# Metabolism of Vitamin K and Vitamin K 2,3-Epoxide via Interaction with a Common Disulfide†

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ABSTRACT: The effects of thiols and sulfhydryl blocking reagents on the reduction of vitamin K to vitamin K hydroquinone and vitamin K 2,3-epoxide to vitamin K and vitamin K hydroquinone catalyzed by rat hepatic microsomes were investigated to determine the mechanism(s) for these reactions. Both vitamin K and vitamin K 2,3-epoxide reductions were catalyzed more effectively with dithiols than with monothiols as the reductant. The sulfhydryl reagent *N*-ethylmaleimide (NEM) inhibited vitamin K and vitamin K 2,3-epoxide reduction much more effectively when microsomes were initially treated with dithiothreitol (prereduced). In prereduced microsomes iodoacetamide was approximately half as effective an inhibitor of vitamin K and vitamin K 2,3-epoxide reduction as NEM, but in microsomes not prereduced it was more effective. Iodoacetic acid was ineffective as an inhibitor. Vitamin K or vitamin K 2,3-epoxide added to prereduced microsomes blocked subsequent inhibition by NEM of vitamin K and vitamin K 2,3-epoxide metabolism, respectively. Vitamin K added to prereduced microsomes also blocked inhibition by NEM of vitamin K 2,3-epoxide metabolism, and vitamin K 2,3-epoxide addition blocked inhibition by NEM of vitamin K metabolism. Vitamin K did not diminish the rate of vitamin K 2,3-epoxide metabolism, however, nor did vitamin K 2,3-epoxide diminish the rate of vitamin K metabolism. These data establish that exogenous thiol compounds promote the reduction of at least one protein disulfide which participates in the metabolism of vitamin K and vitamin K 2,3-epoxide. Presumably, the resultant sulfhydryl groups are reoxidized to the disulfide form during the metabolism of either vitamin which protects them from reaction with NEM.

Vitamin K[1] metabolism is required for postribosomal synthesis of γ-carboxyglutamate residues in the N-terminal region of vitamin K dependent proteins (Suttie, 1980). For coagulation factors II (prothrombin), VII, IX, and X and osteocalcin of bone, the γ-carboxyglutamate residues function in calcium binding (Suttie, 1980; Price et al., 1976, 1981). The metabolic pathways of vitamin K required for its biologic activity are (i) reduction to vitamin K hydroquinone, (ii) oxidation of the hydroquinone to vitamin K 2,3-epoxide concomitant with γ-carboxylation of protein glutamate, and (iii) regeneration of vitamin K by reduction of the epoxide.

Vitamin K reduction to vitamin K hydroquinone in rat liver is catalyzed by DT-diaphorase (EC 1.6.99.2) (Wallin et al., 1978; Wallin & Suttie, 1981) and/or a microsomal reductase which requires sulfhydryl compounds as cofactor and which is very susceptible to inhibition by warfarin (Whitlon et al., 1978; Fasco & Principe, 1980). The microsomal vitamin K 2,3-epoxide reductase is similar to the microsomal vitamin K reductase in that it requires exogenous thiol as cofactor and is inhibitable by warfarin (Bell, 1978; Zimmerman & Matschiner, 1974). In mutant rats resistant to the anticoagulant effects of warfarin, both the vitamin K and vitamin K 2,3-epoxide reductases are less susceptible to inhibition by warfarin, a further point of similarity (Fasco et al, 1982a).

The mechanism(s) of vitamin K and vitamin K 2,3-epoxide reduction has (have) not been elucidated, primarily because the enzyme(s) catalyzing these reactions has (have) not yet been purified. It is not presently known whether these reactions are catalyzed at the same site of a single enzyme, at separate sites on a single enzyme, or at separate sites on two distinct enzymes.

With respect to the mechanism(s) of these reactions, recent data obtained from chemical model studies (Silverman, 1981a,b; Preusch et al., 1982) have provided evidence for sulfhydryl group participation in the reduction of both vitamin K 2,3-epoxide to vitamin K and vitamin K to vitamin K hydroquinone. Each product of vitamin K 2,3-epoxide or vitamin K metabolism, including the recently discovered hydroxyvitamin K (de Metz et al., 1982; Fasco et al., 1983), can be formed by reaction of the appropriate vitamin with thiol compounds (Fasco & Principe, 1980; Silverman, 1981a,b; Preusch et al., 1982).

To test the extent of sulfhydryl group participation in the enzyme-catalyzed reduction of vitamin K and vitamin K 2,3-epoxide, we investigated in hepatic microsomes of rats the effects of sulfhydryl blocking reagents on the metabolism of these two vitamins.

## Experimental Procedures

*Materials.* Vitamin K, DL-dithiothreitol, L-dithiothreitol, dithioerythritol, glutathione (reduced), and lipoic acid (reduced) were purchased from Sigma; NEM (gold label), iodoacetic acid, iodoacetamide, and the remaining sulfhydryl compounds used were from Aldrich Chemical Co.; Emulgen 911 was from Kao Atlas (Tokyo, Japan). The high-performance liquid chromatograph was a Waters Associates (Milford, MA) Model 244 equipped with a Spectra Physics (Santa Clara, CA) Model 4000 recording integrator and WISP automatic injector (Waters Associates). The HPLC column was a radial-pak $C_{18}$ (5-mm i.d.–10-μm particle) housed in a Z-module (Waters Associates). Microsomal protein concentrations were determined by the method of Bradford (1976) using Bio-Rad reagents.

*Preparation of Compounds.* Vitamin K 2,3-epoxide and vitamin K hydroquinone were prepared as described previously (Fasco et al., 1982; Fasco & Principe, 1982a). Aqueous solutions of vitamin K and vitamin K 2,3-epoxide (20 mg/ml) in Emulgen 911 (10% v/v) were also prepared as described

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[1] Abbreviations: NEM, *N*-ethylmaleimide; vitamin K, vitamin $K_1$; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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(Fasco & Principe, 1982a). Water-insoluble sulfhydryl compounds were dissolved in 2-propanol and were added to reaction mixtures in a maximum volume of 0.01 mL.

*Vitamin K and Vitamin K 2,3-Epoxide Reductase Assays.* Male Wistar rats (250 ± 10 g) were fed food and water ad libitum and acclimatized to a 12-h off–on light cycle for at least 1 week. Food was withdrawn 24 h prior to sacrifice with carbon dioxide. Hepatic microsomes were prepared by differential centrifugation as described previously (Fasco & Principe, 1982a). The microsomes pelleted at 100000*g* were resuspended in 0.02 M Tris-HCl–0.15 M KCl buffer, pH 7.4, to a concentration of 20 mg of protein/mL and were stored at −80 °C until use.

The general methods used for the addition of components to reaction mixtures are described below; variations in these methods are described in the figure legends. All procedures were performed at 3–5 °C unless stated otherwise. Each concentration cited is a final concentration. Data are the average of duplicate determinations from a single experiment which was performed at least twice. Less than 10% variation was routinely obtained between duplicate experiments. Concentrations of vitamin K hydroquinone are uncorrected since we have demonstrated previously that under experimental conditions comparable to those used here, less than 5% of the hydroquinone is converted via oxidation to vitamin K (Fasco & Principe, 1980, 1982b).

*Reaction Mixtures Containing Sulfhydryl Blocking Reagents.* Reaction mixtures were in a final volume of 2 mL and contained microsomes (6 mg of protein) and 0.2 M Tris-HCl–0.15 M KCl buffer, pH 7.4. In studies that utilized reduced microsomes, dithiothreitol (0.1 mM) was the reductant and was added to reaction mixtures just prior to incubation at 25 °C. In studies of oxidized microsomes, water was added in place of dithiothreitol. Reaction mixtures were equilibrated at 25 °C by gentle shaking for 1 min. Thereafter at 1-min intervals were added 0.01 mL of 10% (v/v) Emulgen 911 or 0.01 mL of 10% (v/v) Emulgen 911 containing vitamin K or vitamin K 2,3-epoxide (see below), the sulfhydryl blocking reagent (1 mM) in water, dithiothreitol (5 mM) to destroy excess sulfhydryl blocking reagent and provide a source of reducing equivalents for the subsequent reaction, and vitamin K or vitamin K 2,3-epoxide (20 μM). After an additional 10 min, the reaction was terminated by the addition of 4 mL of 2-propanol/hexane (1:1). The vitamins were extracted into the organic phase by a brief vortex mixing, and following a 15-s low-speed centrifugation, a 1.5-mL aliquot of the organic phase was rapidly evaporated to dryness at 30 °C with a stream of oxygen-free nitrogen. The residue was redissolved in 0.1 mL of 2-propanol, and a 0.02-mL aliquot was immediately analyzed by HPLC. The eluting solvent was acetonitrile/2-propanol (9:1) at a flow rate of 1 mL/min. Detection was at 254 nm, and concentrations of vitamin K, vitamin K 2,3-epoxide, and vitamin K hydroquinone were determined by comparison of their peak integrated areas with those of external standards. The approximate elution times were at 730, 450, and 330 s, respectively.

In studies where the effects of vitamin K or vitamin K 2,3-epoxide on the inhibition produced by sulfhydryl blocking reagents were determined, the sequence of additions to reaction mixtures was the same as that described above except that the initial addition of vitamin K or vitamin K 2,3-epoxide (1–20 μM) was after 1 min at 25 °C. After each additional min were added the sulfhydryl blocking reagent (1 mM), dithiothreitol (5 mM), and the final substrate vitamin K or vitamin K 2,3-epoxide (20 μM). Other conditions were as described above. Data are expressed as percentages of rates of metabolism in the absence of the sulfhydryl blocking reagent. In the case where vitamin K 2,3-epoxide was both the initially and finally added substrate, the rates of metabolism were calculated at each time point by subtracting the rate of metabolite formation produced from the initial substrate from that of the combined rate (initially and finally added substrate) at the corresponding time points. The rates of vitamin K hydroquinone formation from vitamin K, when vitamin K was both the initially and finally added substrate, were similarly determined. When the initially added substrate was vitamin K and the finally added substrate was vitamin K 2,3-epoxide, the extent of epoxide metabolism at each time point was determined by subtraction of the initially added vitamin K concentration from the sum of the vitamin K and vitamin K hydroquinone concentrations. The rates of substrate (vitamin K 2,3-epoxide) consumption at the various times of reaction were also determined and agreed well with values calculated by the subtraction method just described. When vitamin K 2,3-epoxide was the initially added substrate and vitamin K the finally added substrate, the rates of vitmain K hydroquinone formation were determined by subtracting the rates of hydroquinone formation from the epoxide (in the absence of vitamin K) from the observed vitamin K hydroquinone formation rates.

*Reactions with Both Vitamin Substrates and No Sulfhydryl Blocking Reagents.* Microsomes (6 mg of protein in 2 mL of buffer) were incubated for 1 min at 25 °C, dithiothreitol (5 mM) was added, and 1 min later vitamin K, vitamin K 2,3-epoxide, or a mixture of the two was added in a constant concentration of Emulgen 911. At various times thereafter, reaction was terminated with 2-propanol/hexane, and the products were extracted and analyzed by HPLC as described above.

## Results

The ability (relative to that of dithiothreitol) of various monothiol and dithiol compounds to serve as a cofactor of vitamin K and vitamin K 2,3-epoxide reduction in hepatic microsomes was determined to assess the cofactor requirements for the two reactions (Table I). In general, the rate of vitamin K or vitamin K 2,3-epoxide reduction in hepatic microsomes was much slower with monothiol than with dithiol compounds as the cofactor. Of the dithiol compounds investigated only 1,6-hexanedithiol was somewhat inactive as a cofactor, which is probably due to its relatively low solubility in water. From the data in Table I, the only apparent structural requirement for cofactor activity was the presence of two thiol groups. Mercaptoethanol differs from 1,2-ethanedithiol only by the substitution of atomic sulfur for oxygen, but it is virtually inactive compared to the dithiol analogue. 1,2-Ethanedithiol was as effective as dithiothreitol in supporting vitamin K hydroquinone formation, and lipoic acid was nearly as effective, thus demonstrating that the distance between the two thiol groups is not critical for cofactor activity. The stereoisomers of DL-dithiothreitol, L-dithiothreitol, and dithioerythritol and the non-hydroxy analogue 1,4-butanedithiol were all about equally effective providing further evidence for the lack of a strict structural requirement for cofactor activity. 4-Butanethiol at 5 mM was more active as a cofactor for the reduction of vitamin K 2,3-epoxide than for the reduction of vitamin K, but at concentrations of 1–2.5 mM (data not shown) it was virtually inactive. In contrast, each of the dithiol compounds was nearly as effective at 1 mM as it was at 5 mM. The data in Table I are consistent with those of Whitlon et al. (1978), which demonstrated that the monothiol compounds cysteine

Table I: Relative Abilities of Various Monothiol and Dithiol Compounds To Function as Cofactors for the Reduction of Vitamin K and Vitamin K 2,3-Epoxide[a]

| sulfhydryl compounds (5 mM) | % of DL-dithiothreitol rate at 5 mM[b] | |
|---|---|---|
| | Vit K → Vit $KH_2$ | Vit KO → Vit K + $KH_2$ |
| monothiols | | |
| mercaptoethanol | 6 | 6 |
| glutathione (red) | 10 | 6 |
| cysteine | 13 | 6 |
| 4-butanethiol | 9 | 64 |
| dithiols | | |
| 1,2-ethanedithiol | 100 | 110 |
| lipoic acid (red) | 90 | 90 |
| 1,4-butanedithiol | 100 | 130 |
| 1,6-hexanedithiol | 4 | 25 |
| L-dithiothreitol | 120 | 100 |
| dithioerythritol | 100 | 103 |

[a] Reaction mixtures were 2 mL final volume and contained 6 mg of microsomal protein and 0.2 M Tris-HCl–0.15 M KCl buffer, pH 7.4. They were incubated for 1 min at 25 °C, and the thiol compound (5 mM) and vitamin K or vitamin K 2,3-epoxide (20 μM) were added sequentially at 1-min intervals. After an additional 10 min of incubation, the reaction was terminated with 2-propanol/hexane (4 mL), the vitamin products were extracted, and their concentrations were determined by high-performance liquid chromatography as described under Experimental Procedures. The water-insoluble thiol compounds were added in 0.01 mL of 2-propanol. [b] At 10 min of incubation, the ratios of vitamin $KH_2$ to vitamin K concentrations were 3.2 for 1,2-ethanedithiol, 0.3 for lipoic acid (red), 2.8 for 1,4-butanedithiol, 2.7 for L-dithiothreitol, and 3.3 for dithioerythritol. The ratio with DL-dithiothreitol was 2.4. No vitamin $KH_2$ was detectable when the other thiol compounds were used as the reductant.

and glutathione exhibited little cofactor activity relative to the dithiol compounds dithiothreitol and lipoic acid for vitamin K or vitamin K 2,3-epoxide dependent γ-carboxylation of hepatic microsomal protein of vitamin K deficient rats. The dithiol compounds used in their investigations also supported the reduction of vitamin K 2,3-epoxide to vitamin K, but the monothiol compounds did not.

Microsomes exposed to 0.5–2 mM of the sulfhydryl blocking reagent NEM for 1 min at 25 °C lost only 10–20% of the vitamin K or vitamin K 2,3-epoxide reductase activity initially present (Figure 1). Reduction of the microsomes with 0.1 mM dithiothreitol prior to their exposure to the same concentrations of NEM for 1 min at 25 °C, however, increased the extent of inhibition of both enzymatic reactions to approximately 80% (Figure 1). Maximum inhibition of both reactions was attained at 0.5 mM or higher NEM concentrations. At a NEM concentration of 1 mM and an exposure for 1 min at 25 °C, prereduction of the microsomes with increasing concentrations of dithiothreitol produced progressively greater extents of inhibition of both vitamin K and vitamin K 2,3-epoxide reduction until, at approximately 0.1 mM, the rate of inhibition was maximal (Figure 2). As for the case of varying NEM concentration, the profile of inhibition over a range of dithiothreitol from 0.02 to 0.2 mM was similar for the two vitamin substrates.

In related experiments (data not shown), some of the mono- and dithiol compounds of Table I were tested for their ability to enhance inhibition by NEM of vitamin K 2,3-epoxide reductase activity by substituting them for dithiothreitol as the initially added reductant. At a concentration of 0.1 mM, and incubation with microsomes for 1 min at 25 °C, butanethiol and mercaptoethanol did not enhance the extent of NEM inhibition over the 10–20% obtained in the absence of any reducing agent. In contrast, 1,4-butanedithiol and 1,2-

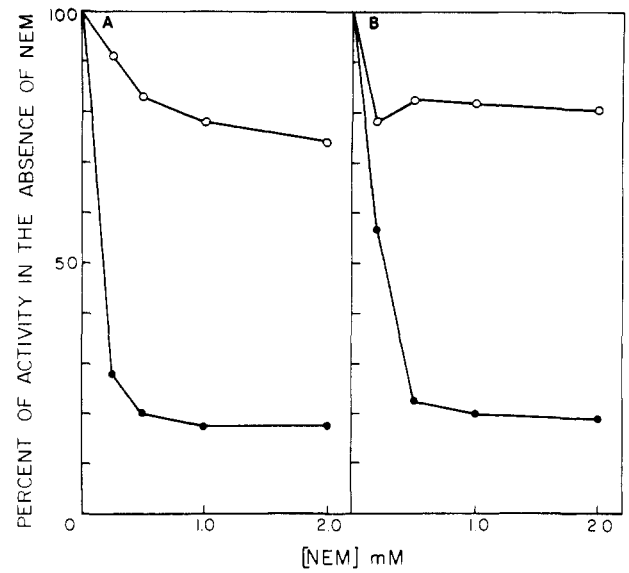


FIGURE 1: Effect of NEM concentration on the rates of vitamin K (A) and vitamin K 2,3-epoxide (B) reduction catalyzed by dithiothreitol (0.1 mM) reduced (●) and oxidized (untreated) (○) rat hepatic microsomes. Reaction mixtures were in a final volume of 2 mL and contained 6 mg of microsomal protein and 0.2 M Tris-HCl–0.15 M KCl buffer, pH 7.4. They were incubated for 1 min with gentle stirring at 25 °C, and the following were added at 1-min intervals: dithiothreitol (0.1 mM) for reduction of the microsomes or the same volume of water; NEM over the range 0.25–2 mM; dithiothreitol (5 mM) to destroy excess NEM and serve as cofactor for the uninhibited enzyme; either vitamin K or vitamin K 2,3-epoxide (20 μM) as the substrate. After an additional 10 min the reactions were terminated by the addition of hexane/2-propanol and the vitamin K products extracted and analyzed by high-performance liquid chromatography as described under Experimental Procedures. Data are expressed as a percent of the rate of vitamin K and vitamin K 2,3-epoxide reduction determined in the absence of NEM.

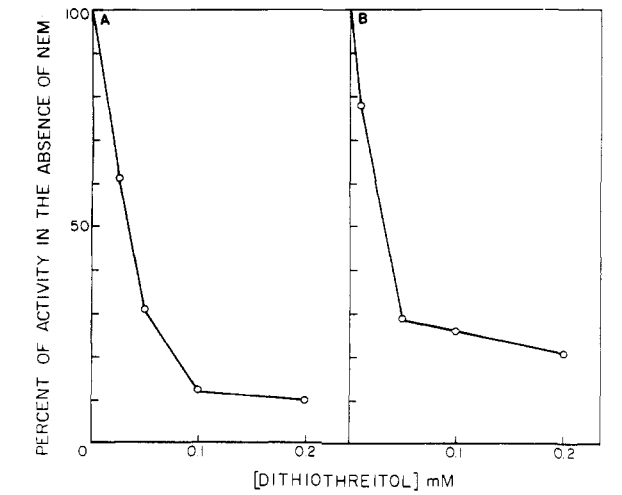


FIGURE 2: Effect of dithiothreitol concentration on the extent of inhibition by NEM of vitamin K (A) and vitamin K 2,3-epoxide (B) reduction catalyzed by hepatic microsomes of rats. Conditions were as described for Figure 1 except that the dithiothreitol concentration added at 1 min of incubation at 25 °C was varied from 0.025 to 0.2 mM.

ethanedithiol enhanced NEM inhibition of the reductase by approximately the same extent as did dithiothreitol. The ability of sulfhydryl compounds to enhance inhibition by NEM thus parallels their ability to function as cofactor, which suggests that both events are interrelated and probably involve reduction of the same disulfide bond.

At concentrations greater than 0.5 mM, iodoacetamide was more effective than NEM as an inhibitor of vitamin K and vitamin K 2,3-epoxide reduction in microsomes which were

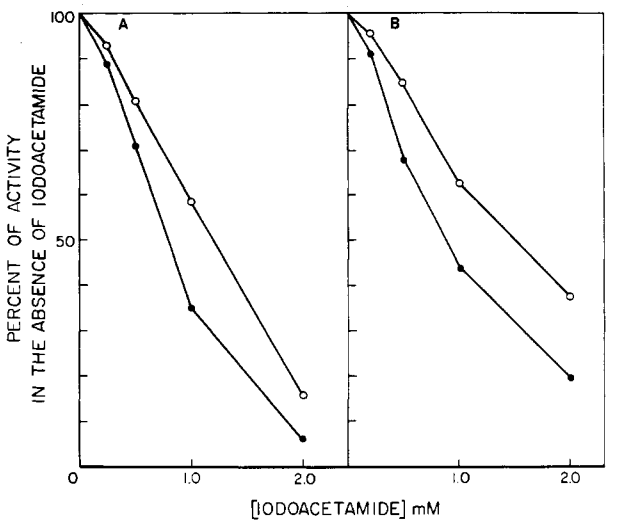


FIGURE 3: Effect of iodoacetamide concentration on the rates of vitamin K (A) and vitamin K 2,3-epoxide (B) metabolism catalyzed by dithiothreitol (0.1 mM) reduced (●) or oxidized (untreated) (○) hepatic microsomes of rats. Conditions were as described for Figure 1 except that iodoacetamide was used in place of NEM as the sulfhydryl blocking reagent.

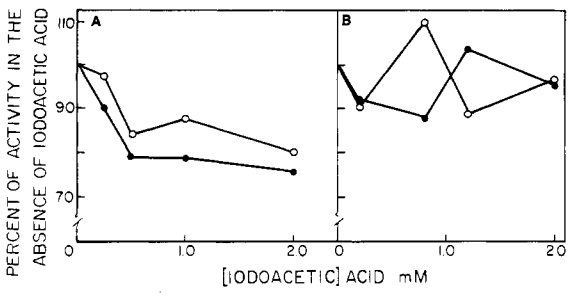


FIGURE 4: Effect of iodoacetic acid concentration on the rates of vitamin K (A) and vitamin K 2,3-epoxide (B) metabolism catalyzed by dithiothreitol (0.1 mM) reduced (●) or oxidized (untreated) (○) hepatic microsomes of rats. Conditions were as described for Figure 1 except that iodoacetic acid was used in place of NEM as the sulfhydryl blocking reagent.

not reduced initially with dithiothreitol (Figure 3). Over the range 0.2–2 mM, the extent of inhibition by iodoacetamide increased steadily to 85% for vitamin K reduction and 62% for vitamin K 2,3-epoxide reduction. Initial reduction of the microsomes with 0.1 mM dithiothreitol enhanced the inhibitory activity of iodoacetamide, but relative to NEM, the differences between the extents of inhibition in oxidized (untreated) and dithiothreitol-reduced microsomes were not as great at any of the inhibitor concentrations investigated (cf. Figures 1 and 3). In microsomes initially reduced with dithiothreitol, the extent of inhibition increased with increasing iodoacetamide concentrations to 2 mM, which also contrasts with results obtained with NEM.

Iodoacetic acid was a weak inhibitor of vitamin K reduction and was inactive as an inhibitor of vitamin K 2,3-epoxide reduction (Figure 4). Reduction of the microsomes prior to their exposure to iodoacetic acid for 1 min at 25 °C had little effect on the rate of vitamin K reduction and no detectable effect on vitamin K 2,3-epoxide reduction. Since iodoacetamide and iodoacetic acid are similar with respect to their structure and mode of action, it is likely that the negative charge on the latter is primarily responsible for its lack of inhibitory activity.

To determine if inhibition of vitamin K and vitamin K 2,3-epoxide reduction by NEM was occurring at one or more sulfhydryl groups which participated in their metabolism,

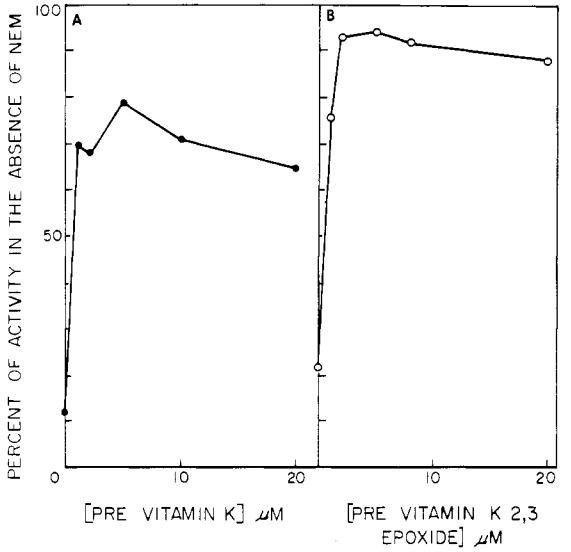


FIGURE 5: Effects of vitamin K or vitamin K 2,3-epoxide on inhibition by NEM of respectively vitamin K (A) and vitamin K 2,3-epoxide (B) reduction in dithiothreitol (0.1 mM) reduced heaptic microsomes of rats. Reaction mixtures were 2 mL final volume and contained 6 mg of microsomal protein, 0.1 mM dithiothreitol, and 0.2 M Tris–HCl–0.15 M KCl buffer, pH 7.4. They were incubated at 25 °C for 1 min, and the following were added at 1-min intervals: initial vitamin K or vitamin K 2,3-epoxide over the range 1–20 μM; NEM (1 mM); dithiothreitol (5 mM); vitamin K or vitamin K 2,3-epoxide (20 μM) substrate which was the same as the initially added vitamin. After an additional 10 min the vitamin products were extracted and their concentrations determined by high-performance liquid chromatography as described under Experimental Procedures.

various concentrations of vitamin K or vitamin K 2,3-epoxide were incubated with dithiothreitol-reduced microsomes for 1 min prior to the addition of NEM. This was followed sequentially at 1-min intervals by the addition of dithiothreitol (5 mM) and the same substrate (20 μM) added initially. Incubation of dithiothreitol-reduced microsomes with 1 μM vitamin K diminished the extent of NEM inhibition of vitamin K reduction from 88% to 30% (Figure 5A). Higher concentrations of the vitamin had little effect on the extent of recovery of reductase activity, and thus, only 70–80% of the original activity was recovered. Incubation of dithiothreitol-reduced microsomes with 1–2 μM vitamin K 2,3-epoxide also diminished the extent of inhibition by NEM of vitamin K 2,3-epoxide reduction from 78% to approximately 10% (Figure 5B), and as for vitamin K, incubation with higher concentrations of the vitamin did not increase enzyme activity. In related experiments, incubation of dithiothreitol-reduced microsomes for 1 min at 25 °C with 1–2 μM vitamin K 2,3-epoxide blocked subsequent NEM inhibition of vitamin K reduction (Figure 6A) as effectively as did preincubation with vitamin K (Figure 5A). Under the same conditions, 1–2 μM vitamin K also effectively diminished NEM inhibition of vitamin K 2,3-epoxide metabolism (Figure 6B). As for the previous cases, initially added vitamin K or vitamin K 2,3-epoxide concentrations greater than 2 μM did not restore complete reductase activity, and thus the maximum levels of activity that could be restored in the presence of NEM were between 70 and 90%. This difference approximated the 10–30% inhibition of vitamin K or vitamin K 2,3-epoxide reductase activity that was produced by incubation of oxidized (untreated) microsomes with NEM.

Since treatment of reduced microsomes with vitamin K or vitamin K 2,3-epoxide could block NEM inhibition of its own or the other vitamin's metabolism, experiments were undertaken to determine if either vitamin affected the metabolism

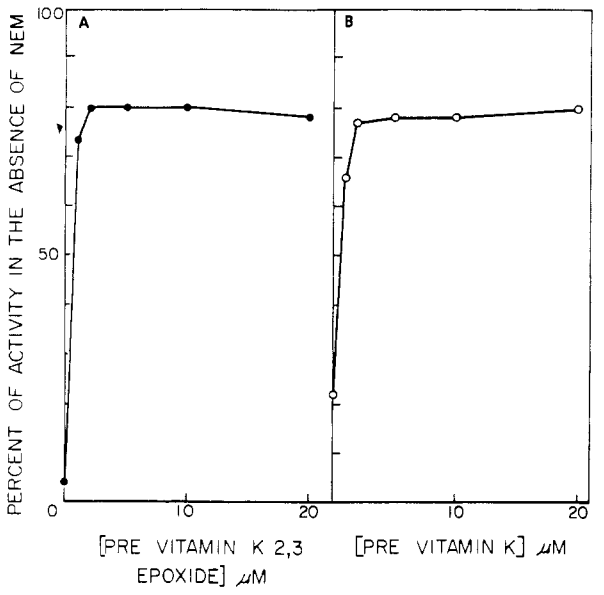


FIGURE 6: Effects of vitamin K 2,3-epoxide or vitamin K on inhibition by NEM of respectively vitamin K (A) and vitamin K 2,3-epoxide (B) reduction in dithiothreitol (0.1 mM) reduced hepatic microsomes of rats. Reaction mixtures were prepared as described in Figure 5 except that the initially added vitamin and the finally added vitamin substrates were different.

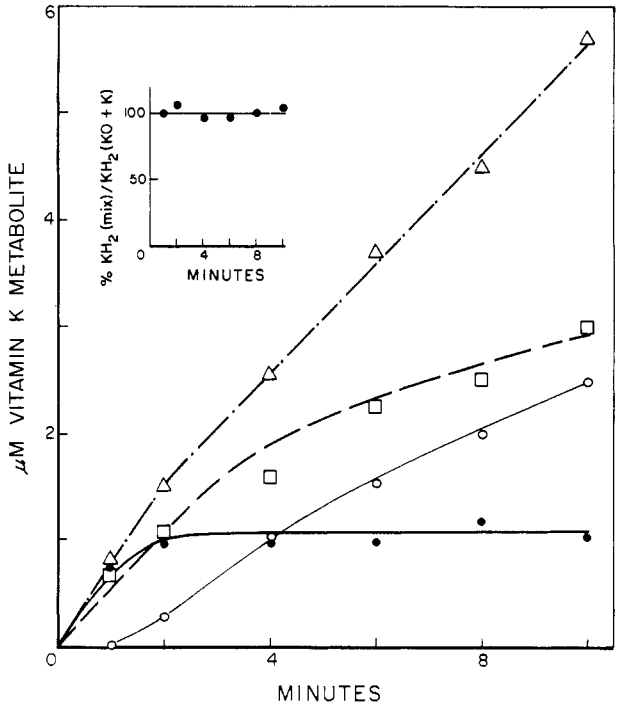


FIGURE 7: Comparison of the concentrations of vitamin K hydroquinone produced by the metabolism of 20 μM vitamin K (□); 20 μM vitamin K 2,3-epoxide (○), and a mixture of 20 μM each of vitamin K and vitamin K 2,3-epoxide (△) with time. The concentrations of vitamin K that were produced from the metabolism of vitamin K 2,3-epoxide at each time point (●) are also included. Conditions were as described under Experimental Procedures. (Insert) Percent vitamin K hydroquinone concentration produced by metabolism of a mixture of vitamin K and vitamin K 2,3-epoxide divided by the sum of the vitamin K hydroquinone concentrations produced by the metabolism of each vitamin as a function of time.

of the other. The time course of vitamin K hydroquinone formation from each vitamin (20 μM) was determined and was compared with that from a mixture (20 μM each) of vitamin K and vitamin K 2,3-epoxide (Figure 7). With vitamin K 2,3-epoxide as substrate, vitamin K was the only product detected during the first minute of reaction; thereafter,

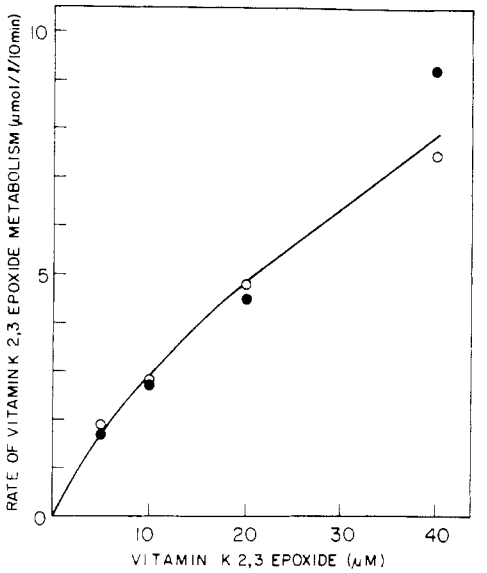


FIGURE 8: Comparison of the metabolism rates of various vitamin K 2,3-epoxide concentrations in the presence (●) and absence (○) of vitamin K (40 μM). Vitamin K and vitamin K 2,3-epoxide were premixed in the appropriate amounts. Reaction mixtures were in a final volume of 2 mL and contained 6 mg of microsomal protein and 0.2 M Tris–HCl–0.15 M KCl buffer, pH 7.4. They were incubated for 1 min at 25 °C with gentle shaking, and dithiothreitol (5 mM) and the substrate mixture were added sequentially at 1-min intervals. The Emulgen 911 was maintained at 0.01% throughout. After an additional 10 min of incubation the reactions were terminated and the vitamin products extracted and analyzed by high-performance liquid chromatography as described under Experimental Procedures.

vitamin K formation attained a steady-state rate, and the rate of vitamin K hydroquinone formation increased (Figure 7). With vitamin K as the substrate, however, vitamin K hydroquinone formation was readily detected at 1 min of reaction (Figure 7). Addition of the mixture of vitamin K and vitamin K 2,3-epoxide to microsomes resulted in a rate of hydroquinone formation at each time point investigated which was equal to that calculated from the contribution of each individual vitamin substrate (Figure 7, insert). Moreover, the extent of hydroquinone formation at 1 min of reaction was indistinguishable from that when vitamin K was the only substrate, which is strong evidence for a lack of interaction.

The rates of vitamin K 2,3-epoxide metabolism over a range of 5–40 μM in the presence and absence of 40 μM vitamin K are presented in Figure 8. Vitamin K had no detectable effect on the rates of vitamin K 2,3-epoxide metabolism even when it was present in 8-fold excess. These data are thus consistent with those of the previous experiment and collectively support the concept that the metabolism of each vitamin occurs independently of the other.

## Discussion

The ability of most of the dithiol compounds investigated to function efficiently as cofactors for the metabolism of vitamin K and vitamin K 2,3-epoxide and to promote inhibition by NEM of both reactions strongly suggests that the reduction of one or more critical disulfide bonds is required for enzymic activity. A plausible explanation for the inability of monothiol compounds to function efficiently as cofactors is that a major proportion of the mixed disulfide initially formed by reaction of the monothiol and reductase disulfide undergoes reoxidation before a second molecule of monothiol can react. In contrast, dithiols, because of the proximity of the second sulfhydryl group, cleave the mixed disulfide at a rate that is sufficiently rapid to produce relatively more of the fully reduced protein disulfide (Cleland, 1964). The concept of a disulfide bond

which must be reduced for enzymic activity is also consistent with our results on NEM inhibition of vitamin K and vitamin K 2,3-epoxide reduction. Since NEM reacts with sulfhydryl groups but not disulfides (Cecil, 1963; Kanaoka et al., 1970), it follows that dithiols promote NEM inhibition of reductase activity by reducing at least one potentially susceptible disulfide bond associated with catalytic activity. Monothiol compounds are unable to reduce this disulfide for the reasons discussed above and thus do not promote NEM inhibition.

The data from these investigations provide strong evidence that vitamin K and vitamin K 2,3-epoxide are metabolized at separate sites which function independently of one another but do not distinguish whether these sites are on the same enzyme or on two separate enzymes. The metabolism of vitamin K 2,3-epoxide is an ordered process in which the first product, vitamin K, must attain a minimum concentration before being further metabolized to vitamin K hydroquinone (Figure 7). Vitamin K formation subsequently attains a steady state while the formation of vitamin K hydroquinone increases. If the metabolism of vitamin K 2,3-epoxide and its metabolite vitamin K occurred at the same catalytic site, it would be expected that the epoxide would inhibit metabolism of vitamin K to the hydroquinone and/or that vitamin K would inhibit metabolism of the epoxide to vitamin K. Inhibition of either vitamin K or vitamin K hydroquinone formation would almost certainly destroy the steady-state condition, however, which was not the case (Figure 7). Moreover, in experiments where various proportions of both vitamin K and vitamin K 2,3-epoxide were added to reaction mixtures, no effect of one on the metabolism of the other was detectable (Figures 7 and 8), supporting the conclusion that both reductions do not occur at a single site.

Two modes of inhibition of vitamin K and vitamin K 2,3-epoxide reduction by sulfhydryl reagents were demonstrated by the present investigations. One occurred with oxidized (untreated) microsomes and could not be blocked by incubation of microsomes with either vitamin K or vitamin K 2,3-epoxide prior to their exposure to the sulfhydryl reagent. Iodoacetamide was more effective than NEM as an inhibitor via this mechanism (cf. Figures 1 and 3). Since reduction was not required for inhibition, there is at least one free nucleophilic group which, when modified, diminishes the rates of both vitamin K and vitamin K 2,3-epoxide reduction. The data from these investigations do not suggest the identity of this group or its relationship to vitamin K and vitamin K 2,3-epoxide metabolism. Iodoacetamide is less specific than NEM toward reaction with nucleophiles other than sulfhydryl groups, however, and its reaction with, for example, an amine could therefore account for the difference in reactivity observed between the two reagents. The second mode of inhibition occurred solely in microsomes that were reduced by dithiol compounds prior to exposure to sulfhydryl reagents. This differed from the first in that incubation of reduced microsomes with low concentrations of either vitamin prior to the addition of NEM or iodoacetamide blocked inhibition of vitamin K and vitamin K 2,3-epoxide reduction. NEM was more useful than iodoacetamide for investigations of inhibition by this mechanism because of its relatively slow reaction with the free nucleophilic group(s) of oxidized microsomes (cf. Figures 1 and 3). On the basis of our present data and those obtained from model reactions, the most probable explanation for the blockage of NEM inhibition by vitamin K or vitamin K 2,3-epoxide is that the sulfhydryl groups produced initially by reduction with dithiols are reoxidized to the nonreactive disulfide form during the metabolism of either vitamin. At the low concentration of dithiol reductant initially present, the rate of metabolism of even 2 $\mu$M vitamin K or vitamin K 2,3-epoxide is sufficiently rapid that the equilibrium between the protein sulfhydryl and disulfide forms lies largley in the direction of the latter. A particularly important result was that incubation of reduced microsomes with vitamin K prior to exposure to NEM blocked inhibition of vitamin K 2,3-epoxide metabolism as effectively as did incubation with vitamin K 2,3-epoxide and that the same was true for vitamin K 2,3-epoxide protection of vitamin K metabolism (Figure 6). The crossover protection against NEM inhibition of enzymic activity would then suggest that the reduction of either vitamin K or vitamin K 2,3-epoxide produces reoxidation of the same NEM-susceptible sulfhydryl groups to the disulfide form. This could occur if the protein sulfhydryl groups produced by reduction of a "common disulfide" by a dithiol compound participated directly in a nucleophilic and/or free radical reaction with both vitamin K and vitamin K 2,3-epoxide. Such a mechanism is incompatible with the data which provided evidence for separate and independent sites of vitamin K and vitamin K 2,3-epoxide metabolism (Figures 7 and 8), however, for the reasons previously discussed. A possible alternate role for a common disulfide in metabolism which is consistent with the concept of separate sites for vitamin K and vitamin K 2,3-epoxide reduction is that in the reduced form its sulfhydryl groups transfer reducing equivalents to some other potentially reactive groups within each catalytic site which then undergo direct reaction with the appropriate vitamin substrate, the reactive group being reoxidized in the process. Because all the currently identified microsomal metabolites of vitamin K and vitamin K 2,3-epoxide can be synthesized under mild conditions by reaction with sulfhydryl-containing compounds, it is possible that the other potentially reactive groups in each of the catalytic sites are also disulfides. However, much additional experimentation is necessary to determine the mechanisms that contribute to the apparent disparity between separate and independent sites of vitamin K and vitamin K 2,3-epoxide metabolism and a common disulfide.

In view of the complexity of the sulfhydryl-dependent reactions involving vitamin K and vitamin K 2,3-epoxide, it is apparent that complete elucidation of the mechanisms involved in their metabolism can be obtained only with highly purified enzyme(s). The enzyme(s) catalyzing these reactions are denatured by a variety of detergents, however, and have not yet been purified to homogeneity. Thus, studies in hepatic microsomes are necessary initially to gain insight into the mode(s) of action of the enzyme(s) and to evaluate subsequently the effects of detergents and stabilizing agents on metabolic activity. Studies using soluble, partially purified hepatic microsomal preparations are currently in progress to characterize more completely the role of various functional groups in the metabolism of vitamin K and vitamin K 2,3-epoxide.

**Registry No.** Vitamin K, 12001-79-5; vitamin K 2,3-epoxide, 25486-55-9; vitamin K hydroquinone, 572-96-3; glutathione, 70-18-8; cysteine, 52-90-4; 4-butanethiol, 109-79-5; 1,2-ethanedithiol, 540-63-6; lipoic acid, 462-20-4; 1,4-butanedithiol, 1191-08-8; 1,6-hexanedithiol, 1191-43-1; L-dithiothreitol, 16096-97-2; dithioerythritol, 6892-68-8; mercaptoethanol, 60-24-2.

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# Direct Observation of Glycine Metabolism in Tobacco Suspension Cells by Carbon-13 NMR Spectroscopy†

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ABSTRACT: Carbon-13 NMR spectroscopy has been applied to the analysis of glycine metabolism in suspension cultured tobacco cells (*Nicotiana tabacum*). Treatment of 0.5 g of cells with glycine-*2*-$^{13}C$, followed by NMR analysis of the intact cells, generated spectra containing seven observable resonances. Comparison of the chemical shifts to authentic samples of glycine and L-serine allowed assignment of three peaks to glycine-*2*-$^{13}C$, serine-*2*-$^{13}C$, and serine-*3*-$^{13}C$. The remaining four resonances, generated by one-bond $^{13}C$–$^{13}C$ coupling ($J$ = 37.5 Hz), were ultimately assigned to serine-*2,3*-$^{13}C$. This was verified by two methods: $^{13}C$–$^{13}C$ spin–spin coupling and two-dimensional homonuclear ($^{13}C$) correlated spectroscopy. The $^{13}C$–$^{13}C$ spin-coupled spectrum of L-serine displayed a serine $C_2$–$C_3$ coupling constant of 37.4 Hz, and the 2-D spectrum, obtained from 3.5 g of tobacco cells treated with 10 mM glycine-*2*-$^{13}C$ for 24 h, established the predicted pattern of correlation. The presence of doubly labeled serine, representing 74% of the total $^{13}C$-labeled serine formed, as well as the two singly labeled serine species, allowed calculations of the intracellular glycine and serine pool sizes and the percent of serine's carbons 2 and 3, which were derived from the glycine-*2*-$^{13}C$ added to the cells. Calculation of intracellular glycine and serine levels was achieved by integration of the individual resonances relative to an external 2.4 M sodium acetate standard. Calibration of the external standard to an internal 0.12 M acetate standard and appropriate adjustments for the individual serine, glycine, and acetate nuclear Overhauser effect values provided the basis for the semiquantitative calculations. The results demonstrate the ability of carbon-13 NMR spectroscopy to monitor the metabolism of glycine in nonphotosynthetic tobacco cells and that serine formation from glycine is the result of tight coupling between glycine decarboxylase and serine hydroxymethyltransferase.

The formation of serine from glycine is an important metabolic pathway in plant cells. In photosynthetic tissues, the glycolate pathway (glycolate → glyoxylate → glycine → serine) is responsible for the photorespiratory production of $CO_2$ by the decarboxylation of glycine and possibly glyoxylate (Oliver, 1979). The primary evidence for the involvement of glycine decarboxylase to produce the photorespired $CO_2$ and methylenetetrahydrofolic acid for serine synthesis comes from studies that have localized glycine decarboxylase and L-serine hydroxymethyltransferase in leaf mitochondria (Woo & Osmund, 1976; Woo, 1979). Studies with $^{14}C$-labeled glyoxylate and glycine have suggested that glyoxylate may directly contribute to the photorespiratory $CO_2$ and formate (Grodzinski, 1978; Oliver, 1979). Gifford & Cossins (1982a,b) presented evidence for the production of serine from $^{3}H$- and $^{14}C$-labeled formate and suggested that formate may be an important source of one-carbon units utilizing the cytosolic enzyme 10-formyltetrahydrofolate synthetase.

Nuclear magnetic resonance spectroscopy has recently provided a novel method for determining biological constituents and processes. Due to its nondestructive nature, NMR[1] spectroscopy can be used to assay living tissues. These tissues can then be recovered, unharmed, for further studies. In addition, because analysis can be performed on the intact tissues, often difficult and time-consuming extraction and separation procedures are eliminated. With the advent of the pulsed Fourier-transform technique and the availability of high magnetic field instruments, NMR spectroscopy can provide a unique and sensitive method of analysis. Furthermore, the

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; 2-D, two dimensional; $T_1$, spin–lattice relaxation time; THF, tetrahydrofolic acid.

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