Molecular and Cellular Biochemistry 61, 17–35 (1984).

Review

The vitamin K-dependent carboxylation reaction

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Summary

Gammacarboxyglutamic acid (Gla) is an abnormal amino acid, which occurs in a number of proteins. It was discovered about 10 years ago in the four vitamin K-dependent blood clotting factors and it could be demonstrated that Gla is formed in a post-translational modification step, which requires a carboxylating enzyme system (carboxylase) and vitamin K. Since at the time of this discovery the earlier mentioned clotting factors were the only proteins known to be synthesized in a vitamin K-dependent way, it has been assumed for many years that the blood clotting system was unique in this respect. Recently it has been demonstrated, however, that vitamin K-dependent carboxylase is not restricted to the liver (the place of synthesis of the clotting factors) but that it is also present in other tissues such as lung, kidney, spleen and testis. Moreover, numerous Gla-containing proteins have been detected, although in most cases their function is not wholly understood. It seems that (like for instance the glycosylation) the vitamin K-dependent carboxylation is a normal post-translational modification, which is required for the correct function of a certain class of Ca^{2+}-binding proteins.

I. Introduction

In the 1930s, when studying the effects of a cholesterol-free diet on chickens, Dam noticed that after some weeks the animals developed a hemorrhagic diathesis. This hemostatic defect could not be counteracted by adding purified cholesterol to the food, but turned out to be related to the deficiency of a fat-soluble vitamin, which was called: vitamin K (1).

Since 1921 a hemorrhagic disease in cattle was known in the U.S.A. and Canada. This bleeding symptom was found to be caused by feeding the animals improperly cured sweet clover hay. The coumarin derivatives in the hay, discovered by Campbell and Link (2, 3), are antagonists of vitamin K. They are not active in this respect in the fresh plants, because there they are bound to glycosides. In the following years a number of substituted 4-hydroxycoumarins have been synthesized and nowadays some of them are frequently used as anticoagulants in rodenticides (rat poison) and in the clinic for the control of thrombogenic episodes (4). Recently it has been demonstrated, that the vitamin K action is not restricted to the coagulation system, but that the vitamin plays a role in the synthesis of numerous proteins in man as well as in other organisms (5, 6).

II. Vitamin K and the formation of gammacarboxyglutamic acid residues

II.1. Discovery of gammacarboxyglutamic acid

After the discovery that vitamin K was essential for the synthesis of a number of proteins, involved in blood coagulation, it lasted more than 30 years before people started to realize at what stage of the protein synthesis vitamin K is involved. A real

breakthrough came in 1963, when Hemker et al. postulated that blood from patients receiving oral anticoagulant therapy contains a mixture of normal and abnormal prothrombin (7). The abnormal prothrombin was called 'Protein Induced by Vitamin K Antagonists' or PIVKA, and it was regarded as a precursor of prothrombin. The hypothesis was based on kinetic experiments which demonstrated in the patient plasma the presence of a competitive inhibitor of the activation of clotting factor X. The abnormal prothrombin was subsequently identified immunochemically in the plasma of dicoumarol-treated patients (8, 9) and cows (10). It was shown to be similar to normal prothrombin with respect to its molecular size, its number of sialic acid residues, its amino acid composition after acid hydrolysis and its antigenic activity (11). However, in the presence of Ca^{2+}, the electrophoretic migration rate of abnormal prothrombin was different from that of normal prothrombin. It turned out, that whereas prothrombin strongly binds Ca^{2+}, the abnormal prothrombin lacks this ability (12, 13, 14).

In the year of 1974 it was reported independently by two groups (15, 16) that the calcium-binding sites in normal prothrombin were gammacarboxyglutamic acid (Gla) residues, and that these residues were normal glutamic acid (Glu) residues in abnormal prothrombin (Fig. 1). Similar observations were made for other vitamin K-dependent proteins and from that time on, the abnormal dicoumarol-induced proteins were designated as descarboxyproteins. Until now the only known physiological significance of Gla residues is related with their binding of Ca^{2+}. This is illustrated by the enhanced activity of the vitamin K-dependent clotting factors in the presence of Ca^{2+} and phospholipids (17). Furthermore vitamin K-dependent proteins are involved in the transport of calcium from the egg shell to developing chicken embryos (18) and in the deposition of calcium in the bone matrix of the human fetus (19).

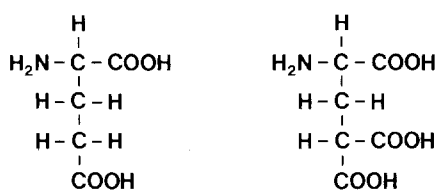

Fig. 1. The structures of glutamic acid (left) and gammacarboxyglutamic acid (right).

II.2. The vitamin K-dependent post-translational modification

All vitamin K-dependent proteins discovered up till now belong to what are called the secretory proteins. These are proteins that are excreted into the extracellular fluid after their maturation has been completed. The mechanism by which these proteins are synthesized has been elucidated by the work of Blobel and Dobberstein (20, 21), Meyer et al. (22) and MacGillivray et al. (23), and it is explained in Fig. 2. Protein synthesis starts with the binding of the m-RNA to a ribosome. When the m-RNA is coded for a secretory protein, the first amino acids which are linked together are not similar to the N-terminal region of the protein to be formed. Instead they form the 'signal sequence', which is a strongly hydrophobic peptide chain, composed of 15–30 amino acids. This signal sequence is recognized by the cytoplasmic 'signal recognition particle', which binds to the ribosomal complex. As a result of this binding any further synthesis of the secretory protein is blocked. A second protein, designated as 'docking protein' is located on the outer side of the endoplasmic reticulum, and it is able to bind to the inactivated ribosomal complex. The result of this binding is, that the ribosomal protein synthesis is re-started. The hydrophobic signal sequence on the N-terminal side of the growing peptide chain will easily penetrate into the phospholipid membrane of the endoplasmic reticulum, thus facilitating the transport of the growing peptide chain to the inner side of the endoplasmic tubular system. Here are located the enzymes involved in post-translational modifications such as the glycosylating enzymes and also the vitamin K-dependent carboxylase. In one of the post-translational steps the signal peptide is cleaved from the maturating protein by a highly specific 'signal peptidase'. After all required modifications have been performed, the protein is transported through the tubular system to be excreted into the extracellular fluid.

Up till now these signal sequences were found in all secretory proteins examined, except albumin which seems to contain an 'internal' signal sequence. Only a small fraction of these proteins con-

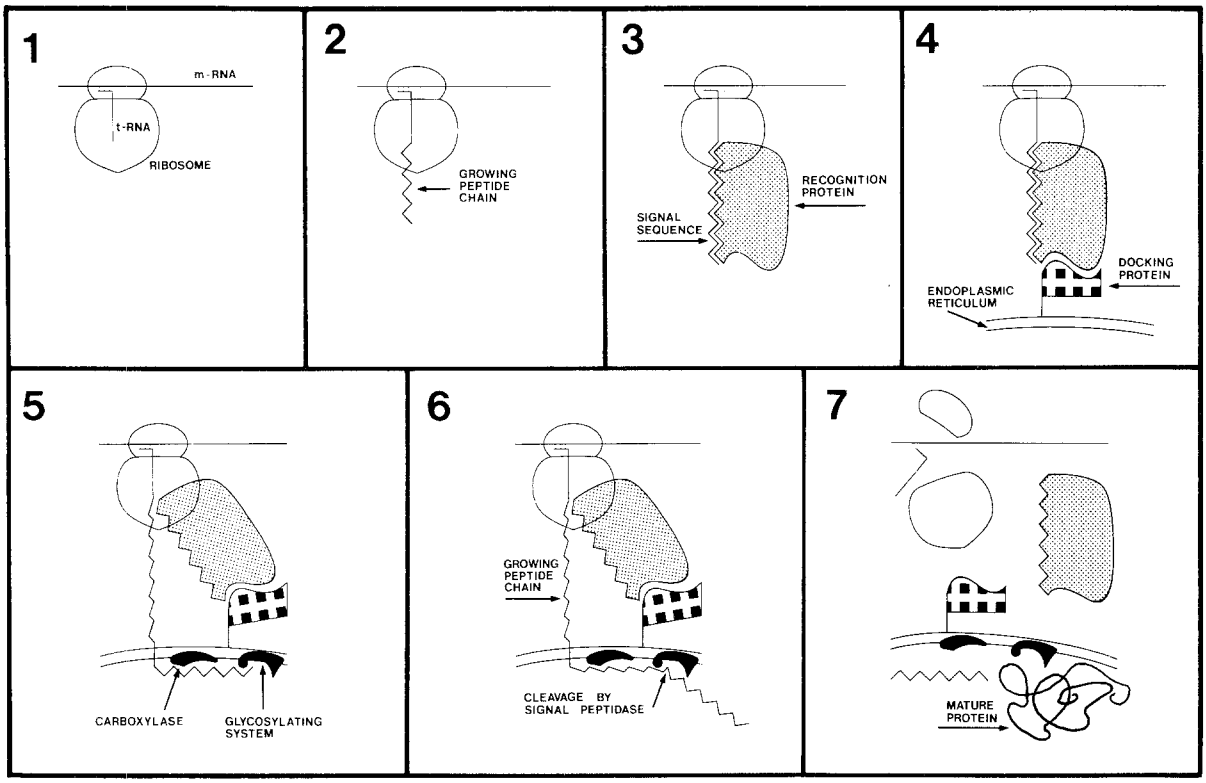

Fig. 2. The synthesis of a secretory protein; 1: formation of the initiation complex; 2: synthesis of the signal sequence; 3: recognition of the completed signal sequence by signal recognition particle, stop of proteins synthesis; 4: binding to the endoplasmic reticulum via the docking protein, restart of protein synthesis; 5: postribosomal modifications on the growing peptide chain; 6: removal of the signal peptide by signal peptidase; 7: termination of protein synthesis, excretion of the mature protein via the endoplasmic tubular system.

tain Gla residues, however. Moreover in vitamin K-dependent proteins the Glu residues are carboxylated at a number of distinct positions only, whereas other Glu residues remain uncarboxylated. The mechanism that is responsible for this selection is still obscure at this moment.

II.3. In vitro *vitamin K-dependent carboxylating enzyme systems*

When comparing the structures of Glu and Gla (Fig. 1) it seems obvious, that vitamin K is involved in a carboxylation reaction, and it was shown by Esmon et al. that the hepatic post-mitochondrial supernatants from vitamin K-deficient rats were able to incorporate $^{14}CO_2$ into an endogenous substrate (24). $NaH^{14}CO_3$ could be used as a CO_2 donor and the incorporation was dependent on the addition of vitamin K. This system directly measures the vitamin K-dependent step and it is highly preferable to earlier systems, in which the generation of coagulation activity was measured (25, 26).

The vitamin K-dependent enzyme system which had been detected in the liver homogenates, appeared to be located in the microsomal fraction and it was mainly concentrated at the luminal side of the rough microsomal membrane (27, 28). Carboxylase could be extracted from these membranes with the aid of several non-ionic surfactants and bile salts (29–31). Since then the vitamin K-dependent carboxylation has been studied most extensively in liver microsomal preparations obtained from vitamin K-deficient or warfarin-treated rats and from warfarin-treated cows (32, 33). Its main characteristics are summarized in Table 1.

When we started with the bovine system, it was known that during warfarin treatment of the cows, non-carboxylated clotting factors (descarboxyfactors) appear in their blood plasma in concentrations similar to those of the normal clotting factors

Table 1. Properties of vitamin K-dependent carboxylase.

Absolute requirements:
- vitamin K quinone + reducing agent (NAD(P)H or DTT) or vitamin K hydroquinone
- O_2
- CO_2
- carboxylatable substrate (endogenous and/or exogenous)

Stimulators
- DTT
- pyridoxalphosphate or Mn^{2+}
- ketones or DMSO

Inhibitors
- chloro-K
- 2,3,5,6-tetrachloro-4-pyridinol
- coumarin anticoagulants
- sulphydryl reagents
- CN

Abbreviations: DTT, dithiothreitol; DMSO, dimethylsulphoxide; chloro-K, 2-chloro-3-phytyl-1,4-naphto-quinone.

in untreated animals (10, 34). This is in contrast to the situation in the rat, where warfarin causes a decrease of the vitamin K-dependent proteins in plasma, but where no descarboxyfactors can be detected (35). Therefore it was unexpected, that the treatment with warfarin caused an accumulation of endogenous substrates for carboxylase in bovine as well as in rat liver (36). We have compared the warfarin-induced accumulation of endogenous hepatic substrates in a number of species (Table 2) and no substantial differences were found. When the plasma concentration of the normal vitamin K-dependent clotting factors is arbitrarily defined as 1 U/ml, the plasma concentration of descarboxyprothrombin was <0.1 U/ml in the rat, 0.8 U/ml in the cow and had intermediate values in the other species. In all cases the amount of normal prothrombin was <0.2 U/ml. So it seems that there is no relation between the accumulation of exogenous substrate in the cell and the amount of excreted descarboxyproteins in plasma.

In an attempt to explain this phenomenon we have put forward the following hypothesis. In a normal liver all prothrombin precursors are carboxylated as soon as they become available. When carboxylase is inhibited, however, the uncarboxylated precursors accumulate (for instance because they are complexed to carboxylase without being carboxylated). This accumulation cannot continue indefinitely and at a certain intracellular concentration (for instance when all enzyme molecules are saturated with the substrate), the precursors are excreted in their non-carboxylated form. The amount of excreted descarboxyprothrombin will than depend on the half-life time of the prothrombin precursors in the liver. In this view it is to be expected, that in the rat – where no descarboxyprothrombin is found in plasma – the intracellular turnover of prothrombin is fast enough to prevent the accumulation of substrate to a level at which a substantial excretion into the extracellular fluid occurs. In the cow, on the other hand, the intracellular breakdown of prothrombin precursors is expected to be slow and after an initial accumulation of these proteins, they are excreted as descarboxyproteins with a similar rate as are the normal proteins in the non-inhibited systems.

Table 2. Effect of warfarin in various species.

Species	Hepatic carboxylase (dpm.mg $^{-1}$.min $^{-1}$)	Endogenous substrate (dpm.mg $^{-1}$)	Ratio b/a	Plasma descarboxyprothrombin (U.ml $^{-1}$)
Cow	3125	7318	2.3	0.8
Goat	1312	3469	2.6	0.5
Horse	986	4813	4.9	0.5
Dog	3309	12650	3.8	0.2
Pig	1222	2954	2.4	0.4
Guinea pig	1050	3827	3.6	0.4
Rat	2862	9416	3.3	<0.1

The various animals (4–10 from each species) were treated with warfarin: rats 18 hr, dogs 4 days and the others 6 days. The animals were subsequently sacrificed and solubilized microsomes were prepared from their respective livers. The amounts of carboxylase and endogenous substrate are measured and quantified as described earlier (36). Descarboxyprothrombin was measured in $BaSO_4$-adsorbed oxalate plasma using Echis carinatus venom (37). The data in this table are the means of at least 4 different animals from each species.

II.4. The relation between carboxylation and epoxidation

In the liver vitamin K is partly present in the form of its 2,3 epoxide (34). The enzyme responsible for the conversion of vitamin K into the epoxide is called epoxidase; it is located in the liver microsomal fraction as is carboxylase (39). The hydroquinone form of vitamin K is the substrate that is converted by epoxidase and the epoxide oxygen is generated by molecular oxygen. The epoxide can be reduced again to vitamin K by vitamin K epoxide reductase (40–42). The reduction of vitamin K quinone, which precedes the epoxidation reaction is carried out by NAD(P)H dehydrogenase (DT-diaphorase, 43, 44) and/or vitamin K reductase. It is not yet sure whether the reduction of vitamin K epoxide and that of vitamin K quinone is carried out by the same enzyme or by two different reductases (42, 45). In this way vitamin K quinone is reduced to the hydroquinone, which is converted to the epoxide, whereas the epoxide, on its turn, can be reduced to vitamin K hydroquinone (possibly via the quinone). This course of events is called the vitamin K cycle (Fig. 3). The reduction of the epoxide is inhibited by coumarins and this seems to be

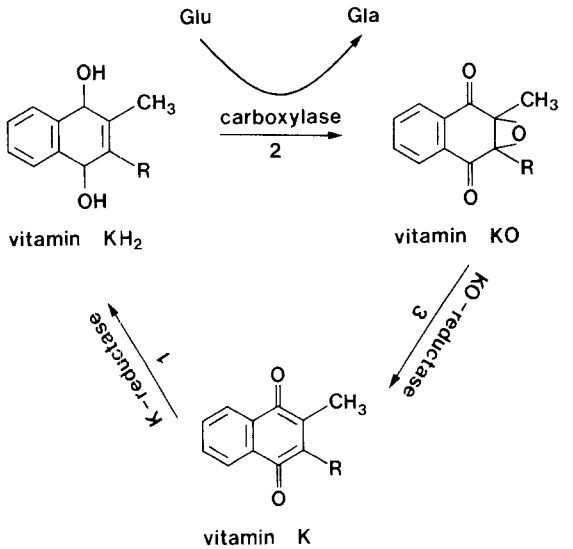

Fig. 3. The vitamin K cycle. Coumarin derivatives inhibit steps 1 and 3. Chloro-K and 2,3,5,6,-tetrachloro-4-pyridinol inhibit step 2. The following abbreviations are used: Glu, glutamic acid; Gla, gammacarboxyglutamic acid, KH_2, vitamin K hydroquinone; KO, vitamin K epoxide.

the biologically important action of these anticoagulants (17).

The biological function of the vitamin K cycle might be the constant generation of vitamin K hydroquinone, which is used in the carboxylation reaction. In this concept the carboxylation reaction is in some way coupled to the formation of vitamin K epoxide and several arguments have been put forward to support this hypothesis. Many requirements for the optimal function of epoxidase and carboxylase are similar (40), the subcellular location of both enzymes is the same (46) and it has been postulated that both reactions proceed via vitamin K hydroperoxide (47, 48). Furthermore it was demonstrated that the formation of vitamin K epoxide is elevated in rat livers, containing increased levels of endogenous substrates for carboxylase (39). Although the carboxylation and the epoxidation reactions seem to be coupled somehow, it was calculated that on a molar base in most cases more epoxide is formed than is CO_2 incorporated (49). By raising the CO_2 concentration equal amounts of vitamin K epoxide and gammacarboxyglutamic acid can be formed (50). Thus epoxidation can take place without carboxylation. On the other hand, carboxylation without epoxidation has never been demonstrated and the concept, that both events are coupled in some way has generally been accepted at this time.

III. Selection of carboxylatable substrates

In the early studies of carboxylase, the enzyme system was invariably obtained from the livers of vitamin K-deficient or warfarin-treated animals. The hepatic microsomal fraction contained both, carboxylase and an endogenous substrate, which had accumulated during the *in vivo* warfarin treatment (24, 36). This endogenous substrate is readily carboxylated upon adding vitamin K to the *in vitro* system.

The hepatic endogenous substrates in the rat and in the cow have been partly characterized. In rat liver several prothrombin precursors, each with a different PI, could be detected (51, 52) but it is not sure whether all these precursors are carboxylated *in vitro*. Using antibodies against rat prothrombin Swanson and Suttie were able to demonstrate that 20–25% of the total carboxylated microsomal pro-

tein precursor pool consists of prothrombin precursors (52). The hepatic endogenous substrates in the cow have been characterized using highly purified immobilized antibodies against one of the clotting factors II (prothrombin), IX and X (33). It resulted, that 21% of the carboxylatable material consisted of prothrombin precursors, which is in good agreement with the rat system. Most of the carboxylatable proteins, however, were factor X precursors (69%) and only 8% were precursors of factor IX (Table 3). The factor X precursor was analyzed on polyacrylamide gels in SDS and demonstrated to be a one-chain molecule. Since the plasma factor X consists of two polypeptide chains, which are linked by disulfide bridges, this result indicates that during the maturation of factor X a proteolytic cleavage of the precursor occurs after the completion of the carboxylation reaction.

Although the use of the endogenous substrates for carboxylase has several advantages (e.g. the correct substrate is present at a site where it can be carboxylated), the drawbacks of this system became more and more clear. It is inherently impossible, for instance, to purify carboxylase to homogeneity. As soon as the substrate is removed from the enzyme, it is obvious that no carboxylase activity can be measured anymore. Secondly, kinetic studies may require that the substrate is present in known amounts or in excess. Therefore a search was started for proteins or peptides that may serve as exogenous substrates for carboxylase. Two lines of research have been described to solve this problem. The first exogenous substrate for carboxylase was reported by Suttie et al. (53), who synthesized a pentapeptide which was similar to the amino acids 5–9 in bovine descarboxyprothrombin (Phe-Leu-Glu-Glu-Val). Later studies showed, that the pentapeptide Phe-Leu-Glu-Glu-Leu was carboxylated even better (54). Although the K_m of this peptide was rather high (4 mM in the rat system and 10 mM in the cow), it could be added in high concentrations because it was readily available and had a low molecular weight. In order to investigate the structural requirements for the carboxylatable substrates, numerous Glu-containing peptides were subsequently synthesized (55), but their relative activity was invariably lower than that of Phe-Leu-Glu-Glu-Leu. The high K_m of all these substrates indicates that, although they are extremely useful in the laboratory practice, they are bad substrates for carboxylase. Since the concentration of the natural substrate in the microsomes is not in the millimolar range, we have to conclude, that up till now these studies have not yielded an exogenous substrate of a quality which is comparable with that of the endogenous substrate. Moreover, it is clear that we still do not know what the structural requirements for a good substrate are.

A second approach was followed by Soute et al. (56), who tried to use highly purified bovine descarboxyprothrombin in their carboxylating system. It resulted, that the intact molecule had a high K_m (0.4 mM), as had descarboxyfragment-1 (this is the N-terminal degradation product which is formed after proteolytic digestion of descarboxyprothrombin with thrombin and which has a M_r of 21 000). Because of this high K_m these substrates had no practical value, but further digestion of descarboxyfragment-1 with subtilisin resulted in a peptide corresponding with the amino acids 13–29 in descarboxyprothrombin. This peptide, which was designated as fragment-Su, had a low K_m (0.001 mM) and hence it was a good substrate for carboxylase (Table 4). The high K_m of the two protein substrates may be explained by assuming that *in vivo* the prothrombin precursors are carboxylated during

Table 3. Immunospecific adsorption of carboxylated reaction products.

Sepharose-linked adsorbant	$^{14}CO_2$ incorporated into adsorbed proteins	
	dpm	% of total
a. Antiprothrombin	1571	21
b. Anti-factor X	5163	69
c. Anti-factor IX	599	8
d. a + b + c	6584	88
e. Albumin	22	0.3

Vitamin K-dependent incorporation of $^{14}CO_2$ was performed in standard reaction mixtures of 1 ml(36). After incubation for 1 hr at 25 °C Sepharose-bound antibodies (0.4 ml slurry) and Triton X-100 (2%, final concentration) were added to the various reaction mixtures and the tubes were rotated end over end overnight at 4 °C. The Sepharose beads were washed alternately 3 times with 1 M NaCl in 0.1 M acetate buffer, pH 4.0 and with 1 M NaCl in 0.1 M borate buffer, pH 8.0. The adsorbed proteins were eluted with 6 M urea in 2% SDS, dialyzed against 0.1 M NaCl and counted. The data are expressed as $dpm.mg^{-1}$. Non-adsorbed reaction mixtures contained 7481 dpm of protein-bound $^{14}CO_2$. In all cases it was verified that adding more insolubilized antibodies did not increase the amount of adsorbed labeled proteins.

Table 4. The kinetic constants of substrates derived form prothrombin.

Substrate	K_m (mM)	V_{max} (dpm.mg $^{-1}$.min $^{-1}$)
Descarboxyprothrombin	0.4	54
Descarboxyfragment-1	0.4	74
Fragment-Su	0.001	174
Phe-Leu-Glu-Glu-Leu	11	108

The K_m and V_{max} were measured in standard reaction mixtures (36) and were calculated from the initial reaction rates at various concentrations of the substrates. The V_{max} is expressed as dpm incorporated per min and per mg microsomal protein.

the ribosomal protein synthesis and that after completion of the peptide chain its tertiary structure causes a sterical hindrance which hampers the carboxylation reaction. A second explanation may be that *in vivo* the carboxylation of prothrombin precursors is followed by a second modification (e.g. glycosylation) which occurs independent of the carboxylation reaction and which hampers the carboxylation of descarboxyprothrombin. Finally it cannot be excluded that the presence of the earlier mentioned signal sequence is a prerequisite for the efficient carboxylation of descarboxythrombin. This signal sequence is absent in descarboxyprothrombin (which is obtained from blood plasma), but may be present in the hepatic substrates. The low K_m of fragment-Su indicates that sterical obstructions are removed and that it is efficiently recognized by carboxylase. It is intriguing that the structure of fragment-Su contains the information which is required for a good substrate, whereas this information is lost in a number of smaller peptides. It may thus be expected that further proteolytic cleavage of fragment-Su will worsen this substrate and hence give us some insight into the structural elements that contribute to the recognition of the substrate by carboxylase.

IV. The purification of hepatic carboxylase

During attempts at purification, carboxylase acts as a typical integral membrane protein. Appreciable concentrations of detergent are required to solubilize the enzyme from the microsomes and it remains bound to the membrane under conditions which are known to remove many peripheral or lumenal microsomal proteins. The detergent that is most frequently used to solubilize carboxylase is Triton X-100, but also others (Lubrol, deoxycholate) may be used (30). Since in solution most detergents form micelles, even at rather low concentrations, the detergent-solubilized carboxylase is trapped in these micelles, together with many other microsomal proteins and it can hardly be purified further. Attempts to partly purify rat carboxylase prior to solubilizing the enzyme were reported by the group of Suttie (57, 58). The purification consisted of the sequential extraction of the microsomal membranes with a number of detergents and chaotropic solutions. This procedure resulted in an increase of the specific activity of carboxylase (about 100-fold) but this increase was not due to the removal of 99% of th contaminating proteins, but to the elimination of an inhibitor of carboxylase. Hence this procedure can hardly be qualified as a purification of the enzyme.

A partial purification of detergent-solubilized bovine carboxylase has been reported by De Metz et al., who were able to extract enzyme/substrate complexes from the solution with the aid of Sepharose-bound highly purified antibodies against clotting factor X (33). Since carboxylase was obtained from the livers of warfarin-treated animals, all carboxylase occurred in the form of enzyme/substrate complexes and as was mentioned before 60–70% of the carboxylatable substrate is a factor X precursor. Therefore this procedure resulted in the extraction of about 60% of the total amount of carboxylase. The non-bound microsomal proteins could be washed away so that the bound enzyme complex, which was designated as Solid-phase carboxylase, had been purified considerably. An advantage of the insolubilized enzyme is that it is stable, even at elevated temperatures. At 37 °C for instance, the carboxylation reaction occurred three times faster than at 25 °C and in the presence of an excess of exogenous substrate, the reaction rate was constant for more than 2 hours (Fig. 4). The Solid-phase enzyme can also be frozen as such and it can be kept at –80 °C for more than 1 year without any loss of enzyme activity. A disadvantge of Solid-phase carboxylase seems to be, that it can hardly be re-solubilized again. Thus far the only way to do so was by allowing the carboxylation reaction proceed in the presence of vitamin K hydroquinone (vitamin K reductase is removed during the washing proce-

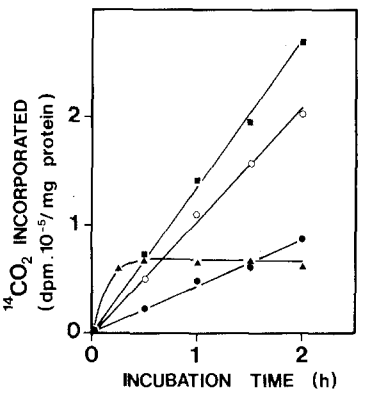

Fig. 4. The activity of Solid-phase carboxylase at various temperatures. Reaction mixtures contained 0.1 ml Solid-phase carboxylase slurry (40 μg protein), 0.01 mCi $NaH^{14}CO_3$, 2 mM dithiothreitol, 4 mM Phe-Leu-Glu-Glu-Leu and 0.2 mM vitamin K hydroquinone. The reaction was stopped by adding 1 ml trichloroacetic acid (5%) and the various reaction mixtures were degassed at elevated temperatures before counting. The carboxylation reaction was monitored at 15 °C (●—●), 25 °C (○—○), 35 °C (■—■) and 45 °C (▲—▲).

dure), Phe-Leu-Glu-Glu-Leu, $NaHCO_3$ and a high concentration of purified factor X. This procedure resulted in the re-solubilization of about 20% of the Solid-phase carboxylase. The re-solubilized enzyme was recovered in the micelles of the detergent, used for solubilizing vitamin K hydroquinone; freezing and thawing of the preparation rapidly destroyed all enzyme activity. Therefore it seems that, although Solid-phase carboxylase is useful in many types of experiments (see also below), the complete purification of carboxylase cannot be achieved via this step.

A real breakthrough in the purification of carboxylase came with the discovery of the detergent 3-([3-cholamidopropyl] dimethylammoniol)-1-propane sulfonate or CHAPS (59). Since the critical micelle concentration of this detergent seems to be rather high, carboxylase can be solubilized from the microsomal membranes without micelles being formed. Using this detergent carboxylase can be fractionated by conventional column chromatography (60) and probably also with the aid of high performance liquid chromatography. It may be expected therefore, that the complete purification of carboxylase will be reported in the near future .

V. Characteristics of carboxylase

As was discussed above, the complete purification of carboxylase has not yet been reported and the only preparation that has been partly characterized with respect to its molecular composition is Solid-phase carboxylase. It was demonstrated that in the carboxylation reaction this enzyme preparation can exclusively use vitamin K hydroquinone and not vitamin K quinone or vitamin K epoxide. Moreover, during the carboxylation reaction the epoxide accumulates in the reaction mixture (61). These results prompted us to the conclusion, that no reductase was present in this preparation any more.

Left aside the Sepharose bead, the anti-factor X and the factor X precursors, Solid-phase carboxylase consists for 40% of phospholipids and for 60% of proteins. The proteins of Solid-phase carboxylase were eluted from the Sepharose and analyzed on polyacrylamide gels (Fig. 5). Moreover we analyzed their vitamin K-binding capacity with the aid of [^{3}H]vitamin K. It resulted, that Solid-phase carboxylase consists of a small number of distinct proteins, but it is not sure which of these proteins belong to the carboxylase complex. When the vitamin K-binding capacity is expressed as dpm per mg protein, the proteins in Solid-phase carboxylase bound approximately 70 times more vitamin K than did the proteins in crude solubilized microsomes. Two vitamin K-binding proteins (45 K and 30 K) were observed in Solid-phase carboxylase (Fig. 5B), whereas on gels containing the crude microsomal proteins an additional peak was observed at 120 K (Fig. 5A). It seems plausible, that at least the vitamin K-binding proteins at 30 K and 45 K are part of the carboxylase complex.

The phospholipids of Solid-phase carboxylase could be identified for more than 95% as phosphatidylcholine (62). When the phospholipids were removed from the carboxylase complex (e.g. with various phosphilipases), the enzyme activity was lost completely, but it could be restored by adding exogenous phosphatidylcholine. Various other phospholipids were assayed for their ability to restore the carboxylase activity and it was observed that several neutral phospholipids were active in this respect (Table 5). Negatively charged phospholipids as well as phosphatidic acid did not restore the enzyme activity. The phospholipids in Solid-phase

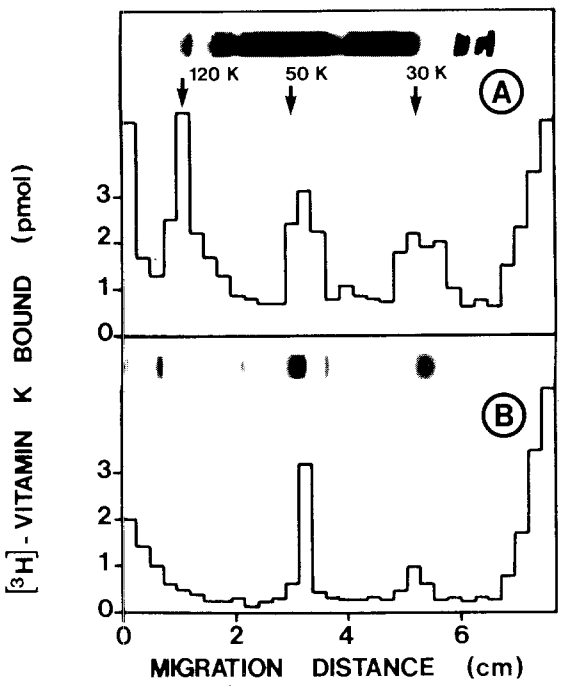

Fig. 5. The binding of [^{3}H] vitamin K to carboxylase. A: Detergent-solubilized microsomes (0.5 mg protein) were incubated with 0.02 mCi [^{3}H] vitamin K (300 Ci/mol) for 2 hr at 25 °C, dialyzed overnight against 0.1% SDS, 0.1 M glycine and 0.05 M Tris-HCl, pH 8.0 and analyzed on polyacrylamide gel. Finally the gel was sliced and counted. A non-labeled sample was run on a parallel gel and stained (top).
B: Solid phase carboxylase (0.01 mg protein) was incubated with [^{3}H] vitamin K, eluted from the Sepharose beads with 6 M urea in 2% SDS and analyzed as in A. All samples analyzed on these gels contained equal amounts of carboxylase activity.

carboxylase could also be exchanged directly by washing the Sepharose beads with a buffer containing the phospholipid of choice and cholate. As is shown in Table 5 the treatment with various neutral phospholipids did not dramatically affect the enzyme activity, but after the exchange with negatively charged phospholipids, carboxylase activity could hardly be detected anymore. So it seems, that neutral phospholipids are an essential part of carboxylase, but it cannot yet be concluded whether these phospholipids actively take part in the carboxylation reaction or merely serve to keep carboxylase (especially the hydrophobic parts of the enzyme) in the proper conformation.

VI. Postulated mechanisms for the carboxylation reaction

A lot of work has been done to elucidate the role of vitamin K in the carboxylation reaction, but at this moment we cannot yet formulate a detailed mechanism, which is compatible with all experiments reported in the literature. Whatever the mechanism will be, it will have to explain the following observations:

1. At 10 °C the equilibration rate between CO_2 and HCO_3^- is rather low, at least in the absence of carbonic anhydrase. Using a microsomal system in the presence of the carbonic anhydrase inhibitor acetazolamide, Jones et al. have demonstrat-

Table 5. The phospholipids in Solid-phase carboxylase.

Phospholipid added	Carboxylase activity (% of control) in	
	Solid-phase carboxylase	Phospholipid-depleted Solid-phase carboxylase
None	100	5
Crude microsomal extract	104	70
Phosphatidylcholine	110	84
Sphyngomyelin	90	50
Phosphatidylethanolamine	50	19
Phosphatidylinositol	3	0
Phosphatidylserine	2	0
Phosphatidylglycerol	2	0
Phosphatidic acid	4	0

Phospholipid-depleted Solid-phase carboxylase was prepared with the aid of phospholipase C (62). The various phospholipids were added to the enzyme preparations in the form of mixed micelles with cholate in a 1:1 ratio (w/w) up to a final concentration of 1 mg/ml. The carboxylase activities are expressed as a percentage of the non-treated enzyme, which was 115 000 dpm per mg of protein after 1 hr incubation at 25 °C.

ed that CO_2 and not HCO_3^- is the active species for carboxylation (63).

2. It has been established by many authors, that molecular oxygen is required for the carboxylation reaction (64, 65). The reaction mechanism should therefore show how oxygen is involved in the carboxylation of a glutamic acid residue.
3. Although it has not yet been proven unequivocally, there is strong evidence that the carboxylation reaction is coupled to the formation of vitamin K epoxide in such a way that no carboxylation can occur without the simultaneous formation of the epoxide (see Section II.4). Therefore every mechanism that is put forward for the carboxylation reaction will have to explain the concurrent formation of vitamin K epoxide.

The first ones who formulated a mechanism that fulfilled the requirements mentioned above were Friedman et al. (65) and Larson et al. (66). Using crude microsomal preparations from rat liver and β,γ-[^{3}H]Glu-labeled Phe-Leu-Glu-Glu-Leu these authors demonstrated, that the cleavage of the γC–H bond is dependent on vitamin K hydroquinone and that the amount of vitamin K-dependent hydrogen release exceeds the amount of CO_2 fixed. Hence there is a vitamin K-dependent exchange of γC–^{3}H with non-labeled hydrogen. It was also demonstrated, that at saturating CO_2 concentrations equal amounts of vitamin K epoxide and gamma-carboxyglutamic acid are formed. When the CO_2 concentration was decreased, this ratio shifted to a large excess of epoxide. The mechanism that is proposed by these authors is explained in Fig. 6. In the first step the reduced vitamin K is oxygenated to an intermediate, which is probably but not necessarily a hydroperoxide. This intermediate subsequently activates the γ-position of glutamic acid and is converted into vitamin K epoxide. The activated glutamyl residue may be either a carbanion or a carbon radical. In the latter case an additional mechanism is required to explain the formation of a CO_2 radical. It will be clear, that in this view the carboxylation reaction occurs in a number of subsequent steps in which first the γC–H is released and in a later stage CO_2 is bound to the 'activated' γC atom. The data argue against a concerted mechanism for the cleavage of the γC–H bond and carboxylation.

A more detailed concept of a reaction mechanism was put forward by De Metz et al. (48) who used the

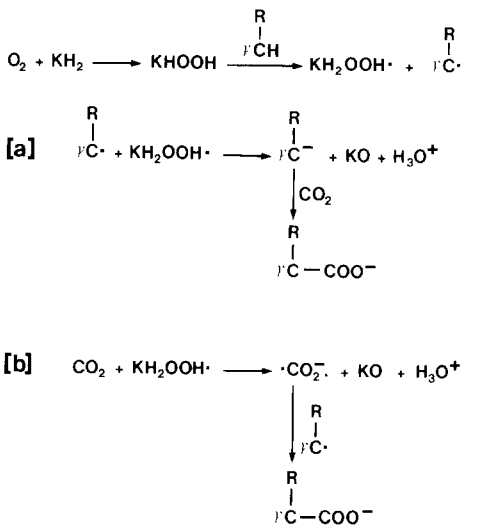

Fig. 6. The non-concerted mechanism for the carboxylation reaction. Oxygenated vitamin K hydroquinone induces the formation of a γC radical. The resulting vitamin K radical may lead either to the formation of a γC carbanion (pathway a) or to the formation of a CO_2 radical (pathway b). In both ways the reaction products are a Gla residue, vitamin K epoxide and water.

partly purified Solid-phase carboxylase for their experiments. The proposed mechanism is based on the observation that in the presence of disodiumsulfite a) the carboxylation reaction is enhanced, and b) that under these conditions vitamin K hydroquinone is not converted to the epoxide but to hydroxyvitamin K. This metabolite was detected in the reaction mixture with the aid of high performance liquid chromatography (HPLC). The carboxylation reaction is thought to occur in two steps. In the first step vitamin K hydroquinone is oxidized into a hydroperoxide (Fig. 7). This reaction does not necessarily take place on the enzyme, because it already proceeds when vitamin K hydroquinone is solubilized in water. Since, in general, hydroquinones rapidly form radicals, and since radical scavengers are inhibitors of carboxylase, it seems that the hydroperoxide is formed in the way shown in Fig. 7. The role of carboxylase may now be a) that it mediates in the proper alignment of the reaction components, and b) that it couples the heterolytic cleavage of the peroxide bond to the carboxylation of a glutamic acid residue (Fig. 7). The shift of the electron pair thus results in the formation of vitamin K epoxide, a Gla residue and water. A similar

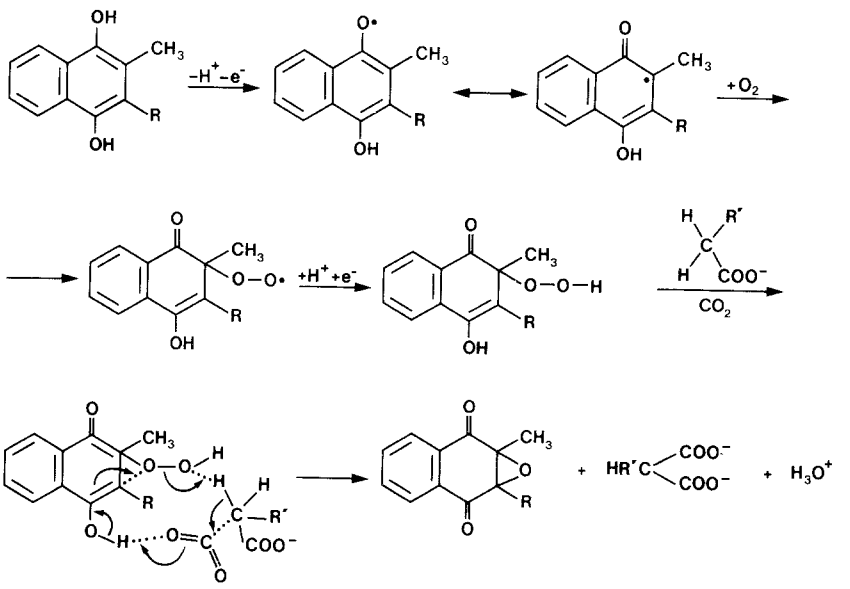

Fig. 7. The concerted mechanism for the carboxylation reaction. Vitamin K hydroquinone is converted into a hydroperoxide. The breakage of the peroxide bond is the driving force for the removal of the γ–C hydrogen, which is replaced by carbon dioxide. Further details are explained in the text.

mechanism may be proposed in which a homolytic cleavage of the peroxide initiates the carboxylation but, as in the model of Friedman, here too an additional mechanism has to be proposed for the formation of a CO_2 radical. The stimulation by sulfite is thought to occur via a weakening of the peroxide bond by a nucleophilic interaction. This interaction might stimulate the electron shift and thus the carboxylation reaction. At the same time the vitamin K-bound oxygen is reduced via a two-electron transfer and subsequently protonated to hydroxyvitamin K. At limiting CO_2 concentrations more vitamin K epoxide and hydroxyvitamin K are formed than CO_2 is incorporated. In the 'uncoupled' reaction a proton may take the place of CO_2, thus giving rise to the exchange of the γC hydrogen with water and the concurrent formation of vitamin K epoxide.

So at present two alternative theories are operative to explain the mechanism of the vitamin K-dependent reaction. Whereas one theory predicts the concerted exchange of the γC hydrogen by CO_2, this possibility is excluded by the other one. Up till now we cannot decide whether one of the proposed reaction mechanisms is correct or that a third model has to be developed.

Another approach to elucidate some details of the reaction mechanism was reported by De Metz et al. (67), who demonstrated that some organic solvents (ketones and dimethyl sulphoxide) stimulate the carboxylation reaction. The formation of vitamin K epoxide was stimulated simultaneously and the stimulation was independent of the physical state (gel or liquid cristalline) of the phospholipids in carboxylase. In order to demonstrate the effect of the organic solvents we incubated carboxylating reaction mixtures in the presence and absence of acetone. The enzyme used in these experiments was Solid-phase carboxylase, which had been reconstituted with a purified phospholipid of a well-defined melting point (23 °C). When the results of these experiments are processed in the form of Arrhenius plots (Fig. 8) three conclusions may be drawn. The first conclusion is that both, in the absence and presence of acetone the plots are linear between 10 and 30 °C, indicating once more that the physical state of the phospholipid does not influence the carboxylation rate. In the second place it is obvious that in case vitamin K hydroquinone is used as a coenzyme, acetone decreases the slope of the curve and hence the activation energy. Therefore the lower the temperature is, the more pronounced will be the effect of acetone. Finally it is clear, that when a vitamin K analogue, lacking the long phytyl side-

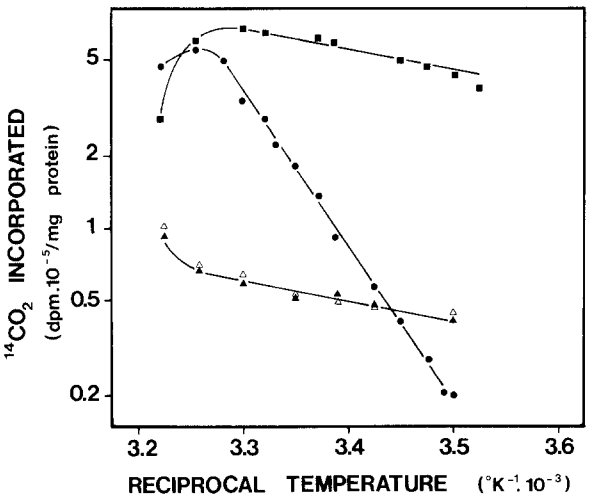

Fig. 8. Arrhenius plots of Solid-phase carboxylase. Solid-phase carboxylase was depleted of its natural phospholipids and reconstituted with di-C14:O phosphatidylcholine (58). The carboxylase activity was measured in the presence of vitamin K hydroquinone and in the absence (●—●) or presence (○—○) of acetone (10% v/v). The carboxylase activity was also measured when vitamin K was replaced by 3-DTT menadiol (vitamin K hydroquinone in which the phytyl side-chain is replaced by dithiothreitol (68)), in the absence (▲—▲) and presence (△—△) of acetone.

chain was used as a coenzyme, the activation energy of the carboxylation reaction was low, both in the absence and presence of acetone.

These data may easily be explained by assuming that the organic solvents facilitate the transport of vitamin K hydroquinone from the detergent micelles to the carboxylase enzyme complex. In that case we would expect a decrease of the amount of vitamin K required for a maximal enzyme activity. Since the K_m for the vitamin remaines unchanged by acetone, this explanation cannot be correct. Another explanation might be, that the organic solvents interact with the site on carboxylase that is responsible for the binding of vitamin K. This site will probably have a hydrophobic character, and organic solvents might interact with this part of the enzyme without changing the K_m for the vitamin. Since it seems that phospholipids are not involved in the stimulation of carboxylase by organic solvents, this hydrophobic region will probably be situated on the protein part of carboxylase. Speculating, one might ascribe a carrier function to the phytyl chain of vitamin K, the function of which is analogous to that of the side-chain of biotin and lipoic acid: the phytyl chain might serve as a swinging arm, which remains bound to one site of carboxylase, but which is able to carry the functional naphtoquinone group from one site to the next. The organic solvents might facilitate that carrier function by increasing the mobility of the vitamin. Vitamin K analogues lacking the long hydrophobic side-chain might bypass the carrier function and obviously would not be affected by organic solvents.

VII. Non-hepatic carboxylases

It has been thought for many years, that vitamin K-dependent carboxylase in liver was the only enzyme system that made use of vitamin K for the production of the Gla residues in the vitamin K-dependent clotting factors. Consequently it was assumed, that the synthesis of these factors could exclusively be inhibited by vitamin K antagonists. During the last few years, however, a number of investigators has reported the presence of vitamin K-dependent carboxylase in non-hepatic tissues, such as kidney, spleen, lung and bone (6, 19, 69, 71). The importance of these carboxylases remained obscure untill now, because we do not know the function of the carboxylated reaction products. In an attempt to make an inventory of the carboxylating systems in different types of bovine tissue, we prepared the microsomal fractions of 20 different types of tissue from warfarin-treated cows. The presence of carboxylase and endogenous substrate were investigated in these preparations and the results are summarized in Table 6. In all tissues where we were able to detect carboxylase, we also observed that during the warfarin treatment of the animals an endogenous substrate had accumulated in the microsomes. These endogenous substrates were not present in similar preparations from non-treated animals and the non-hepatic substrates shared only few (if any) antigenic determinants with the hepatic clotting factor precursors (data not shown). It is not known whether all non-hepatic carboxylating systems are similar to the hepatic one. It is also possible, that 'carboxylase' may be subdivided into a number of isoenzymes. In the latter case one would expect a certain substrate specificity: carboxylase from liver would recognize the precursors of the clotting factors, but not the

Table 6. Microsomal carboxylase and substrate in tissues from warfarin-treated cows.

Tissue	Amount of carboxylase (dpm.mg $^{-1}$.min $^{-1}$)	Amount of substrate (dpm.mg $^{-1}$)
Liver	63	2205
Testis	132	2867
Kidney	46	265
Spleen	44	904
Lung	40	132
Thyroid	20	154
Pancreas	7	135
Thymus	7	62
Arteries	39	221
Veins	0	0
Brain	0	0
Leucocytes	0	0
Heart muscle	0	0
Skeletal muscle	0	0
Diaphragm	0	0
Epiphysis	15	220

Microsomes were prepared from the various tissues as described earlier (36), except for the epiphysis, which was powdered and dissolved in 0.5 M EDTA, pH 8.0 before it was homogenized. All reactions were performed under standard conditions in the presence of 0.5% CHAPS. The data are the means of four different animals.

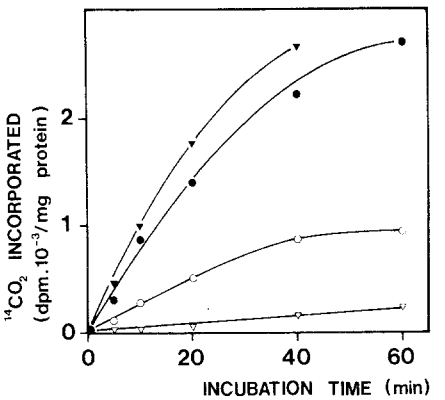

Fig. 9. The carboxylation of non-hepatic substrates by hepatic carboxylase. B.S. was prepared from bovine bone by extracting bovine bone powder with 1 M EDTA, pH 8.0 overnight. The extract was desalted on a Sephadex G-25 column and thermally decarboxylated (72). A.S. was prepared from the microsomal supernatant of bovine arteries, which was dialyzed against distilled water and concentrated by lyophilization. The two substrates were tested under standard conditions in solubilized microsomes from cows that had not been treated with warfarin. Therefore no endogenous substrate was present in this carboxylase preparation. Neither B.S. (●—●) nor A.S. (○—○) were purified before use. In this figure they are compared with the pentapeptide Phe-Leu-Glu-Glu-Leu (▲—▲) and with no substrate present (△—△). Blanc values (minus vitamin K) have been subtracted.

substrates from bone or arteries. We have tested this possibility by preparing the substrates from bone (B.S.) and arteries (A.S.) and testing these substrates in bovine-liver carboxylase. The results are shown in Fig. 9 and it has to be concluded that both non-hepatic substrates are carboxylated in the liver system. Although this experiment shows, that there is no absolute substrate specificity, it does not rule out the possibility of quantitative differences in the affinity of certain substrates for different enzyme systems. Whether this is the case or not can only be found out by purifying a number of hepatic and non-hepatic substrates and determining their respective K_m values in the various carboxylating systems. This type of research is in current progress in our laboratory.

Now it has been established, that vitamin K-dependent carboxylases are found in many non-hepatic tissues, the question raises: what are the functions of the non-hepatic Gla containing proteins and what is their physiological importance? At present no experimental data are available that may help us to answer this question. A number of carboxylated proteins have been detected, some of them have even been purified and extensively characterized, but this information does not necessarily lead to the elucidation of their function. A possible role for some of these proteins is discussed in the last section of this paper.

A second question that has to be answered is: are the non-hepatic carboxylases inhibited during the treatment of patients with vitamin K antagonists (oral anticoagulant therapy)? In most experiments reported until now, warfarin is administrated to experimental animals in dosages of about 5 mg per kg body weight. The dose used for therapeutical purposes in man is about 100-fold lower (73) and it remains to be demonstrated, that the non-hepatic carboxylases are inhibited *in vivo* by this therapy in a similar way as is the hepatic system. Using the rat as an experimental model system we have done some experiments that may give an answer to this question. Warfarin was added to their drinking water in a concentration of 2 mg per liter, which resulted in a daily intake of 200–250 μg per kg body weight. Each successive day 6 rats were sacrificed,

blood was taken for a clotting assay and a number of organs were excised for the production of the microsomal fraction. The results are shown in Fig. 10. In the liver the accumulation of endogenous substrate did not occur at its maximal rate (dotted line), indicating that the intake of warfarin was marginal. Nevertheless it is obvious, that in all investigated tissues the accumulation of endogenous substrates starts as soon as the warfarin is given to the animals. After 7 days the substrate level had increased about 10-fold in all tissues, indicating that during the intake of very low amounts of warfarin not only hepatic carboxylase is partly inhibited but also that under these conditions the non-hepatic carboxylases are inhibited to a similar extent. Although these results were obtained with rats, there is no reason to believe that the situation is different in man. When we regard the inhibition of the non-hepatic carboxylases as a side-effect of oral anticoalgulant therapy, we have to explain why, despite of the fact that coumarin derivatives have been used on a large scale for many years, clinical symptoms of this side-effect are hardly known (except for chondrodysplasia punctata, the fetal warfarin syndrome, which has been documented very

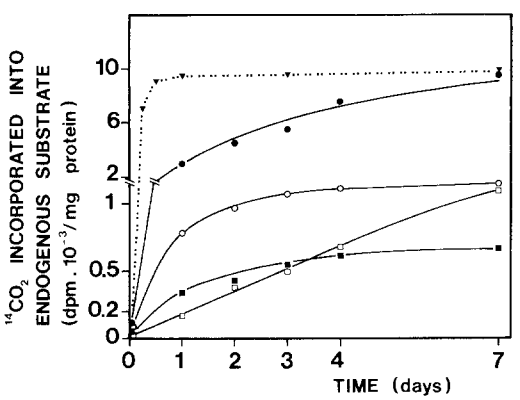

Fig. 10. The effect of low doses of warfarin on non-hepatic tissues. Rats were treated with warfarin, which was present in the drinking water in a concentration of 2 mg/l and its effect was demonstrated in the microsomal fractions of various tissues by measuring the accumulation of endogenous substrates. After the indicated intervals 6 rats were sacrificed and the corresponding organs were pooled before the microsomes were prepared. The following organs were used: liver (●—●), lung (○—○), spleen (■—■) and testis (□—□) and they are compared with the liver from rats which had first received 1.5 mg of warfarin by an intraperitoneal injection (dotted line). Under these conditions it is assumed that the accumulation of endogenous substrate occurs at its maximal rate.

well and which is described in Section IX.1). We might assume, for instance, that all vitamin K-dependent proteins are produced in great excess, so that a 90% decrease of their synthesis does not lead to clinical symptoms. On the other hand it may also be that, because we do not know the function of the non-hepatic vitamin K-dependent proteins, less conspicuous symptoms have been overlooked. It is our opinion, that the best approach of this problem will be found in the close cooperation between biochemists (characterizing the Gla-containing proteins and speculating about their possible function) and clinicians (searching for possible side-effects of coumarin derivatives in patients). Only than we may come to an understanding of the physiological importance of the non-hepatic Gla-containing proteins.

VIII. Human carboxylase

Up till now we have only discussed the vitamin K-dependent carboxylase obtained from experimental animals. It is evident that the importance of the data, obtained in these model systems is greatly enhanced, when they can be verified in tissues from human origin. A first attempt was made by Friedman et al. (74) who prepared carboxylase from human placenta. Compared with rat liver carboxylase the activity of this system was very low, however. Carboxylase from human liver was described for the first time by Soute et al. (5). It should be realized, that human tissues are less easily available than are tissues from animal origin, and that the medical treatment of the donors (administration of drugs, the time lapse between the death of the patient and the excision of the organs etc.) cannot be adapted to optimal conditions for the experiments. Therefore conclusions should only be drawn with great care. In order to illustrate the presence of carboxylase, we prepared the microsomal fractions from six human livers, which were compared with similar fractions from normal (Fig. 11A) and warfarin-treated cows (Fig. 11B). The human livers were obtained from three donors who had died from severe brain damage after various traffic accidents (Fig. 11C) and from three donors who had been hospitalized for at least one week and who had died from various diseases (Fig. 11D). It was ascertained, that none of the donors had received anti-

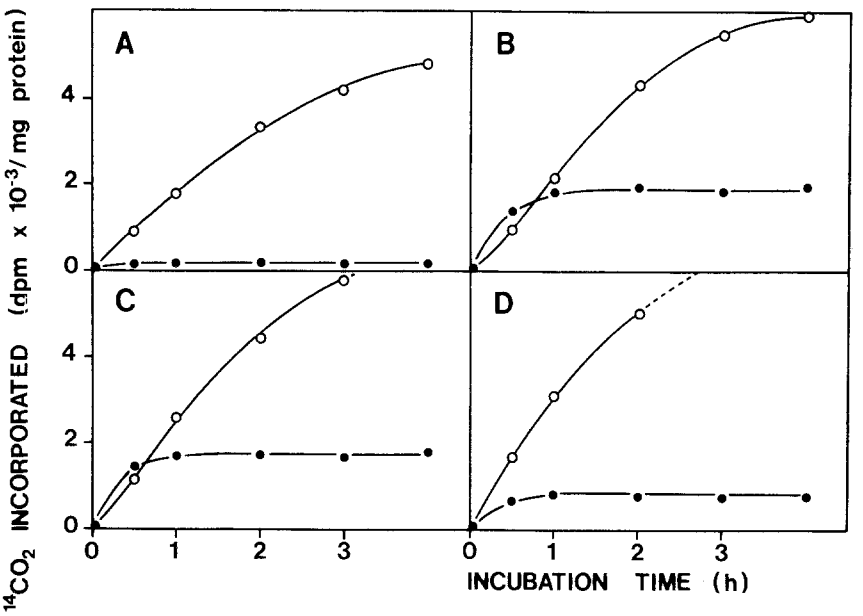

Fig. 11. Comparison of human and bovine liver carboxylase. Carboxylase was measured in the absence (●—●) and presence (○—○) of Phe-Leu-Glu-Glu-Leu. Microsomes were prepared from the liver of normal cows (A), warfarin-treated cows (B), human donors who had died after traffic accidents (C) and human donors who had died after various diseases (D). For further details see text.

vitamin K drugs, but nevertheless we found high levels of endogenous substrates in the human preparations. These levels were comparable with those in warfarin-treated cows or in vitamin K-deficient rats. In order to investigate whether this apparent vitamin K deficiency in man is also found in tissues from normal, healthy individuals, we prepared carboxylase from human placenta and from the arteries of umbilical cord. Although the carboxylase content in these preparations ranged from 40–60% of that in liver microsomes, no trace of a carboxylatable endogenous substrate was observed. We think, therefore, that the abnormal high level of clotting factor precursors in human liver is a result of the condition of the donors at the moment that the livers were obtained. The victims of traffic accidents, for instance, all suffered from severe brain traumas, massive blood loss and diffuse intravascular coagulation. After hospitalization it lasted 4–8 hr before their livers could be taken. Because blood loss, a decreased plasma concentration of clotting factors and clotting factor activation products all have been reported to stimulate the *de novo* synthesis of clotting factors (75, 76), it is likely that in the livers used for our experiments the synthesis of clotting factors had been strongly enhanced. It has been suggested that the carboxylation reaction might be the rate-limiting step during the synthesis of the clotting factors (5) and this would explain the accumulation of precursors of these factors during periods of increased ribosomal protein synthesis. Since there are no further experimental data available to support this hypothesis, other explanations may be given for the high amount of endogenous substrate in human liver. On the other hand we may conclude, that the presence of vitamin K-dependent carboxylase has been convincingly demonstrated in human liver, placenta and arterial vessel wall and that there are no arguments to believe that it would be absent in other tissues such as lung, spleen and kidney.

IX. Possible functions of vitamin K-dependent proteins

At present we know that in many species the treatment with anti-vitamin K drugs causes an accumulation of non-carboxylated proteins in the microsomal fraction of many types of tissue. In the liver, for instance, precursors of the vitamin K-de-

pendent clotting factors are – at least partly – retained instead of being excreted in the blood stream. This example also illustrates, that the place were these proteins are synthesized is not necessarily the same as where they exert their biological function. Therefore we would like to know a) where we can find the carboxylated vitamin K-dependent proteins, and b) what they are doing there. Unfortunately, except for the proteins involved in blood coagulation, we do not know the functions of these proteins and when we try to shed some light on these two questions, it should be realized that our discussion is thus merely based on speculations.

Nevertheless we would like to finish this review with an overview of what is known and what might be expected about the role of non-hepatic vitamin K-dependent proteins. Before starting these speculations, we should keep in mind that up till now the only known function of Gla residues is the strong and selective binding of Ca^{2+}. All functions described to the various Gla-containing proteins should therefore be dependent on or related with the binding or the transport of Ca^{2+}.

IX.1. Bone Gla-protein (B.G.P.)

One of the best documented side-effects of coumarin derivatives is the fetal warfarin syndrome, the abnormal bone growth in the fetus during pregnancy (19). It could be demonstrated that bone contains a vitamin K-dependent protein (Bone Gla Protein, or osteocalcin), which is synthesized by the bone cells (77, 78). Osteocalcin appears in the developing bone coincident with the start of mineralization and reaches its highest concentrations in the rapid growing parts of the bone (79, 80). Many attempts have been made to relate the γ-carboxylation of osteocalcin (and thus its ability to interact with Ca^{2+}) with some function of this protein, but as yet no biological role has been unambiguously demonstrated. *In vitro* osteocalcin strongly inhibits the precipitation of hydroxyapatite from supersaturated solutions of calcium and phosphate. When the protein would have a similar function *in vivo*, we might expect that it prevents excessive mineralization in developing bone. Indeed, the previously mentioned fetal warfarin syndrome is characterized by islands of calcification within what is normally the uncalcified growth plate (81). Recently it has been reported, that a similar effect could be obtained by the treatment of new-born rats for 8 months with warfarin (82). After this period the animals even showed a complete fusion of the proximal tibial growth plate resulting in a cessation of the longitudinal growth. Although it seems highly probable that these defects are related to the function of osteocalcin, up till now no conclusive evidence has been given to support this hypothesis. It should also be emphasized, that no skeletal abnormalities have been reported in man even after many years of oral anticoagulant therapy. Nevertheless it would be interesting to investigate the occurrence of a number of bone diseases in patients receiving vitamin K antagonists. In this respect it is suggestive, that it has been reported that in postmenopausal patients with osteoporosis the loss of calcium was found to be reduced 18 to 50% by daily treatment with vitamin K (83).

IX.2. Arterial Gla Protein (A.G.P.)

It has been shown in Table 6 that there is a striking difference between the carboxylase content of arteries and that of veins. Bovine arterial vessel wall contains a high amount of carboxylase, whereas in veins the enzyme could not be detected. Similar results were obtained with the arteries and veins from human umbilical cord, and the possibility cannot be excluded, that arterial carboxylase is somehow involved in the process of atherosclerosis, which also occurs in arteries and not in veins. In this respect it is suggestive, that a Gla-containing protein (atherocalcin) has been identified in hardened atherosclerotic plaque (84). Whether the product of arterial carboxylase, A.G.P., is similar to atherocalcin is not sure at this moment.

IX.3. Renal Gla-Protein (R.G.P.)

The occurrence of vitamin K-dependent carboxylase has been described by several authors. Obviously one of the most probable functions of the carboxylated R.G.P. might be that it plays a role in the transport and/or excretion of Ca^{2+}. Recently a Gla-containing protein has been described, which occurs in renal stones (85). Again, though probable, it is not sure whether this protein is similar to or related with R.G.P. The function of R.G.P. remains obscure at this moment. When the protein is involved in the excretion of Ca^{2+}, it might be ex-

pected that during anticoagulant therapy (when descarboxy-R.G.P. is formed instead of R.G.P.) the Ca^{2+} concentration in the kidney is increased, resulting in a higher frequency of renal stones. On the other hand, R.P.G. might also serve as nuclei for the cristallization of calcium salts. In this view the inhibition of R.P.G. synthesis would lead to a decreased frequency of renal stones. On beforehand neither of these two possibilities can be excluded and we would like to plead for a statistical analysis of the medical records from patients on long-term anticoagulant therapy.

IX.4. Testicular Gla Protein (T.G.P.)

Vitamin K-dependent carboxylase has been detected in the testis of several species. Since most of the proteins, synthesized in the testis are used for the production of sperm cells, we have tried to demonstrate the presence of a vitamin K-dependent protein in sperm. Washed human sperm cells were homogenized in 50 mM EDTA, pH 7.0 and the resulting proteins were thermally decarboxylated. This procedure converts Gla residues into Glu without destructing the rest of the protein. Finally this preparation was used as a substrate in bovine liver carboxylase. As is shown in Fig. 12, $^{14}CO_2$ is

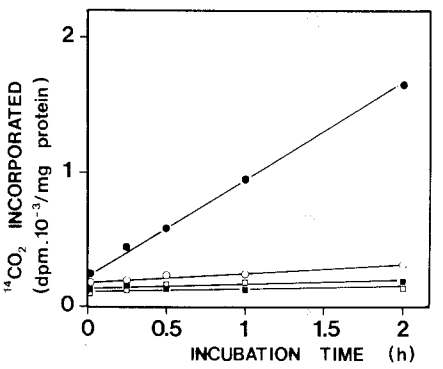

Fig. 12. The carboxylation of sperm proteins. Fresh human sperm cells were washed with 0.15 M NaCl and homogenized in 50 mM EDTA, pH 7.0. The membrane fragments were spun down and the resulting supernatant was dialyzed against distilled water, lyophilized and decarboxylated (72). The proteins were subsequently dissolved in 0.15 M NaCl and used as a substrate for bovine carboxylase, under similar conditions as described in the legend of Fig. 9. The substrate was tested in the presence (●—●) and absence (○—○) of vitamin K hydroquinone. Control experiments were: non-decarboxylated substrate (■—■) and no substrate (□—□), both in the presence of vitamin K hydroquinone.

incorporated into at least one of the sperm proteins and this incorporation strictly depends on the presence of vitamin K. This example not only demonstrates, that a Gla-containing protein is present in human sperm, it also shows how we may detect Gla-containing proteins in other cells or tissues. At this moment it is difficult to speculate about the function of T.G.P. One of the possibilities is that T.G.P. is related to acrosin, a trypsin-like serine protease, the known part of which has an amino acid homology of about 50% with thrombin and which is required for the penetration of the sperm chromosomes into the ovum (86). Like prothrombin, acrosin also occurs in the form of a zymogen (pro-acrosin), which probably would contain the Gla residues.

The fact, that also in testis endogenous substrates accumulate *in vivo* during treatment with low doses of warfarin demonstrates, that under these conditions the production of T.G.P. is affected, which might influence the fertility of the sperm cells. Most patients, treated with vitamin K antagonists are over 50 years old, and maybe this is the reason why – as far as we know – their fertility has never been checked. At this moment we have been informed about three patients who were capable of begetting offspring after more than one year of continuous oral anticoagulant therapy. We would suggest, however, to statistically analyze the fertility of large groups of patients.

X. Concluding remarks

From the moment of its discovery, people have been optimistic about the purification of hepatic carboxylase. Since the enzyme is firmly bound to the microsomal membranes, its purification still presents serious problems. Unfortunately these problems also hamper the final elucidation of the mechanism of the carboxylation reaction.

Vitamin K-dependent carboxylase seems to occur in many different types of tissue. It is not known whether carboxylase consists of a number of isoenzymes, each with its own substrate specificity, or that it occurs in one form in all these tissues. Since the vitamin K dependency is universal for all these enzyme systems, it was to be expected, that all carboxylases are inhibited to the same extent by vitamin K antagonist. Nevertheless it might be

desirable to develop drugs that specifically inhibit one type of carboxylase without affecting others. Obviously these type of inhibitors should not be analogues of the common coenzyme vitamin K, but they should be analogues of the substrate, the carboxylation of wich is to be inhibited. Therefore it is very promising, that recently the first two inhibitors of this type have been synthesized (87). Both inhibitors were structural analogues of the peptide Phe-Leu-Glu-Glu-Leu and they inhibited the carboxylation of endogenous and exogenous substrates in a liver microsomal system. Whether these inhibitors preferentially inhibit hepatic carboxylase remains to be seen, however.

Acknowledgements

The author wishes to thank the following people: Dr H. C. Hemker, Dr M. de Metz, Dr M. A. C. de Boer-van den Berg, B. A. M. Soute, M. Ulrich, H. Hendrix and M. C. Roncaglioni*, who all contributed to the results presented in this review, and Mrs M. Molenaar-van de Voort, who has typed this manuscript. Our research was supported by grant MD 82145 from the Thrombosestichting Nederland.

References

1. Dam, H., 1935. Biochem. J. 29: 1273–1285.
2. Campbell, M. A. and Link, K. P., 1941. J. Biol. Chem. 138: 21–33.
3. Link, K. P., 1959. Circulation 19: 97–107.
4. O'Reilly, R. A., 1976. Ann. Rev. Med. 27: 245–261.
5. Soute, B. A. M., De Metz, M. & Vermeer, C., 1982. FEBS Lett. 146: 365–368.
6. Vermeer, C., Hendrix, H. and Daemen, M., 1982. FEBS Lett. 148: 317–320.
7. Hemker, H. C., Veltkamp, J. J., Hensen, A. and Loeliger, E. A., 1963. Nature 200: 589–590.
8. Josso, F., Lavergne, J. M. Gouault, M., Prou-Wartelle, C. and Soulier, J. P., 1968. Thromb. Diath. Haemorrhag. 20: 88–98.
9. Ganrot, P. O. and Nilehn, J. E., 1968. Scand. J. Clin. Lab. Invest. 22: 23–28.
10. Reekers, P. P. M., Lindhout, M. J., Kop-Klaassen, B. H. M. and Hemker, H. C., 1973. Biochim. Biophys. Acta 317: 559–562.

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11. Stenflo, J., 1970. Acta Chem. Scand. 24: 3762–3763.
12. Nelsestuen, G. L. and Suttie, J. W., 1972. Biochemistry 11: 4961–4964.
13. Stenflo, J. and Ganrot, P. O., 1973. Biochem. Biophys. Res. Commun. 50: 98–104.
14. Bjørk, J. and Stenflo, J., 1973. FEBS Lett. 32: 343–346.
15. Stenflo, J., Fernlund, P., Egan, W. and Roepstorff, P., 1974. Proc. Natl. Acad. Sci. U.S.A. 71: 2730–2733.
16. Nelsestuen, G. L., Zytkovicz, T. H. and Howard, J. B., 1974. Proc. Natl. Acad. Sci. U.S.A. 71: 2730–2733.
17. Suttie, J. W. and Jackson, C. M., 1977. Physiol. Rev. 57: 1–70.
18. Tuan, R., 1979. J. Biol. Chem. 245: 1356–1364.
19. Gallop, P. M., Lian, J. B. and Hauschka, P. V., 1980. N. Engl. J. Med. 302: 1460–1466.
20. Blobel, G. and Dobberstein, B., 1975. J. Cell. Biol. 67: 835–851.
21. Walter, P. and Blobel, G., 1982. Nature 299: 691–698.
22. Meyer, D. I., Krause, E. and Dobberstein, B., 1982. Nature 297: 647–650.
23. MacGillivray, R. T. A., Chung, D. W. and Davie, E. W., 1980. In: Vitamin K Metabolism and Vitamin K-Dependent Proteins. (Suttie, J. W. ed.), pp. 546–552, Univ. Park Press, Baltimore.
24. Esmon, C. T., Sadowski, J. A. & Suttie, J. W., 1975. J. Biol. Chem. 250: 4744–4748.
25. Lowenthal, J. and Jaeger, V., 1977. Biochem. Biophys. Res. Commun. 74: 25–32.
26. Vermeer, C., Soute, B. A. M. and Hemker, H. C., 1978. Biochim. Biophys. Acta 523: 494–505.
27. Helgeland, L., 1977. Biochim. Biophys. Acta 499: 181–193.
28. Carlisle, T. L. and Suttie, J. W., 1980. Biochemistry 19: 1161–1167.
29. Esmon, C. T. and Suttie, J. W., 1976. J. Biol. Chem. 251: 6238–6243.
30. Mack, D. O., Suen, E. T., Girardot, J. M., Miller, J. A., Delaney, R. and Johnson, B. C., 1976. J. Biol. Chem. 251: 3269–3276.
31. Girardot, J. M. and Johnson, B. C., 1982. Anal. Biochem. 121: 315–320.
32. Vermeer, C., De Metz, M., Soute, B. A. M. and Hemker, H. C., 1980. In: Protides of Biological Fluids, 28th Colloquium (Peeters, H., ed.), Vol. 28, pp. 249–252, Pergamon Press, Oxford.
33. De Metz, M., Vermeer, C., Soute, B. A. M., van Scharrenburg, G. J. M., Slotboom, A. J. and Hemker, H. C., 1981. FEBS Lett. 123: 215–218.
34. Lindhout, M. J., Kop-Klaassen, B. H. M., Reekers, P. P. M., and Hemker, H. C., 1976. J. Mol. Med. 148: 140–144.
35. Carlisle, T. L., Shah, D. V., Schlegel, R. and Suttie, J. W., 1975. Proc. Soc. Exptl. Biol. Med. 148: 140–144.
36. Vermeer, C., Soute, B. A. M., De Metz, M. and Hemker, H. C., 1982. Biochim. Biophys. Acta 714: 361–365.
37. Vermeer, C., Soute, B. A. M. and Hemker, H. C., 1977. Thrombos. Res. 10: 495–507.
38. Matschiner, J. T., Bell, R. G., Amelotti, J. M. and Knauer, T. E., 1970. Biochim. Biophys. Acta 201: 309–315.
39. Willingham, A. K. and Matschiner, J. T., 1974. Biochem. J. 140: 435–441.
40. Sadowski, J. A., Schnoes, H. K. and Suttie, J. W., 1979. Biochemistry 16: 3856–3863.

41. Zimmerman, A. and Matschiner, J. T., 1974. Biochem. Pharmacol. 23: 1033-1040.
42. Matschiner, J. T., Zimmerman, A. and Bell, R. G., 1974. Thrombos. Diathes. Haemorrh. Suppl. 57: 45-52.
43. Whitlon, D. W., Sadowski, J. A. and Suttie, J. W., 1978. Biochemistry 17: 1371-1377.
44. Martins, C., Ganser, R. and Viviani, A., 1975. FEBS Lett. 59: 13-14.
45 Wallin, R., Gebhardt, O. and Prydz, H., 1978. Biochem. J. 169: 95-101.
46. Sherman, P. A. and Sander, E. G., 1981. Biochem. Biophys. Res. Commun. 103: 997-1005.
47. Larson, A. E. and Suttie, J. W., 1978. Proc. Natl. Acad. Sci. U.S.A. 75: 5413-5416.
48. De Metz, M., Soute, B. A. M., Hemker, H. C., Fokkens, R., Lugtenburg, J. and Vermeer, C., 1982. J. Biol. Chem. 257: 5413-5416.
49. Suttie, J. W., Geweke, L. O., Marin, S. L. and Willingham, A. K., 1980. FEBS Lett. 109: 267-270.
50. Larson, A. E., Friedman, P. A. and Suttie, J. W., 1981. J. Biol. Chem. 256: 11032-11035.
51. Grant, G. A. and Suttie, J. W., 1976. Biochemistry 15: 5387-5393.
52. Swanson, J. C. and Suttie, J. W., 1982. Biochemistry 21: 6011-6018.
53. Suttie, J. W., Hageman, J. M., Lehrman, S. R. and Rich, D. H., 1976. J. Biol. Chem. 251: 5827-5830.
54. Houser, R. M., Carey, D. J., Dus, K. M., Marshall, G. R. and Olson, R. E., 1977. FEBS Lett. 75: 226-230.
55. Rich, D. H., Lehrman, S. R., Kawai, M., Goodman, H. L. and Suttie, J. W., 1981. J. Med. Chem. 24: 706-711.
56. Soute, B. A. M., Vermeer, C., De Metz, M., Hemker, H. C. and Lijnen, H. R., 1981. Biochim. Biophys. Acta 676: 101-107.
57. Canfield, L. M., Sinsky, T. A. and Suttie, J. W., 1980. Arch. Biochem. Biophys. 202: 515-524.
58. Wallin, R. and Suttie, J. W., 1982. Arch. Biochem. Biophys. 214: 155-163.
59. Girardot, R. and Johnson, B. C., 1982. Anal. Biochem. 121: 315-320.
60. Girardot, J. M., 1982. J. Biol. Chem. 257: 15008-15011.
61. De Metz, M., 1982. Ph. D. Thesis, University of Limburg, Maastricht.
62. De Metz, M., Vermeer, C., Soute, B. A. M. and Hemker, H. C., 1981. J. Biol. Chem. 256: 10843-10846.
63. Jones, J. P., Gardner, E. J., Cooper, T. G. and Olson, R. E., 1977. J. Biol. Chem. 252: 7738-7742.
64. Sadowski, J. A., Esmon, C. T. and Suttie, J. W., 1976. J. Biol. Chem. 251: 2770-2776.
65. Friedman, F. A., Shia, M. A., Gallop, P. M. and Griep, A. E., 1979. Proc. Natl. Acad. Sci. U.S.A. 76: 3126-3129.
66. Larson, A. E., Friedman, P. A. and Suttie, J. W., 1981. J. Biol. Chem. 256: 11032-11035.
67. De Metz, M., Soute, B. A. M., Hemker, H. C. and Vermeer, C., 1983. Biochem. J. 209: 719-724.
68. Mack, D. O., Wolfensberger, M., Girardot, J. M., Miller, J. A. and Johnson, B. C., 1979. J. Biol. Chem. 254: 2656-2664.
69. Vermeer, C. and Ulrich, M., 1982. Thrombos. Res. 28: 171-178.
70. Bell, R. G., 1980. Arch. Biochem. Biophys. 203: 58-64.
71. Lian, J. B. and Friedman, P. A., 1978. J. Biol. Chem. 253: 6623-6626.
72. Poser, J. W. and Price, P. A., 1979. J. Biol. Chem. 254: 431-436.
73. Sixty Plus Reinfarction Group, 1980. Lancet II: 989-994.
74. Friedman, P. A. Hauschka, P. V., Shia, M. A. and Wallace, J. K., 1979. Biochim. Biophys. Acta 583: 261-265.
75. Shah, D. V., Nyari, L. Y., Swanson, J. C. and Suttie, J. W., 1980. Thrombos. Res. 19: 111-118.
76. Graves, C. B., Munns, T. W., Carlisle, T. L., Grant, G. A. and Strauss, A. W., 1981. Proc. Natl. Acad. Sci. U.S.A. 78: 9063-9068.
77. Hauschka, P. V. and Reid, M. L., 1978. J. Biol. Chem. 253: 9063-9068.
78. Poser, J. W., Esch, F. S., Ling, N. C. and Price, P. A., 1980. J. Biol. Chem. 255: 8685-8691.
79. Price, P. A., Lothringer, J. W., Bankol, S. A. and Reddi, A. H., 1981.
80. Lian, J. B., Glimcher, M. J., Roufosse, A. H., Hauschka, P. V., Gallop, P. M., Cohen-Solal, L. and Reit, B., 1982. J. Biol. Chem. 257: 4999-5003.
81. Hall, J. G., Pauli, R. and Wilson, K., 1980. Am. J. Med. 68: 122-140.
82. Price, P. A., Williamson, M. K., Haba, T., Dell, R. B. and Jee, W. S. S., 1982. Proc. Natl. Acad. Sci. U.S.A. 79: 7734-7738.
83. Tomita, A., 1971. Clin. Endocrinol. 19: 731-736.
84. Levy, R. J., Lian, J. B. and Gallop, P., 1979. Biochem. Biophys. Res. Commun. 91: 41-49.
85. Lian, J. B., Prien, Jr., E. L., Glimcher, M. J. and Gallop, P. M., 1977. J. Clin. Invest. 59: 1151-1157.
86. Müller-Esterl, W. and Fritz, H., 1981. Meth. Enzymol. 80: 621-632.
87. Rich, D. H., Kawai, M., Goodman, H. L., Engelke, J. and Suttie, J. W., 1983. FEBS Lett. 152: 79-82.

Received 14 June 1983.