) responsible for the conversion of the vitamin to the oxide have increased activity during vitamin deficiency.

Discussion

In view of these data, the hypothesis that the coumarins exert their anticoagulant effect by increasing the oxide:K_1 ratio in the liver, and that the oxide of the vitamin is the true inhibitor of prothrombin synthesis, does not seem tenable. The data clearly show (Table III) that in the absence of warfarin, prothrombin synthesis can occur over a 15-min period during which the ratio of oxide to K must have ranged from infinity (that injected) to 2.3 at 15 min. Previous studies, which have supported the K oxide theory of warfarin action, have indicated that any ratio above 1.0 should have inhibited prothrombin formation. The slower response which was seen following injection of the oxide would be expected as some of it would presumably have to be converted to the vitamin before it could exert an effect.

Although not as conclusive, the data obtained by comparing the effects of different coumarins on vitamin K metabolism and on the inhibition of prothrombin synthesis are also inconsistent with the vitamin K oxide theory. These data indicated that there was not a good correlation between the effect of these compounds as anticoagulants, and their ability to increase the tissue levels of vitamin K oxide. The oxide:K_1 ratios seen when compounds II and III were injected were considerably below that which has previously been associated with inhibition even though they were as active as warfarin as an anticoagulant. Compound IV had some anticoagulant action even though the oxide:K_1 ratio in the liver was indistinguishable from control animals.

These data again point out that the coumarins, in addition to their effect on prothrombin synthesis, have profound effects on the metabolism of vitamin K. Not only was the interconversion of vitamin and oxide interferred with, but coumarin administration caused an increase in the amount of vitamin K metabolites which could not be identified as either vitamin K or vitamin K oxide. In normal rats, roughly 50% of the activity from injected vitamin K can be identified as these two compounds (Table II), and coumarin administration reduces this to about 25%. The other metabolites have not been identified, but are presumably more polar, as they are not extracted from the tissues by hexane.

Although these data do indicate that vitamin K oxide is not the antagonist of vitamin K when coumarins are administered, they do not furnish any indication of what the significance of this compound might be. The presence of an oxidase and reductase in liver, and the observations (Willingham and Matschiner, 1974) that the level of at least the epoxidase may change with vitamin K status of the animal would suggest that this interconversion between the two forms of the vitamin might in some way be related to the physiological actions of the vitamin.

References

Bell, R. G., and Matschiner, J. T. (1970), *Arch. Biochem. Biophys. 141,* 473–476.

Bell, R. G., and Matschiner, J. T. (1972), *Nature (London) 237,* 32–33.

Bell, R. G., Sadowski, J. A., and Matschiner, J. T. (1972), *Biochemistry 11,* 1959–1961.

Campbell, H. A., and Link, K. P. (1941), *J. Biol. Chem. 138,* 21–33.

Lowenthal, J., and Birnbaum, H. (1969), *Science 164,* 181–183.

Mameesh, M. S., and Johnson, B. C. (1959), *Proc. Soc. Exp. Biol. Med. 101,* 467–469.

Matschiner, J. T., Bell, R. G., Amelotti, J. M., and Knauer, T. E. (1970), *Biochim. Biophys. Acta 201,* 309–315.

Matschiner, J. T., Zimmermann, A., and Bell, R. G. (1974), *Thromb. Diath. Haemorrh., Suppl.* (in press).

Olson, R. R. (1970), *in* The Fat-Soluble Vitamins, DeLuca, H. F., and Suttie, J. W., Ed., Madison, Wis., University of Wisconsin Press, pp 463–489.

Shah, D. V., and Suttie, J. W. (1972), *Arch. Biochem. Biophys. 150,* 91–95.

Shapiro, S. S., and Waugh, D. F. (1966), *Thromb. Diath. Haemorrhag. 16,* 469.

Suttie, J. W. (1975), *Thromb. Diath. Haemorrh., Suppl.* (in press).

Willingham, A. K., and Matschiner, J. T. (1974), *Biochem. J. 140,* 435–441.

Clusters in Lipid Bilayers and the Interpretation of Thermal Effects in Biological Membranes†

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ABSTRACT: The partitioning of the spin label, 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo), has been studied in a number of aqueous phospholipid dispersions. In dioleoyllecithin bilayers the formation of quasicrystalline clusters has been detected at temperatures below *ca.* 30°, as shown by the exclusion of Tempo from the clusters. A pure complex of dioleoyllecithin with the [Ca^{2+}; Mg^{2+}]ATPase from sarcoplasmic reticulum has been prepared, and a break in the Arrhenius plot observed at 29°. Similar evidence is presented for cluster formation in native sarcoplasmic reticulum membranes at temperatures below *ca.* 25°. The possibility is discussed that breaks in Arrhenius plots at *ca.* 20° for a number of other enzyme systems also correspond to cluster formation rather than to a lipid liquid-crystalline to crystalline phase transition.

In the last few years much attention has been focused on the effects of temperature on biological membranes, in the hope of being able to correlate known changes in the physical properties of the lipid component with a corresponding change in some membrane property. The major change occurring in a lipid–water system as the temperature is varied is the crystalline to liquid crystalline transition, also called the order–disorder transition, corresponding to a "melting" of the lipid–hydrocarbon chains. This change has been detected by thermal calorimetry, X-ray diffraction, electron spin resonance, and nuclear magnetic resonance (see, for example, Chapman *et al.,* 1967; Levine, 1972). In the liquid crystalline state, a lipid has considerable freedom of motion, with extensive internal motion (Hubbell and McConnell, 1971; Levine *et al.,* 1972), rapid anisotropic rotation of the whole lipid molecule (Lee *et al.,* 1974b), and rapid diffusion in the plane of the bilayer (Träuble and Sackmann, 1972; Devaux and McConnell, 1972; Lee *et al.,* 1973), probably by a vacancy diffusion process (Lee *et al.,* 1974a). Although less is known about mobility in lipids below the crystalline to liquid-crystalline transition temperature, it is clear that many of these motions are severely restricted (Lee *et al.,* 1974a).

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