Stimulation of the vitamin K-dependent carboxylase from bovine liver

Menno DE METZ, Berry A. M. SOUTE, H. Coenraad HEMKER and Cees VERMEER*
*Department of Biochemistry, State University of Limburg, P.O. Box 616, 6200 MD Maastricht,
The Netherlands*

(Received 31 August 1982/Accepted 18 November 1982)

Vitamin K-dependent carboxylase from bovine liver is stimulated not only by reducing agents and bivalent metal ions (especially Mn^{2+}), but also by several organic solvents (dimethyl sulphoxide, ketones and acetonitrile). The organic solvents stimulated both the carboxylation of glutamic acid residues and the formation of vitamin K epoxide. This stimulation by organic solvents was independent of the physical state of the phospholipid; it was highest at low temperatures and could only be demonstrated with vitamin K₁ and not with 3-DTT-MK-O (the thioether adduct of menadione and dithiothreitol) or t-butyl hydroperoxide, which normally can substitute for vitamin K. We suggest that organic solvents exert their effect by changing the mobility of the isoprenoid side chain of vitamin K₁ within the carboxylase complex.

The conversion of glutamic acid residues into γ -carboxyglutamic acid residues is a vitamin K-dependent reaction that has been demonstrated in various species and organs (Suttie, 1980; Gallop *et al.*, 1980). It is generally believed that the carboxylation reaction is coupled to the oxidation of vitamin K quinol to vitamin K 2,3-epoxide (Larson *et al.*, 1981). A hydroperoxide form of vitamin K has been postulated as an intermediate (Larson & Suttie, 1978; De Metz *et al.*, 1982a).

In vitro, vitamin K activity appears to be related to 2-methyl-1,4-naphthoquinone (menadione) with some neutral side chain in the 3-position, e.g. an isoprenoid compound, an *O*-ether or a thioether (Olson *et al.*, 1978; Mack *et al.*, 1979; Suttie, 1980; Johnson, 1980). The length of the side chain required for optimal activity lies between two and six isoprenoid units (Jones *et al.*, 1976; Friedman & Shia, 1976; Johnson, 1980). The side chain does not seem to be essential for proper catalytic function, but it is probably related to the ability of the vitamin to bind to a functional site of carboxylase. It has been suggested that this site had a partly hydrophobic character and that short-chain homologues of vitamin K might be active without entering this hydrophobic area (Jones *et al.*, 1976).

Most studies on the vitamin K-dependent carboxylation are performed with a liver microsomal

fraction of anticoagulated or vitamin K-deficient rats. Endogenous substrates for the vitamin K-dependent carboxylase, e.g. precursors of clotting factors (Willingham *et al.*, 1980), are accumulated in those fractions. Apparently the carboxylase is a thiol enzyme and is stimulated by the presence of reducing agents, e.g. dithiothreitol (Johnson, 1980; Friedman & Shia, 1976). Furthermore, pyridoxal phosphate (Dubin *et al.*, 1979; Suttie *et al.*, 1980) and Mn^{2+} (Larson *et al.*, 1981; Larson & Suttie, 1980) have been reported to stimulate the carboxylation reaction, but the mechanism of stimulation is unknown. Up till now, carboxylase could not be demonstrated to be a metalloenzyme (Larson & Suttie, 1980).

We have isolated carboxylase from the livers of normal and anticoagulated cows (Vermeer *et al.*, 1980). The properties of carboxylase from a cow system are similar to those from a rat system (Vermeer *et al.*, 1982; Uotila & Suttie, 1982). With the livers of anticoagulated cows we achieved a considerable purification by immunoadsorption of the endogenous substrate, which forms a tight complex with the carboxylating enzyme (De Metz *et al.*, 1981a). The purified enzyme is still attached to Sepharose beads and therefore called 'solid-phase carboxylase'. The properties of solid-phase carboxylase are similar to those of a non-purified microsomal preparation of bovine or rat liver (De Metz, 1982; De Metz *et al.*, 1981b, 1982a). In the present paper we describe the stimulation of solid-phase carboxylase by some bivalent metal ions and organic solvents. Implications of the stimulation by organic solvents with respect to the function of the side chain of vitamin K are discussed.

Abbreviations used: Me₂SO, dimethyl sulphoxide; PtdCho, phosphatidylcholine; 3-DTT-MK-O, the thioether adduct of menadione and dithiothreitol; fatty acids are denoted by C_{x:y}, where *x* is the chain length and *y* is the number of double bonds.

* To whom all correspondence and requests for reprints should be addressed.

Materials and methods

Chemicals and buffers

Vitamin K was obtained from Hoffmann-LaRoche (Basel, Switzerland). Menadione (as a water-soluble bisulphite salt), warfarin and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $\text{NaH}^{14}\text{CO}_3$ (40 Ci/mol) and Atomlight were purchased from New England Nuclear Corp. (Dreieich, Germany), the synthetic peptide Phe-Leu-Glu-Glu-Leu from Vega Fox (Tucson, AZ, U.S.A.) and pyridoxal phosphate from Boehringer (Mannheim, Germany). Butanone, pentan-2-one and pentan-3-one were obtained from Aldrich (Beerse, Belgium) and the synthetic phospholipids from Calbiochem (La Jolla, CA, U.S.A.). Ultrogel AcA 22 was obtained from LKB (Bromma, Sweden). All other chemicals were from Merck (Darmstadt, Germany). Vitamin K quinol and menadiol were prepared by adding 0.5 mg of NaBH_4 to 1 ml of a solution containing 10 mg of the quinone. Vitamin K epoxide was prepared as described by Fieser *et al.* (1941) and 3-DTT-MK-O was prepared as described by Mack *et al.* (1979). Buffer A comprised 150 mM-NaCl, 20 mM-Tris/acetic acid, pH 7.5, and 0.5 mM-EDTA.

Preparation of carboxylase

Solubilized microsomes (microsomal fractions) and solid-phase carboxylase were prepared from the livers of warfarin-treated cows as described previously (De Metz *et al.*, 1981a). The enzyme could be resolubilized from the Sepharose beads by allowing the carboxylation reaction proceed in buffer A containing 2 mM-dithiothreitol, 0.2 mM-vitamin K quinol, 4 mM-Phe-Leu-Glu-Glu-Leu and 5 mM- NaHCO_3 for 5 h at 25°C. After incubation, 1 M-NaCl was added and the eluted carboxylase was dialysed against buffer A before use.

Phospholipid-depleted solid-phase carboxylase was prepared with the aid of phospholipase C as described by De Metz *et al.* (1981b). The various phospholipids were added to the depleted carboxylase as mixed micelles with cholate in a 1:1 (w/w) ratio. The final phospholipid concentration was 1 mg/ml. Removal of cholate was accomplished by dialysis against buffer A at 4°C for 48 h.

Measurement of carboxylase activity

The vitamin K-dependent incorporation of $^{14}\text{CO}_2$ was measured by incubating reaction mixtures (0.25 ml) at 25°C in buffer A containing carboxylase (solubilized microsomes, 1 mg of protein; solid-phase carboxylase, 40 µg of protein; resolubilized carboxylase, 10 µg of protein), 20 µCi of $\text{NaH}^{14}\text{CO}_3$, 2 mM-dithiothreitol and 0.2 mM-vitamin K quinol or 3-DTT-MK-O quinol. The t-butyl-hydroperoxide-driven reaction was measured

under similar conditions as described for the vitamin K-dependent reaction, except that t-butyl hydroperoxide (5 mM) was added instead of vitamin K quinol. When the effect of metal ions was studied, buffer A without EDTA was used. The reaction was stopped with 2 ml of ice-cold trichloroacetic acid (10%, w/v) and the samples were degassed at elevated temperatures before counting in Atomlight in a Packard Tri-Carb scintillation counter.

Determination of protein, vitamin K quinol, vitamin K quinone and vitamin K epoxide

Protein concentrations were determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard. The various forms of vitamin K were extracted from 1 ml of carboxylating reaction mixtures and analysed by means of high-pressure liquid chromatography as described previously (Vermeer *et al.*, 1982).

Results

The effect of organic solvents on solid-phase carboxylase

During our efforts to resolubilize the carboxylase from the solid phase with organic solvents we observed that the enzyme activity was irreversibly lost in most cases. However, some more-or-less water-soluble organic solvents stimulated the vitamin K-dependent carboxylation without affecting the K_m of the various reaction components. At 25°C the stimulation was only found with solid-phase carboxylase or resolubilized solid-phase carboxylase and not with the less-purified solubilized microsomal fraction. Ketones and Me_2SO were especially

Table 1. Stimulation of the vitamin K-dependent carboxylation and epoxidation in solid-phase carboxylase

The vitamin K-dependent incorporation of $^{14}\text{CO}_2$ and the formation of vitamin K epoxide were measured in standard reaction mixtures (1 ml), containing 100 µM-warfarin. After incubation for 120 min at 25°C, a 0.4 ml sample was used for determining the amount of incorporated CO_2 and 0.4 ml for assessing the amount of epoxide. The control values (without addition) were 260 000 d.p.m. of incorporated $^{14}\text{CO}_2$ per mg of protein and 96 nmol of vitamin K epoxide per mg of protein.

Addition	$^{14}\text{CO}_2$ incorporation (% of control)	Formation of vitamin K epoxide (% of control)
None	100	100
MnCl_2 (20 mM)	250	120
Me_2SO (20%, v/v)	490	500
Acetone (10%, v/v)	440	350
Acetonitrile (5%, v/v)	160	150
MnCl_2 (20 mM) + Me_2SO (20%, v/v)	1220	530

Fig. 1. Time course of the carboxylation reaction. The incorporation of $^{14}\text{CO}_2$ was measured with solid-phase carboxylase as described in the Materials and methods section after various incubation times without any addition (●), in the presence of acetone (10%, v/v) (○) and in the presence of Me_2SO (20%, v/v) (■).

potent stimulators (Table 1). Alcohols and aldehydes, when miscible with water, were inhibitory. The addition of Me_2SO and acetone to the reaction mixture enhanced not only the vitamin K-dependent carboxylation but also the epoxidation. The stimulation of the carboxylation reaction by organic solvents was cumulative with the stimulation by metal ions. A time course of the reaction with and without either Me_2SO or acetone is shown in Fig. 1. Both in the presence and in the absence of these solvents the reaction proceeds at a constant rate. The influence of the chain length of the ketones on the stimulation is shown in Fig. 2. The amount of organic solvent required for an optimal stimulation decreased with increasing chain length of the ketones. The maximal stimulation was not dependent on the chain length. All ketones and Me_2SO inhibited the carboxylation reaction at high concentrations.

Stimulation by organic solvents of solid-phase carboxylase containing different phospholipids

Since we know that phospholipids (especially PtdCho) are necessary for enzyme activity (De Metz *et al.*, 1981b), the effects of organic solvents might be explained by the interaction of the solvents with the phospholipid moiety. We tried to test this

Fig. 2. Stimulation of the vitamin K-dependent $^{14}\text{CO}_2$ incorporation by several ketones and Me_2SO .

The incorporation of $^{14}\text{CO}_2$ was measured with solid-phase carboxylase as described in the Materials and methods section after 120 min incubation at 25°C in the presence of pentan-3-one (●), pentan-2-one (○), butanone (■), acetone (□) and Me_2SO (▲). The incorporation is expressed as a percentage of the value without solvents (260 000 d.p.m./mg of protein).

hypothesis as follows: solid-phase carboxylase was depleted of its phospholipids and reconstituted with PtdCho containing fatty acid residues of different chain length and saturation (see the Materials and methods section). We used di- $\text{C}_{12:0}$, di- $\text{C}_{14:0}$, di- $\text{C}_{16:0}$, di- $\text{C}_{18:1}$, di- $\text{C}_{18:1}$ -*cis* and di- $\text{C}_{18:1}$ -*trans* PtdCho with melting points at 0, 23, 42, 58, -20 and 9.5°C respectively. Solid-phase carboxylases, reconstituted with the different phosphatidylcholines, were all stimulated about 4-fold by Me_2SO or acetone at 25°C . At this temperature, bilayer conformations of di- $\text{C}_{12:0}$, di- $\text{C}_{14:0}$ and di- $\text{C}_{18:1}$ PtdCho are in the liquid-crystalline phase, whereas di- $\text{C}_{16:0}$ and di- $\text{C}_{18:0}$ PtdCho are in the crystalline phase. So the physical state of the phospholipid moiety does not seem to be relevant for the stimulation by organic solvents of the phospholipid-enzyme complex.

Stimulation of solid-phase carboxylase by organic solvents at different temperatures

The effect of the temperature on enzyme activity was measured with solid-phase carboxylase reconstituted with di- $\text{C}_{14:0}$ PtdCho (Fig. 3). The data are presented as an Arrhenius plot and the activation energy can be calculated from the slope of this curve. A change in the activation energy at 23°C (the melting point of di- $\text{C}_{14:0}$ PtdCho) could not be demonstrated, indicating that the physical state of the phospholipid does not influence the enzyme activity. With Me_2SO (not shown) and acetone in the reaction mixture the activation energy decreased. It is obvious that, at temperatures above

Fig. 3. Effect of temperature upon the carboxylation reaction measured with solid-phase carboxylase, reconstituted with di- $C_{14:0}$ PtdCho

Solid-phase carboxylase was depleted of phospholipids and reconstituted with di- $C_{14:0}$ PtdCho as described in the Materials and methods section. The carboxylase activity was measured at various temperatures under standard conditions after 120 min of incubation. The incorporation of $^{14}CO_2$ is shown as an Arrhenius plot against the reciprocal of the absolute temperature, without addition (●) and in the presence of 10% (v/v) acetone (○).

Fig. 4. Effect of temperature upon the vitamin K-dependent carboxylation reaction, measured with solid-phase carboxylase

The carboxylase activity was measured at various temperatures under standard conditions after 120 min incubation. The incorporation of $^{14}CO_2$ is shown as an Arrhenius plot against the reciprocal of the absolute temperature, without additions (●), in the presence of acetone (10%, v/v) (○), with 0.2 mM-3-DTT-MK-O quinol instead of vitamin K quinol (■), and with 0.2 mM-3-DTT-MK-O quinol instead of vitamin K quinol in the presence of acetone (10% v/v) (□).

30°C, the organic solvents have hardly any effect on carboxylase, whereas the stimulation of the carboxylation reaction increasingly pronounced at lower temperatures. The same effect could also be demonstrated with the native solid-phase carboxylase (Fig. 4). We therefore investigated whether the non-purified carboxylase could also be stimulated at low temperatures. When measured with a solubilized microsomal preparation under standard conditions at 25°C, the incorporation of $^{14}CO_2$ was 3300 d.p.m./h per mg and was not stimulated by organic solvents, but we observed that at 5°C the incorporation of $^{14}CO_2$ increased from 425 d.p.m./h per mg to 1980 d.p.m./h per mg by the addition of acetone (15%, v/v).

Stimulation of solid-phase carboxylase by Mn^{2+} and organic solvents using various forms of vitamin K

Although the oxidation of vitamin K quinol is probably driving the carboxylation reaction, vitamin

Table 2. Effect of various coenzymes on the carboxylation reaction in solid-phase carboxylase

The incorporation of $^{14}CO_2$ was measured in standard reaction mixtures (0.25 ml) at 25°C and expressed as a percentage of the rate of incorporation in the presence of vitamin K quinol (2200 d.p.m./min per mg of protein). Acetone (if added) was present at a concentration of 10% (v/v). The concentration of $MnCl_2$ (if added) was 20 mM, except for the 3-DTT-MK-O-driven reaction, in which case we added 2 mM.

Coenzyme	$^{14}CO_2$ incorporation (% of control)		
	Without addition	+ Acetone	+ $MnCl_2$
Vitamin K quinol	100	440	250
Vitamin K quinone	15	82	35
Vitamin K epoxide	11	57	19
3-DTT-MK-O quinol	20	28	50
t-Butylhydroperoxide	18	16	3

K and vitamin K epoxide can be used as a vitamin source when vitamin K (epoxide) reductase is present. The carboxylase activity is lower with these more-oxidized forms of vitamin K, but the stimulation by organic solvents and Mn^{2+} could be demonstrated with all forms of the vitamin (Table 2). Homologues of vitamin K with a short hydrophobic side chain at the 3-position can also be used as a vitamin source. We used the adduct of menadione and dithiothreitol (3-DTT-MK-O), which can be easily prepared and has a good activity (Olson *et al.*, 1978; Mack *et al.*, 1979). With 3-DTT-MK-O the stimulation by Mn^{2+} could still be demonstrated. The stimulation by Me_2SO and acetone with 3-DTT-MK-O was very low, however, when compared with the stimulation by these solvents in the presence of vitamin K₁ (Table 2). The stimulation by both Mn^{2+} and organic solvents was also low when the *t*-butyl hydroperoxide-driven incorporation of $^{14}CO_2$ was measured.

It has been proposed (Jones *et al.*, 1976, 1977) that short-chain homologues of vitamin K are relatively better coenzymes at low temperatures, i.e. the activation energy of the carboxylation reaction decreases with a decreasing isoprenyl chain length. Indeed, this decrease could be demonstrated when the effect of the temperature on the activity of 3-DTT-MK-O was studied (Fig. 4). The activation energy with 3-DTT-MK-O was considerably lower than that with vitamin K₁, but was similar to the activation energy with *t*-butyl hydroperoxide (results not shown).

Discussion

It is known from the rat and bovine microsomal system that reducing agents, pyridoxal phosphate and bivalent metal ions (especially Mn^{2+}) stimulated vitamin K-dependent carboxylation. The way in which pyridoxal phosphate influences the carboxylation reaction is not understood. Moreover, when measured with the aid of solid-phase carboxylase, the stimulating effect of pyridoxal phosphate was very low. The stimulating effect of reducing agents, which was measured in microsomal preparations as well as in solid-phase carboxylase, probably reflects the necessity for reduced thiol groups in carboxylase and vitamin K reductase (Johnson, 1980). Alternatively the reducing agents might simply function either to prevent the non-enzymic oxidation of vitamin K or to recycle the vitamin K epoxide that is formed during the reaction. The stimulating effect of Mn^{2+} could also be demonstrated both in the crude microsomal extract and in solid-phase carboxylase. Because only the carboxylation and not the epoxidation was stimulated, we concluded that, in the presence of Mn^{2+} , a better coupling exists between the epoxid-

ation of vitamin K quinol and the carboxylation reaction. The ratio of vitamin K epoxide formed/ CO_2 fixed decreases from 10 (De Metz *et al.*, 1982b) to 4 when Mn^{2+} is added. When vitamin K or vitamin K epoxide are used as vitamin source, vitamin K quinol will probably be formed before carboxylation can occur. Hence the same principle will probably underly the stimulation by Mn^{2+} with all three forms of vitamin K. The *t*-butyl hydroperoxide-driven carboxylation reaction was inhibited by Mn^{2+} , most probably because Mn^{2+} acts as a catalyst for the rapid breakdown of the peroxide.

Several organic solvents stimulated the vitamin K-dependent carboxylation and epoxidation reaction. The stimulating solvents were all miscible or partly miscible with water and were poor solvents for phospholipids. Solvents like alcohols, which do solubilize phospholipids, may inhibit the carboxylation reaction, because the phospholipids are essential for enzyme activity (De Metz *et al.*, 1981b). The observed inhibition by aldehydes could be explained by their high reactivity compared with ketones (e.g. the formation of a Schiff base with free amino groups). Although the stimulating agents were miscible with water, the hydrophobicity seems to be relevant for their action. This could be demonstrated by the effect of ketones with different chain length on the carboxylating activity.

With the purified enzyme the stimulation by organic solvents was highest at low temperatures and, with a non-purified microsomal preparation, stimulation by organic solvents was observed only at low temperatures (when the stimulation with solid-phase carboxylase is also highest). It is possible that in a non-purified preparation the proteins and lipids surrounding the carboxylase complex interact with the organic solvent and hence suppress the stimulating effect on the carboxylase activity. On the other hand, the difference between solid-phase carboxylase and a non-purified preparation might reflect a difference in the state of the enzyme complex itself, i.e. the activity of solid-phase carboxylase might be suboptimal.

Jones *et al.* (1976) reported that homologues of vitamin K with short chains at the 3-position have low activation energies. This was confirmed with 3-DTT-MK-O. In the same publication, Jones *et al.* (1976) observed a change in the activation energy of the carboxylating enzyme at 30°C, the 'melting point' of the microsomal membrane. With solid-phase carboxylase reconstituted with $di-C_{14:0}$ PtdCho the activation energy was constant between 10 and 35°C, although the phospholipid, when present in the bilayer conformation, changes from the crystalline to the liquid-crystalline state at 23°C. It is not known if the physical state of the phospholipid adjacent to the protein of the carboxylase also changes over this temperature

interval, since those phospholipids might be immobilized by interaction with the carboxylase protein. Anyway, the physical state of the phospholipid, as it can be measured in the bilayer conformation, seems not to influence the enzyme activity. A possible explanation for the change in activation energy reported by Jones *et al.* (1976) is the lability of the carboxylating activity in microsomal preparations from rat liver at high temperatures (Suttie *et al.*, 1979).

When coenzymes with a low activation energy are used (3-DTT-MK-O or t-butyl hydroperoxide), stimulation by Me₂SO and ketones is not observed. These observations indicate that the stimulation by organic solvents is related to the side chain of vitamin K at the 3-position. The site on carboxylase that binds this side chain of vitamin K will probably have a hydrophobic character, and organic solvents might interact with this part of the enzyme without changing the K_m of vitamin K. As we could not find evidence for the involvement of phospholipids in the stimulation by organic solvents, we suggest the presence of a hydrophobic region on the protein part of carboxylase at the binding site of the side chain of vitamin K. Although most experimental evidence is lacking until now, it is tempting to ascribe a carrier function to the side chain of vitamin K analogous to the function of the side chain of biotin and that of lipoic acid. The side chain of vitamin K could serve as a swinging arm carrying the naphthoquinone head group from one site to the next. Speculating, one might assume that organic solvents might facilitate the carrier function by increasing the mobility of the vitamin. Short-chain homologues of vitamin K may bypass the carrier function.

This research was supported in part by a grant (no. MD 82145) from the 'Thrombosesichting Nederland'.

References

- De Metz, M. (1982) Ph.D. Thesis, Maastricht
- De Metz, M., Vermeer, C., Soute, B. A. M., Van Scharrenburg, G. J. M., Slotboom, A. J. & Hemker, H. C. (1981a) *FEBS Lett.* **123**, 215–218
- De Metz, M., Vermeer, C., Soute, B. A. M. & Hemker, H. C. (1981b) *J. Biol. Chem.* **256**, 10843–10846
- De Metz, M., Soute, B. A. M., Hemker, H. C., Fokkens, R., Lugtenburg, J. & Vermeer, C. (1982a) *J. Biol. Chem.* **257**, 5326–5329
- De Metz, M., Soute, B. A. M., Hemker, H. C. & Vermeer, C. (1982b) *FEBS Lett.* **137**, 253–256
- Dubin, A., Suen, E. T., Delaney, R., Chiu, A. & Johnson, B. C. (1979) *Biochem. Biophys. Res. Commun.* **88**, 1024–1029
- Fieser, L. F., Tishler, M. & Sampson, W. L. (1941) *J. Biol. Chem.* **137**, 659–692
- Friedman, P. A. & Shia, M. (1976) *Biochem. Biophys. Res. Commun.* **70**, 647–654
- Gallop, P. H., Lian, J. B. & Hauschka, P. V. (1980) *N. Engl. J. Med.* **302**, 1460–1466
- Johnson, B. C. (1980) *Methods Enzymol.* **67**, 165–180
- Jones, J. P., Fausto, A., Houser, R. M., Gardner, E. J. & Olson, R. E. (1976) *Biochem. Biophys. Res. Commun.* **72**, 589–597
- Jones, J. P., Gardner, E. J., Cooper, T. G. & Olson, R. E. (1977) *J. Biol. Chem.* **252**, 7738–7742
- Larson, A. E. & Suttie, J. W. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5413–5416
- Larson, A. E. & Suttie, J. W. (1980) *FEBS Lett.* **118**, 95–98
- Larson, A. E., Friedman, P. A. & Suttie, J. W. (1981) *J. Biol. Chem.* **256**, 11032–11035
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **137**, 265–275
- Mack, D. O., Wolfensberger, M., Girardot, J. M., Miller, J. A. & Johnson, B. C. (1979) *J. Biol. Chem.* **254**, 2656–2664
- Olson, R. E., Houser, R. H., Searcey, M. T., Gardner, E. J., Scheinbuks, J., Subba Rao, G. N., Jones, J. P. & Hall, A. L. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 2610–2641
- Suttie, J. W. (1980) *C.R.C. Crit. Rev. Biochem.* **8**, 191–223
- Suttie, J. W., Lehrmann, S. R., Geweke, L. O., Hageman, J. M. & Rich, D. H. (1979) *Biochem. Biophys. Res. Commun.* **86**, 500–507
- Suttie, J. W., Geweke, L. O., Finnan, J. L., Lehrmann, S. R. & Rich, D. H. (1980) in *Vitamin K Metabolism and Vitamin K Dependent Proteins* (Suttie, J. W., ed.), pp. 450–454, University Park Press, Baltimore
- Uotila, L. & Suttie, J. W. (1982) *Biochem. J.* **201**, 249–258
- Vermeer, C., De Metz, M., Soute, B. A. M. & Hemker, H. C. (1980) *Protides Biol. Fluids Proc. Colloq.* **28**, 249–252
- Vermeer, C., Soute, B. A. M., De Metz, M. & Hemker, H. C. (1982) *Biochim. Biophys. Acta* **714**, 361–365
- Willingham, A. K., Martin, S. L., Graves, C. B., Grabau, G. G. & Munns, T. W. (1980) in *Vitamin K Metabolism and Vitamin K Dependent Proteins* (Suttie, J. W., ed.), pp. 553–559, University Park Press, Baltimore