Purification of a vitamin K epoxide reductase that catalyzes conversion of vitamin K 2,3-epoxide to 3-hydroxy-2-methyl-3-phytyl-2,3-dihydronaphthoquinone

(beef liver microsomes/warfarin/dithiothreitol)

INDRANI MUKHARJI AND RICHARD B. SILVERMAN*

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60201

Communicated by Irving M. Klotz, January 11, 1985

ABSTRACT An enzyme from bovine liver microsomes that catalyzes the reduction of vitamin K 2,3-epoxide to 2- and 3-hydroxy-2-methyl-3-phytyl-2,3-dihydronaphthoquinone was purified 1152-fold to apparent homogeneity. Microsomes were solubilized with 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), and the enzyme was purified by chromatography on PBE-94 ion exchanger, hydroxylapatite, and DEAE-cellulose, and then gel filtration on Sephacryl S-200. The homogeneity of the final preparation was established by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate. The molecular weight of the native enzyme is 25,000 and that of denatured enzyme is 12,400, which suggests that the enzyme is a dimer with identical subunits. No chromophoric cofactors are associated with the enzyme. Dithiothreitol and CHAPS are essential for activity, but high concentrations of glycerol reduces the activity. The enzyme is not inhibited by warfarin, a potent inhibitor of the vitamin K epoxide reductase, which catalyzes the conversion of vitamin K 2,3-epoxide to vitamin K. Evidence is presented indicating that the purified enzyme is not simply a fragment of the warfarin-sensitive vitamin K epoxide reductase.

Vitamin K (2-methyl-3-phytyl-1,4-naphthoquinone) is required for the biosynthesis of active prothrombin and other plasma clotting factors (1-3). Its primary function is involved in the post-translational γ -carboxylation of certain glutamic acid residues in proteins that are converted to coagulation factors II, VII, IX, and X (3). This carboxylation reaction, which is catalyzed in microsomes by a vitamin K-dependent carboxylase, requires vitamin K hydroquinone, molecular oxygen, carbon dioxide (4), and a peptide-bound glutamyl residue (5). Concomitant with γ -glutamate carboxylation is the epoxidation of vitamin K hydroquinone; both reactions are believed to be catalyzed by the same enzyme (6, 7). The vitamin K 2,3-epoxide generated by this epoxidase/carboxylase is converted back to vitamin K by vitamin K epoxide reductase so that the cycle can continue (8, 9). This reaction requires dithiothreitol for activity (the physiological reductant is unknown), and it is potently inhibited by warfarin and other oral anticoagulants (10-12). The vitamin K hydroquinone required for carboxylation of the γ -glutamyl residues is generated by reduction of vitamin K, catalyzed by certain NAD(P)H-linked dehydrogenases (13, 14) as well as a dithiothreitol-dependent reductase, which is sensitive to warfarin and may be the vitamin K epoxide reductase involved in the vitamin K cycle (9, 15-17).

A new metabolite of vitamin K, 2(or 3)-hydroxy-2-methyl-3-phytyl-2,3-dihydronaphthoquinone (structure 1)[†] was identified as an *in vitro* product of vitamin K 2,3-epoxide metabolism in hepatic microsomes of warfarin-resistant rats,

but not of normal rats (18). More recently, however, this compound was isolated and identified as an *in vivo* metabolite of vitamin K epoxide in both normal and warfarin-resistant rats (20). It was concluded that this metabolite was not a product of vitamin K epoxide reductase *in vivo*, and it may result from a non-enzymatic reaction (20). In a chemical model study for the conversion of vitamin K epoxide to vitamin K, it was observed that an intermediate in this dithiothreitol-dependent reduction was 2(or 3)-hydroxy-2,3-dihydro(vitamin K) (21). As a result of these studies and other model studies (22), it was suggested that 3-hydroxy-2,3-dihydro(vitamin K) is an intermediate in the vitamin K epoxide reductase-catalyzed conversion of vitamin K epoxide to vitamin K (18, 21). Detection of 2(or 3)-hydroxy-2,3-dihydro(vitamin K) also has been reported during the carboxylase reaction by using a partially purified vitamin K-dependent carboxylating enzyme system from bovine liver microsomes of warfarin-treated cows (19). In this case, it was suggested that the new metabolite was derived from a reduction of 2(or 3)-peroxy-2-methyl-3-phytyl-2,3-dihydronaphthoquinone, the hypothesized intermediate in the epoxidase (carboxylase) reaction. We describe here the isolation and purification to apparent homogeneity of an enzyme in bovine liver microsomes of untreated cows that catalyzes the conversion of vitamin K 2,3-epoxide to 3- and 2-hydroxy-2,3-dihydro(vitamin K) and that is distinct from the enzyme that reduces vitamin K 2,3-epoxide to vitamin K.

MATERIALS AND METHODS

Materials. Beef liver was obtained from a local slaughterhouse and was stored frozen at -70°C until ready for use.

Abbreviation: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

*To whom reprint requests should be addressed at the Department of Chemistry.

[†]A generic name previously given to this metabolite was hydroxyvitamin K (18-20). However, this name implies a substitution of hydroxyl for hydrogen somewhere in the molecule, which is not the case. The new metabolite is actually the product of an addition of a molecule of water across the 2-3 double bond of vitamin K, and it is more accurately termed "hydrated vitamin K" even though this name also is ambiguous as to what is hydrated. Throughout the text, Structures 1a and 1b are referred to as 2- and 3-hydroxy-2,3-dihydro(vitamin K).

Potassium phosphate (monobasic and dibasic), Tris, glycerol, DL-dithiothreitol, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), vitamin K, *N,N,N',N'*-tetramethylethylenediamine, ammonium persulfate, NaDodSO₄, warfarin, and the molecular weight standard kits were obtained from Sigma. Acrylamide and bis(acrylamide) were purchased from Bio-Rad. Ultrapure sucrose was the product of Schwarz/Mann, and EDTA disodium salt was obtained from Fisher. Ion exchanger PBE-94 was purchased from Pharmacia. Hydroxylapatite fast flow dry powder was obtained from Calbiochem. Whatman DE-52 microgranular preswollen anion exchanger was supplied by American Scientific Products, McGaw, IL.

Vitamin K epoxide was prepared from vitamin K and alkaline hydrogen peroxide according to the method of Tishler *et al.* (23). 3-Hydroxy-2,3-dihydro(vitamin K) and the 1:3 mixture of 2- and 3-hydroxy-2,3-dihydro(vitamin K) were obtained by published procedures (18, 21).

Methods. Compounds prepared as described above were purified by HPLC using a 10- μ m C₁₈ reversed-phase semipreparative column (Alltech, Deerfield, IL; 50 cm $\times$ 10 mm) and eluting with methanol at 4 ml/min. The retention times for Structures 1a and 1b are 17.9 and 18.8 min, respectively, whereas those for vitamin K epoxide and vitamin K are 33.0 and 53.2 min, respectively. All HPLC work was carried out with a Beckman model 330 HPLC with an analytical UV detector (254 nm). The NMR spectra were recorded on a JEOL JNM FX 270 MHz spectrometer, the UV spectra were recorded on a Perkin-Elmer 330 UV-VIS spectrophotometer, and the mass spectra were obtained on a Hewlett Packard 5985 GC/mass spectrometer. Polyacrylamide slab gel electrophoresis was carried out on 12% acrylamide/0.32% bisacrylamide or 10% acrylamide/0.27% bisacrylamide gels 1.5 mm thick in the presence of 0.2 NaDodSO₄ in Tris acetate buffer (pH 7.4). *N,N,N',N'*-Tetramethylethylenediamine (0.067%) was used for gel polymerization. The gels were run in an LKB 2001 vertical electrophoresis unit and a 2002 power supply at 60 V for 1 hr, then at 90 V for 4 hr. The gels were developed by a modification of the silver staining method of Morrissey (24), in which the glutaraldehyde fixation step was omitted. Protein concentrations were determined by the method of Lowry *et al.* (25) with bovine serum albumin as standard.

Enzyme Purification. *Step 1: Isolation and solubilization of microsomes.* Frozen beef liver was thawed and homogenized with 3 times its volume of 50 mM potassium phosphate buffer (pH 7.4) containing 250 mM sucrose, 0.5 mM EDTA (homogenizing buffer) in a Waring Blender (six 20 sec pulses) at 4°C and made into a smooth suspension with 20 additional strokes of a Teflon cap hand homogenizer. The homogenate was centrifuged at 12,000 rpm (17,000 $\times$ g) for 30 min to precipitate the cell debris. The supernatant was treated with one-fifth its volume of 100 mM calcium chloride, and after 1 hr, it was centrifuged at 18,000 rpm (39,000 $\times$ g) for 30 min. The microsomal fragments that precipitated were stable for months when stored frozen at -70°C. The microsomal pellet was solubilized by resuspension in 100 ml of the homogenizing buffer containing 1% CHAPS and 0.5 M sodium chloride, using an all glass hand homogenizer. The smooth suspension obtained with 20 strokes was stirred gently at 4°C for 2 hr. Centrifugation at 20,000 rpm (48,000 $\times$ g) for 90 min yielded the supernatant that contained the solubilized enzyme activity.

Step 2: Chromatography on PBE-94 ion exchanger. The solubilized microsomal supernatant was dialyzed overnight against (3 $\times$ 500 ml) of 25 mM Tris-CH₃COOH, pH 8.3/10% (vol/vol) glycerol/0.5 mM EDTA and then was applied to a PBE-94 column (2 $\times$ 25 cm) equilibrated with the same buffer. After sample application, the column was washed with the equilibrating buffer until all of the enzyme activity

eluted in the wash. The active eluant was dialyzed against (3 $\times$ 500 ml) of 50 mM Tris phosphate, pH 7.8/10% (vol/vol) glycerol.

Step 3: Hydroxylapatite chromatography. The dialyzed protein (100 ml) was applied to a hydroxylapatite column (2.8 $\times$ 11.5 cm) equilibrated with 50 mM Tris phosphate, pH 7.8/10% glycerol. After sample application, the column was washed with the equilibrating buffer until all of the enzyme activity was collected. To this active eluant (95 ml) was added CHAPS to make the final concentration 1.5%.

Step 4: DEAE-cellulose chromatography. The hydroxylapatite eluant containing 1.5% CHAPS was then applied to a DEAE-cellulose column (2 $\times$ 15 cm) equilibrated with 50 mM Tris phosphate, pH 7.8/10% glycerol/0.5 mM EDTA/1.5% CHAPS. The column was washed at a flow rate of 16 ml/hr with one column volume of the equilibrating buffer, and it was developed with a linear concentration gradient (250 ml) of 0–1.0 M sodium chloride in the pH 7.8 buffer. Fractions of 8 ml were collected and assayed. The active fractions were pooled and concentrated by ultrafiltration (Amicon).

Step 5: Sephacryl S-200. The concentrated DEAE-cellulose eluant was applied to a Sephacryl S-200 column (2.8 $\times$ 72 cm) equilibrated with 50 mM Tris phosphate, pH 7.4/10% glycerol/0.5 mM EDTA/0.25 M sodium chloride/1.0% CHAPS. The proteins were eluted at a flow rate of 20 ml/hr and 5-ml fractions were collected and assayed.

Enzyme Assays. The enzyme preparation was incubated at 25°C in a total volume of 0.5 ml of 50 mM Tris phosphate, pH 7.4/10% glycerol/3 mM dithiothreitol/1.5% CHAPS/20 μ M vitamin K 2,3-epoxide in ethanol for 5 min, during which time the reaction was linear. The final concentration of ethanol in the assay was 4%. The reaction was terminated by gently mixing in a Vortex with 2 ml of an isopropanol/hexane (3:2) mixture followed by 2 ml of hexane. The organic extracts were combined and the solvents were removed under vacuum in a Savant Speed Vac concentrator. The residue was dissolved in 0.05 ml of isopropanol and 0.02 ml of this was analyzed by HPLC, eluting with methanol at 2 ml/min on an analytical 10- μ m C₁₈ reversed-phase column (25 $\times$ 4.6 mm). The retention times for 2(or 3)-hydroxy-2,3-dihydro(vitamin K) and vitamin K 2,3-epoxide are 4.45 and 7.10 min, respectively, and these products were quantitated against standard curves.

RESULTS AND DISCUSSION

In our attempts to purify the vitamin K epoxide reductase from bovine liver microsomes, which reduces vitamin K 2,3-epoxide to vitamin K and possibly to vitamin K hydroquinone in the presence of dithiothreitol (17), we consistently observed the formation of 2- or 3-hydroxy-2,3-dihydro(vitamin K). Hildebrandt *et al.* (26) reported the artefactual formation of this metabolite in their assays with crude rat liver microsomes at pH 9.0 or above. We found that in Tris phosphate buffer at pH 7.4–7.8 in the presence of 1–5 mM dithiothreitol, the nonenzymatic formation of 2- or 3-hydroxy-2,3-dihydro(vitamin K) was negligible and that in crude bovine liver microsomes vitamin K 2,3-epoxide was enzymatically converted both to vitamin K as well as to 2- or 3-hydroxy-2,3-dihydro(vitamin K). Two distinct enzymes have been separated that catalyze the conversion of the epoxide to its metabolites. The enzyme activity that converted vitamin K epoxide to 2- or 3-hydroxy-2,3-dihydro(vitamin K) was very low in solubilized microsomes, but it was enhanced 6-fold when 1 M sodium chloride was added to the buffer (a gradual increase in activity was observed up to 2 M sodium chloride). The sodium chloride may be dissociating the enzyme from an endogenous inhibitor, because no enhanced activity by sodium chloride was

FIG. 1. DEAE-cellulose chromatography. Protein concentration (----) and enzyme activity (○) were monitored.

observed with the purified enzyme. A 16-fold purification, which was achieved on a PBE-94 ion exchanger, also may have resulted from the removal of endogenous inhibitor(s) of the enzyme, because only 70% of the proteins were removed on this column. The PBE-94 ion exchanger and hydroxylapatite columns removed 84% of the solubilized microsomal proteins, which did not require CHAPS to remain in solution. Therefore, to reduce consumption of the expensive detergent, CHAPS, the first two columns were run without it. CHAPS, however, had to be included in the DEAE-cellulose and Sephacryl S-200 chromatographies to keep the remaining 16% of the proteins in solution for further purification. The enzyme activity was slightly retarded on DEAE-cellulose (Fig. 1); the active fractions were concentrated by ultrafiltration and then applied directly onto Sephacryl S-200 for size

exclusion chromatography (Fig. 2). The enzyme activity eluted as a symmetrical peak at 1.7 times the void volume. To reduce the adsorption properties of the high matrix density of Sephacryl, 250 mM sodium chloride was included in the elution buffer and the pH was reduced from 7.8 to 7.4. The enzyme at this point was purified 1152-fold from microsomes, with an overall yield of 69% (Table 1). Calibration of the Sephacryl S-200 with standard proteins indicated that the molecular weight of the enzyme was 25,000. Polyacrylamide slab gel electrophoresis in the presence of NaDodSO₄ gave a single band corresponding to a molecular weight of 12,400 (Fig. 3), therefore suggesting that the enzyme is a dimer with two identical subunits. Coomassie blue staining was not effective for this protein, but a silver stain (24) was quite adequate to visualize the band. The UV-visible spectrum of

FIG. 2. Sephacryl S-200 chromatography. Protein concentration (----) and enzyme activity (○) were monitored.

Table 1. Purification of warfarin-insensitive vitamin K epoxide reductase

	Total vol, ml	Total protein, mg	Total activity, $\mu\text{mol/min}$	Specific activity, $\mu\text{mol/min per mg}$	% yield	-Fold purification
Resuspended microsomes	120	3900	0.795	0.000204		
Solubilized microsomes	120	2400	2.33	0.000971		4.8
PBE-94 eluant	100	720	11.5	0.0159	100	78
Hydroxylapatite eluant	95	394	9.78	0.0248	85	121
DE-52 eluant	100	138.6	9.5	0.0685	82.6	336
S-200 eluant	60	34.0	7.98	0.235	69.4	1152

the purified enzyme showed no absorbances above 282 nm, and, therefore, chromophoric cofactors can be excluded. The apparently homogeneous enzyme catalyzes the reduction of vitamin K 2,3-epoxide to 2- and 3-hydroxy-2,3-dihydro(vitamin K). The identification of the structure of the major reaction products was made based on the work of Fasco *et al.* (18) and Preusch and Suttie (20). Semipreparative HPLC of the reaction products revealed a 1:5 mixture of 2- and 3-hydroxy-2,3-dihydro(vitamin K) when compared with the retention times of these compounds prepared non-enzymatically (18). These two major enzyme-generated products were isolated together and their structure was identified by comparison of their NMR, UV, and mass spectra to those reported (18). Furthermore, after removal of the solvent, the concentrated product mixture was allowed to stand at room temperature for 1 hr, after which time it was shown by HPLC to have completely decomposed to vitamin K.

The mechanism proposed for formation of 3-hydroxy-2,3-dihydro(vitamin K) as an intermediate in the vitamin K epoxide reductase-catalyzed reaction (18) was based on chemical model studies by Silverman (22) and by Preusch and Suttie (21), and it is quite reasonable. However, as shown here, the derivation of the 3-hydroxy-2,3-dihydro(vitamin K)

in microsomes is in question. It was recently reported that 2- and 3-hydroxy-2,3-dihydro(vitamin K) were *in vivo* metabolites of vitamin K epoxide, but their source was an enigma because the products, which were derived from racemic vitamin K epoxide, were optically inactive (20). Previously, these metabolites were observed in a stereospecific reaction catalyzed only by hepatic microsomes of warfarin-resistant rats (18). It cannot yet be concluded, however, that the nonstereospecific product is attributed to the new enzyme isolated here, whereas the stereospecific reaction results from a mutational change in the vitamin K epoxide reductase of warfarin-resistant rats.

The new enzyme described here is distinct from the vitamin K epoxide reductase that converts vitamin K epoxide to vitamin K. Unlike solubilized microsomes, the homogeneous enzyme contains no activity for the conversion of the epoxide to vitamin K. Also, incubation of purified enzyme with 2- and 3-hydroxy-2,3-dihydro(vitamin K) produced no vitamin K above background (detection limit was <1% conversion). Whereas 1 μM and 10 μM warfarin inhibit by >90% the vitamin K epoxide to vitamin K activity in a partially purified enzyme mixture (unpublished results) and in solubilized microsomes, respectively, both the purified enzyme reported here and the vitamin K epoxide to 3-hydroxy-2,3-dihydro(vitamin K) activity in solubilized microsomes are unaffected by 80 μM warfarin. Since both enzymes are vitamin K epoxide reductases that are dithiothreitol dependent and reduce the epoxide to molecules of equivalent oxidation states, one is referred to as the warfarin-insensitive enzyme and the homogeneous enzyme prepared here is referred to as the warfarin-insensitive vitamin K epoxide reductase. These two activities also are strongly dependent on the thiol reducing agent. 2-Mercaptoethanol, reduced glutathione, 1,2-ethanedithiol, and reduced lipoic acid did not impart any activity for the warfarin-insensitive enzyme; only dithiothreitol was effective as an electron donor. The warfarin-sensitive enzyme, however, is active in the presence of 4-butanethiol and several dithiols (27). It is not clear what the physiological electron donor is. Enzyme activity was dependent on glycerol concentration. When the glycerol concentration was increased over a range of 5%–30%, the warfarin-insensitive enzyme activity, in both partially purified as well as in purified form, was inhibited by 88%, whereas the activity of the warfarin-sensitive enzyme was increased by 16%. No band corresponding to the warfarin-insensitive enzyme was observed when NaDodSO₄ gel electrophoresis was carried out on a partially purified preparation of warfarin-sensitive vitamin K epoxide reductase (unpublished results), even though the gel was grossly overloaded (Fig. 4). The amount of protein added to the gel was such that if the protein corresponding to warfarin-insensitive enzyme were present in 0.01% of the total solubilized microsomal proteins, it would have been observed; the warfarin-insensitive enzyme is 0.87% of the total proteins in microsomes (Table 1). Therefore, this experiment is good evidence to support the exclusion of two possibilities regarding the warfarin-insensitive enzyme: first, that the warfarin-insensitive enzyme is simply a dimeric subunit of the warfarin-sensitive enzyme and second, that the first half

FIG. 3. Molecular weight determination by NaDodSO₄/polyacrylamide slab gel electrophoresis. Lane 1 contains 0.16 μg of protein and lane 2 contains 0.32 μg . The molecular weight standards used were bovine plasma albumin (66,000), ovalbumin (45,000), pepsin (34,700), trypsinogen (24,000), β -lactoglobulin (18,400), and lysozyme (14,300).

FIG. 4. NaDodSO₄/polyacrylamide slab gel electrophoresis of a partially purified warfarin-sensitive vitamin K epoxide reductase. Protein (13 μ g) was applied (lane 1) to a 12% acrylamide/0.32% bis(acrylamide) gel and was electrophoresed. In lane 2, 0.12 μ g of purified warfarin-insensitive vitamin K epoxide reductase was electrophoresed concurrently.

of the overall reaction is catalyzed by the warfarin-insensitive dimeric subunit, whereas the dehydration step (18, 21, 22) is catalyzed by another subunit. This would be similar to tryptophan synthetase whose two different pairs of identical subunits can be separated and the two halves of the overall reaction uncoupled (28, 29). The properties of the purified enzyme also indicate that it is neither the microsomal epoxide hydrolase, which is a monomer of molecular weight 54,000 and metabolizes a wide range of structurally diverse epoxides to their dihydroxy derivatives (30, 31), nor a glutathione *S*-epoxide transferase, which uses glutathione as its cofactor and is found primarily in the cytosol (32).

The mechanism proposed for the warfarin-sensitive vitamin K epoxide reductase, which has yet to be purified to homogeneity, may involve an enzyme-bound intermediate resembling that of Structure 1b (18, 21, 22). Therefore, the purified warfarin-insensitive enzyme should be an excellent model enzyme to study the mechanism for the enzyme-catalyzed reduction of vitamin K epoxide.

We thank John S. Oliver for taking the 270 MHz NMR spectra and for photographing the gels. The financial support of this work by the

National Institutes of Health (Grant HL 27108) is gratefully acknowledged. R.B.S. is recipient of an Alfred P. Sloan Research Fellowship (1981–1985) and a National Institutes of Health Research Career Development Award (1982–1987).

1. Suttie, J. W. & Jackson, C. M. (1977) *Physiol. Rev.* **57**, 1–70.
2. Olson, R. E. & Suttie, J. W. (1978) *Vitam. Horm. (N.Y.)* **35**, 59–108.
3. Suttie, J. W. (1980) *Vitamin K Metabolism and Vitamin K Dependent Proteins* (Univ. Park Press, Baltimore).
4. Jones, J. P., Gardner, E. J., Cooper, T. G. & Olson, R. E. (1977) *J. Biol. Chem.* **252**, 7738–7742.
5. Suttie, J. W. (1980) *CRC Crit. Rev. Biochem.* **8**, 191–223.
6. Suttie, J. W., Geweke, L. O., Martin, S. L. & Willingham, A. K. (1980) *FEBS Lett.* **109**, 267–270.
7. Larson, A. E., Friedman, P. A. & Suttie, J. W. (1981) *J. Biol. Chem.* **256**, 11032–11035.
8. Zimmerman, A. & Matschiner, J. T. (1974) *Biochem. Pharmacol.* **23**, 1033–1040.
9. Whitton, D. S., Sadowski, J. A. & Suttie, J. W. (1978) *Biochemistry* **17**, 1371–1377.
10. Matschiner, J. T., Bell, R. G., Amelotti, J. M. & Knauer, T. E. (1970) *Biochim. Biophys. Acta* **201**, 309–315.
11. Sadowski, J. A. & Suttie, J. W. (1974) *Biochemistry* **13**, 3696–3699.
12. Bell, R. G. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 2599–2604.
13. Wallin, R., Gebhart, O. & Prydz, H. (1978) *Biochem. J.* **169**, 95–101.
14. Wallin, R. & Suttie, J. W. (1981) *Biochem. J.* **194**, 983–988.
15. Fasco, M. J. & Principe, L. M. (1980) *Biochem. Biophys. Res. Commun.* **97**, 1487–1492.
16. Fasco, M. J. & Principe, L. M. (1982) *J. Biol. Chem.* **257**, 4894–4901.
17. Fasco, M. J., Hildebrandt, E. F. & Suttie, J. W. (1982) *J. Biol. Chem.* **257**, 11210–11212.
18. Fasco, M. J., Preusch, P. C., Hildebrandt, E. F. & Suttie, J. W. (1983) *J. Biol. Chem.* **258**, 4372–4380.
19. deMetz, M., Soute, B. A. M., Hemker, H. C., Fokkens, R., Lugtenberg, J. & Vermeer, C. (1982) *J. Biol. Chem.* **257**, 5326–5327.
20. Preusch, P. C. & Suttie, J. W. (1984) *J. Nutr.* **114**, 902–910.
21. Preusch, P. C. & Suttie, J. W. (1983) *J. Org. Chem.* **48**, 3301–3305.
22. Silverman, R. B. (1981) *J. Am. Chem. Soc.* **103**, 5939–5941.
23. Tishler, M., Fieser, L. F. & Wendler, N. L. (1940) *J. Am. Chem. Soc.* **62**, 2866–2871.
24. Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
26. Hildebrandt, E. F., Preusch, P. C., Patterson, J. L. & Suttie, J. W. (1984) *Arch. Biochem. Biophys.* **228**, 480–492.
27. Lee, J. J. & Fasco, M. J. (1984) *Biochemistry* **23**, 2246–2252.
28. Kumagai, H. & Miles, E. W. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1271–1278.
29. Yanofsky, C. & Crawford, I. P. (1972) in *The Enzymes*, ed. Boyer, P. (Academic, New York), 3rd Ed., Vol. 7, pp. 1–31.
30. Lu, A. Y. H., Ryan, D., Jerina, D. M., Daly, J. W. & Levin, W. (1975) *J. Biol. Chem.* **250**, 8283–8288.
31. Lu, A. Y. H., Jerina, D. W. & Levin, W. (1977) *J. Biol. Chem.* **252**, 3715–3723.
32. Boyland, E. & Williams, K. (1965) *Biochem. J.* **94**, 190–197.