Vitamin K Dependent Formation of Factor VII by a Cell-Free System from Rat Liver*

Bernard M. Babior† and Ruby S. Kipnes

ABSTRACT: Incubation of cell-free rat liver homogenates in the presence of Ca^{2+} and Earle's base (a mixture of salts) results in the appearance of factor VII activity in the reaction mixture. The appearance of factor VII activity is not due to an effect of the reaction mixture *per se* on the clotting assay, nor to activation of preformed clotting factors during the incubation, but appears to represent the formation of new clotting factor during the course of the incubation. Homogenates obtained from rats given warfarin fail to generate factor VII activity; vitamin K restores the ability of the homogenate to form the clotting factor if it is given *in vivo*, but not *in vitro*. The factor VII forming system is located in the soluble fraction of the homogenate (the 105,000*g* supernatant).

Vitamin K has long been known to be necessary for the production of a variety of clotting factors by the liver (Dam, 1966). In 1966, it was shown that the vitamin K dependent production of a number of these clotting factors, including prothrombin and factors VII and X, is not affected by inhibitors of protein synthesis (Babior, 1966; Olson *et al.*, 1966). This result has been interpreted as indicating that the vitamin is involved in the conversion of a preformed polypeptide precursor into the active clotting factor. Although this interpretation has not been universally accepted (Suttie, 1967), more recent experiments have tended to support it (Lowenthal and Birnbaum, 1969; Hill *et al.*, 1968; Ranhotra and Johnson, 1969; Suttie, 1969).

Apart from some recent work with warfarin-resistant rats which has implicated ribosomes as a possible site of action of vitamin K (Suttie, 1969), little additional information has been provided concerning the mechanism of action of vitamin K in clotting factor synthesis. This paucity of new information probably reflects the experimental difficulties involved in obtaining biochemical data from a whole-cell system. Because of these difficulties, a cell-free system was developed in which the vitamin K dependent synthesis of factor VII could be studied more easily.

Materials and Methods

Biochemicals, including ATP, reduced glutathione, and phenazine methosulfate were obtained from Sigma. Alhydrox (Cutter) for the adsorption of factors II, VII, IX, and X from normal human plasma was the generous gift of Dr. R. H. Aster. Tissue culture media and components were obtained from Grand Island Biologicals. Other reagents were the best grade commercially available, and were used without further purification.

Livers for homogenization were obtained from 150- to 180-g male Charles River rats. Where indicated, the rats were poisoned 18 hr prior to sacrifice by the intraperitoneal injection of warfarin at a dose of 2.5 mg/100 g. The rats were anesthetized and the livers freed of blood by perfusion as previously described (Babior, 1966), using as the initial perfusate 30 ml of warm saline (0.154 M) and as the final perfusate 20 ml of either cold tissue culture medium of the type used in the incubation (Figures 2 and 3) or cold saline (all other experiments). To prepare the homogenate, a mixture of minced liver and 0.25 M sucrose (1:9 w/v) was ground at 0° in a Potter–Elvehjem homogenizer with a Teflon pestle. Whole cells and nuclei were then removed by a 5-min centrifugation at 900*g*. For experiments with subcellular fractions, mitochondria, microsomes, and soluble supernatant were prepared by a modification of the method of Hogeboom (Hogeboom, 1955) in which the homogenate was centrifuged for 10 min at 2400*g* to precipitate mitochondria, after which microsomes were spun down by a 1-hr centrifugation at 105,000*g*.

Factor VII was assayed by a one-stage method based on the correction of the coagulation defect of plasma from a factor VII deficient patient (Pechet, 1964). The material to be assayed (either plasma or reaction mixture) was diluted with an equal volume of 0.25 M imidazole·HCl buffer (pH 7.3). This mixture (50 μl) was then placed in a small polyethylene vessel together with 25 μl of citrated factor VII deficient human plasma and 25 μl of citrated normal human plasma which had been adsorbed for 5 min at room temperature with 0.1 volume of Alhydrox. This mixture was brought to 37°, and coagulation was initiated by the addition of 0.2 ml of a 1:1 mixture of tissue thromboplastin (Dade) and 0.02 M $CaCl_2$. The time from the addition of thromboplastin to the formation of a clot (the "factor VII time") was measured automatically with a fibrometer (Baltimore Biological Laboratories). A standard curve, prepared by measuring factor VII levels in dilutions of citrated normal rat plasma, is shown in Figure 1. The factor VII content of normal rat plasma was arbitrarily set at 100 units/ml. In the results reported below,

* From the Thorndike Memorial Laboratory, Harvard Medical Unit, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02118. *Received January 7, 1970.* This research was supported in part by Public Health Service Grants AM-09115, FR-0076, and AM-5413.

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TABLE I: Effect of Homogenates[a] on the Factor VII Assay.

Expt	Warfarin	Incubation Time (hr)[a]	Factor VII Time (sec) Saline	Reaction Mixture + Saline	Plasma + Saline	Reaction Mixture + Plasma
1	−	0	40.7	36.2	16.4	16.4
		3		32.5		15.0
2	−	0	37.0	37.4	15.5	15.7
		3		33.2		15.4
3	+	0	38.2	36.7	16.7	16.7
		3		36.2		16.5
4	+	0	39.7	40.5	18.2	17.5
		3		37.5		17.5

[a] Homogenates were prepared as described in Materials and Methods, using livers from 2 normal rats and from 2 warfarin-treated rats. Reaction mixtures contained 0.5 ml of homogenate, 0.5 ml of tissue culture medium (Gibco Medium 199), 2 μmoles of $CaCl_2$, 0.2 μmole of phenazine methosulfate, 2 μmoles of ATP, and 1.6 μmoles of reduced glutathione in a total volume of 1.5 ml. Incubations were conducted at 37° for the times noted. The reactions were terminated by chilling in melting ice. Factor VII levels were then determined on 0.05-ml aliquots of the following: 0.077 M NaCl; a mixture of equal volumes of reaction mixture and 0.154 M NaCl; a mixture of equal volumes of citrated rat plasma and 0.154 M NaCl; and a mixture of equal volumes of reaction mixture and citrated rat plasma.

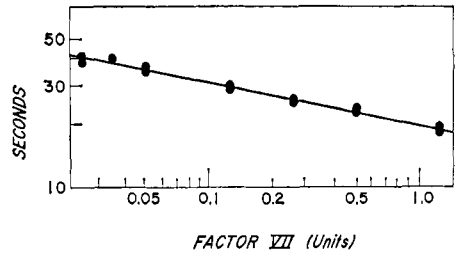

FIGURE 1: Standard curve for the factor VII assay.

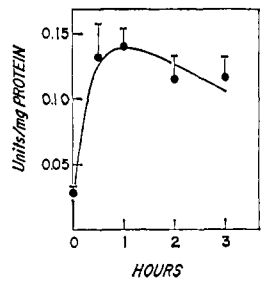

FIGURE 2: Appearance of factor VII activity in a cell-free homogenate. The reaction mixtures contained 0.5 ml of homogenate, 0.5 ml of tissue culture medium (Gibco Medium 199), 2 μmoles of $CaCl_2$, 0.2 μmole of phenazine methosulfate, 2 μmoles of ATP, and 1.5 μmoles of reduced glutathione in a total volume of 1.5 ml. Incubations were conducted at 37° for the times noted. The reactions were then terminated by chilling in melting ice and factor VII activity was assayed immediately as described in the text. The figure shows the mean + 1 SE for 8 homogenates.

the factor VII level represents the experimental factor VII level minus the factor VII level of a blank in which the material to be assayed was replaced by 0.154 M saline.

Protein was assayed by the biuret method (Layne, 1957), using bovine serum albumin as standard. The protein concentration of the whole homogenates varied between 15 and 35 mg per ml.

Figures 2 and 3 show results obtained with unselected homogenates. In other experiments, results are reported for those homogenates which showed substantial factor VII forming ability in appropriate control incubations. This selection resulted in the elimination of about 25% of all homogenates tested.

Results

Vitamin K Dependent Generation of Factor VII Activity by a Cell-Free Liver Homogenate. When a cell-free homogenate from the liver of a normal weanling rat is incubated under the conditions described, factor VII activity appears in the reaction mixture (Figure 2). The activity reaches a maximum between 0.5 and 1 hr after the start of the reaction, and by 3 hr has begun to decline. On the average, the maximum value represents a fivefold increase over the initial level.

The results presented in Table I show that this increase in factor VII activity is not due to a nonspecific effect of the homogenate on the assay. In these experiments, the factor VII content of plasma from normal rats was determined after 1:1 dilution with either saline or incubation mixture. The homogenates used in the incubations were obtained from both normal and warfarinized rats, and each homogenate was tested both before and after a 3-hr incubation under the conditions described in Figure 2. The results show that in each case, the factor VII time of the mixture of plasma and incubation mixture is about equal to the factor VII time of the saline-diluted plasma. Thus, there does not appear to be either an activator or an inhibitor of the factor VII assay in any of the reaction mixtures, either before or after incubation.

The appearance of factor VII activity is completely abolished if the rat from which the liver is obtained is poisoned with warfarin 18 hr prior to sacrifice (Figure 3). However, administration of vitamin K *in vivo* will restore the factor VII forming capacity of homogenates from such warfarin-treated rats. In the group of animals treated with vitamin K, plasma factor VII levels at the time of sacrifice averaged 30% of normal (±5% SE). Nevertheless, the quantities of factor VII in the incubation mixtures were greater than the quantities found at the corresponding times in incubations with homogenate from normal rats (*cf.* Figure 2). In contrast, inhibition of factor VII formation was not reversed by the addition of vitamin K directly to reaction mixtures containing homogenates from warfarinized rats.

Some Properties of the Factor VII Forming System. In most of the foregoing experiments, the reaction mixtures contained several constituents which might be expected to be necessary for the vitamin K dependent formation of factor VII, including ATP, glutathione, and phenazine methosulfate, an

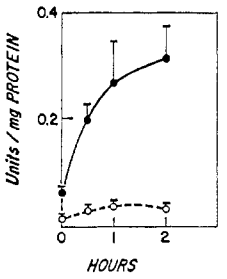

FIGURE 3: Vitamin K dependence of factor VII formation. Homogenates were prepared from livers obtained from rats which had been poisoned with warfarin as described in the text. Where noted, the animals were given vitamin K (Aquamephyton, 2 mg by tail vein) 2 hr before killing. In experiments with animals which had not been given vitamin K, the reaction mixtures contained 0.5 ml of homogenate, 0.5 ml of Eagle's medium, 2 μmoles of $CaCl_2$, 0.2 μmole of phenazine methosulfate, 2 μmoles of ATP, and 1.6 μmoles of reduced glutathione in a total volume of 1.5 ml. Phenazine methosulfate, ATP, and glutathione were omitted from reaction mixtures containing homogenates from the vitamin K treated animals, and the volume of these reaction mixtures was reduced to 1.2 ml; subsequent results show that these modifications have no significant effect on the formation of factor VII. The experiments were conducted as described in Figure 2. The mean + 1 SE is shown for 6 homogenates from animals which had received no vitamin K, and for 3 homogenates from animals which had been treated with vitamin K.

electron-transport dye. The results of Table II show that these constituents are in fact not required for the formation of factor VII, since their omission from the incubation mixture had no effect on the reaction. However, both calcium ion and tissue culture medium are required for the reaction, since elimination of the latter significantly decreased the formation of the clotting factor, while omission of the former abolished it completely (see Table III).

Investigations of the effect of the various components of tissue culture medium on the appearance of factor VII activity

TABLE III: Requirements for Calcium and for Various Components of Eagle's Medium for Factor VII Formation.[a]

Omission	Factor VII (units/mg of protein)
None	0.123 ± 0.028
Earle's base[b]	0.069 ± 0.022
Amino acids	0.113 ± 0.033
Vitamins	0.137 ± 0.029
$CaCl_2$[c]	0.027 ± 0.013

[a] The reaction mixtures contained 0.5 ml of homogenate, 0.5 ml of Eagle's medium, and 2 μmoles of $CaCl_2$, with omissions as noted, in a total volume of 1.2 ml. Incubations were conducted for 0.5 hr at 37°. The reactions were terminated by chilling in melting ice, and factor VII activity was assayed immediately as described in the text. For each homogenate, an initial factor VII level was obtained from a complete incubation mixture prepared at 0° immediately before assay. The table shows the mean ± 1 SE for 5 homogenates. The values shown represent the observed values minus the mean initial factor VII level (mean ± 1 SE of initial levels = 0.029 ± 0.006). [b] Earle's base is the mixture of salts that comprises one component of Eagle's medium. [c] For this experiment, $CaCl_2$ was also omitted from Earle's base.

TABLE II: Requirements for Factor VII Formation.[a]

Omission	Factor VII (units/mg of protein)
None	0.120 ± 0.052
Eagle's medium	0 ± 0.020
ATP	0.108 ± 0.044
Glutathione	0.136 ± 0.036
Phenazine methosulfate	0.126 ± 0.036

[a] The reaction mixtures contained 0.5 ml of homogenate, 0.5 mg of Eagle's medium, 2 μmoles of $CaCl_2$, 0.2 μmole of phenazine methosulfate, 2 μmoles of ATP, and 1.6 μmoles of reduced glutathione, with omissions as noted, in a total volume of 1.5 ml. Incubations were conducted for 0.5 hr at 37°. The reactions were terminated by chilling in melting ice, and factor VII activity was assayed immediately as described in the text. For each homogenate, an initial factor VII level was obtained from a complete incubation mixture prepared at 0° immediately before assay. The table shows the mean ± 1 SE for 4 homogenates. The values shown represent the observed values minus the mean initial factor VII level (mean ± 1 SE of initial levels = 0.058 ± 0.012).

TABLE IV: Requirements for Various Ions for Factor VII Formation.[a]

Omission	Factor VII (units/mg of protein)
None	0.056 ± 0.009
NaCl	0.065 ± 0.013
KCl	0.069 ± 0.011
NaH_2PO_4	0.080 ± 0.003
$MgSO_4$	0.065 ± 0.013
$MgCl_2$	0.072 ± 0.008
$MgSO_4$ + $MgCl_2$	0.045 ± 0.018
$CaCl_2$	0.031 ± 0.009

[a] The reaction mixtures contained 0.5 ml of homogenate, 0.5 ml of Earle's base, and 2 μmoles of $CaCl_2$, with omissions as noted, in a total volume of 1.2 ml. Incubations were conducted for 0.5 hr at 37°. The reactions were terminated by chilling in melting ice, and factor VII activity was assayed immediately as described in the text. For each homogenate, an initial factor VII level was obtained from a complete incubation mixture prepared at 0° immediately before assay. The table shows the mean ± 1 SE for 3 homogenates. The values shown represent the observed values minus the mean initial factor VII level (mean ± 1 SE of initial levels = 0.025 ± 0.003).

TABLE V: Distribution of Factor VII Forming Activity by Subcellular Fraction.[a]

Fraction	Factor VII (units/mg of homogenate protein)								
	1	2	3	4	5	6	7	8	9
Homogenate	0.15	0.25	0.25	0.27	0.23	0.13	0.25	0.23	0.19
Mitochondria	0	0.01	0			0.01	0	0.02	0.02
9000*g* supernatant	0.16	0.21	0.25	0.30	0.30				
Microsomes				0.09	0	0.01	0.04	0.04	0.04
Soluble supernatant				0.14	0.24	0.09	0.21	0.20	0.05
Microsomes + soluble supernatant				0.21	0.20				

[a] Fractions were prepared from a cell-free rat liver homogenate as described in the text. The pellet from the 9000*g* centrifugation was suspended in a quantity of 0.25 M sucrose equal to the original volume of the cell-free homogenate from which the pellet was obtained. The 105,000*g* pellet was suspended in a quantity of 0.25 M sucrose equal to 0.2 times the volume of the 9000*g* supernatant from which the pellet was obtained. Except as noted below, reaction mixtures contained 0.5 ml of homogenate or cell fraction, 0.5 ml of Eagle's medium, and 2 μmoles of $CaCl_2$ in a total volume of 1.2 ml. In the incubations containing the 105,000*g* pellet, only 0.1 ml of the tissue fraction was used, adding 0.4 ml of 0.25 M sucrose to make the composition of these reaction mixtures comparable with that of the rest. The incubations with the 105,000*g* pellet plus the 105,000*g* supernatant contained 0.1 ml of the former and 0.5 ml of the latter, bringing the final volumes of these mixtures to 1.3 ml. Incubations were conducted for 0.5 hr at 37°. The reactions were terminated by chilling in melting ice, and factor VII activity was assayed immediately as described in the text. Initial factor VII levels were also obtained from each reaction mixture. The values shown in the table represent the difference between the final and initial factor VII levels.

are shown in Table III. For these experiments, Eagle's medium was used, since this could be obtained commercially in the form of separate components consisting of vitamins, salts, and amino acids, each of which could be omitted individually from the reaction mixture. It should be noted that in addition to the Eagle's salts, the incubation mixtures were also supplemented with $CaCl_2$. The complete Eagle's medium was found to be as effective as Gibco Medium 199 in promoting the formation of factor VII. Omission of either vitamins or amino acids had no effect on the reaction. Upon omitting the salt mixture, however, there was a small but consistent fall in the amount of factor VII activity appearing in 0.5 hr. There is only an insignificant fall in factor VII formation upon omitting the individual components of the salt mixture (Table IV), suggesting that the fall in activity observed when all the salts are omitted may reflect an effect of ionic strength rather than a requirement for a specific ion.

Table III shows that there was a striking fall in factor VII formation when $CaCl_2$ was omitted. The dependence of clotting factor production on calcium ion concentration is shown in Figure 4. These results indicate that there is an optimum calcium concentration for the formation of factor VII. This concentration is in the vicinity of 1 mM. In the presence of either greater or lesser concentrations of Ca^{2+}, clotting factor formation declines.

Further characteristics of the reaction are presented in Figure 5 and Table V. With regard to the time course of factor VII appearance, previous experiments have indicated that production frequently stops after 0.5 hr and has almost invariably stopped after 1 hr (*cf.* Figure 2). However, the data of Figure 5 show that for the first 0.5 hr the amount of factor VII in the reaction mixture increases at an approximately constant rate, and in addition, that the amount of factor VII present at the end of a 0.5-hr incubation is roughly proportional to the concentration of protein in the incubation mixture. Finally, the experiments reported in Table VI indicate that neither mitochondria or microsomes are required for the formation of factor VII. All of the clotting factor forming ability was found to reside in the 105,000*g* supernatant.

Activation of Factor VII during the Incubation. The assay for factor VII depends on the shortening of the factor VII time of a factor VII deficient system by the material being tested. This time could be shortened not only by the formation of new clotting factor during the incubation, but also by the activa-

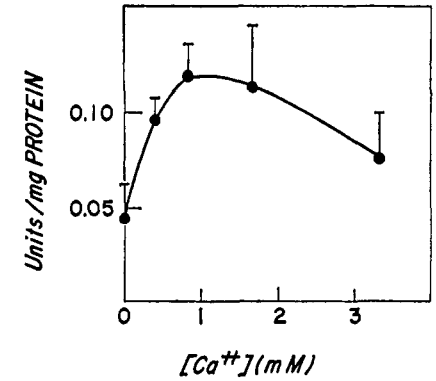

FIGURE 4: Effect of Ca^{2+} concentration on the formation of factor VII. The reaction mixtures contained 0.5 ml of homogenate, 0.5 ml of Eagle's medium (modified by the omission of $CaCl_2$ from the salts), and $CaCl_2$ at the concentrations noted, in a volume of 1.2 ml. Incubations were conducted for 0.5 hr at 37°. The reactions were terminated by chilling in melting ice, and factor VII activity was assayed immediately as described in the text. For each homogenate, an initial factor VII level was obtained from an incubation mixture ($CaCl_2$ concentration 1.7 mM) prepared at 0° immediately before assay.

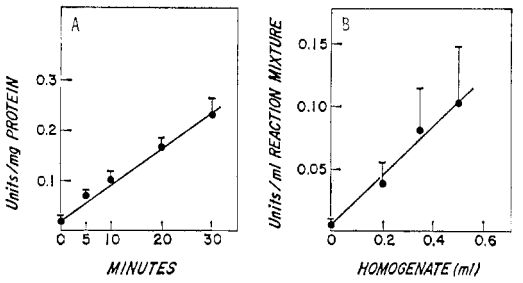

FIGURE 5: Factor VII formation. (A) As a function of time. The reaction mixtures contained 0.5 ml of homogenate, 0.5 ml of Eagle's medium, and 2 μmoles of $CaCl_2$ in a total volume of 1.2 ml. Incubations were conducted at 37° for the times noted. The reactions were terminated and factor VII was determined as described in Figure 4. The figure shows the mean + 1 SE for 3 homogenates. (B) Factor VII formation as a function of protein concentration. The reaction mixtures contained homogenate as shown, plus sufficient 0.25 M sucrose to bring the volume of homogenate plus sucrose to 0.5 ml, 0.5 ml of Eagle's medium, and 2 μmoles of $CaCl_2$ in a total volume of 1.2 ml. Incubations were conducted as described in Figure 4. Initial levels were obtained as described in Figure 4, using incubations containing 0.5 ml of homogenate. The figure shows the mean + 1 SE for 3 homogenates. The values shown represent the observed value minus (mean initial level times the volume of homogenate/0.5). The mean ± 1 SE of the initial levels = 0.007 ± 0.001.

tion during the incubation of clotting factors already present in the reaction mixture.

In determining the extent to which such activation of preexisting clotting factors was responsible for the observed shortening of factor VII times, advantage was taken of the fact that tissue thromboplastin is only necessary for the first step of the extrinsic coagulation cascade, namely, the activation of factor VII (Biggs and MacFarlane, 1962; Williams, 1964; Nemerson, 1966) while the remainder of the cascade requires only Ca^{2+} and phospholipid (Biggs and MacFarlane, 1962; Nemerson, 1966; Jobin and Esnouf, 1967). Activation of clotting factors during the incubation could therefore be determined by beginning the coagulation assay with calcium plus phospholipid, rather than with calcium plus tissue thromboplastin (see Materials and Methods), since the presence of activated clotting factors would lead to a shortening of the clotting time in the absence of tissue thromboplastin. In Table VI is shown a series of experiments with soluble supernatant in which the appearance of factor VII as determined by the usual assay is compared with the "factor VII appearance" according to the assay using calcium plus phospholipid. It is apparent that only a small fraction of the factor VII activity present in the reaction mixture at the end of the incubation can be accounted for by the activation of preformed clotting factors.

TABLE VI: Activation of Factor VII during the Course of the Incubation.[a]

Expt	Factor VII (units/mg of homogenate protein)	
	Calcium + Phospholipid	Calcium + Thromboplastin
6	0.02	0.09
7	0.04	0.21
8	0.04	0.20
9	<0.01	0.05

[a] Factor VII levels were determined on the 105,000g supernatants from expt 6–9 of the preceding table, using calcium plus phospholipid [Inosthin (Associated Concentrates)] rather than calcium plus thromboplastin to initiate coagulation. The phospholipid emulsion was prepared by sonicating a suspension of 2 g of inosthin in 100 ml of distilled water at maximum power for 5 min at room temperature, using a Branson Model W140D cell disruptor. The final concentration of phospholipid present in the assay mixture was 110 μg/ml. A slight prolongation of the factor VII time was observed when the concentration of phospholipid was either doubled or halved, indicating that the amount used in the experiment was optimal. The figures in the table represent the difference between the final and initial factor VII levels.

Discussion

Homogenates from the livers of young rats appear to carry out the vitamin K dependent formation of factor VII. Since the vitamin K dependent step has previously been shown to be unaffected by inhibitors of protein synthesis, the factor VII formed in the present experiments is presumably being generated by the modification of a polypeptide precursor already present in the liver.

In addition to vitamin K, this process, like other processes connected with coagulation, requires calcium. The requirement for calcium in the formation of vitamin K dependent clotting factors had previously been reported by Prydz (1964). Although the incubation mixtures in which factor VII formation took place contained both tissue thromboplastin (present in the liver homogenate) and Ca^{2+}, and therefore were capable of activating clotting factors, two lines of evidence indicate that the observed appearance of factor VII represented formation from a precursor rather than activation of preexisting clotting factor: (a) the fact that the activity appears in the 105,000g supernatant suggests that its appearance is due to formation of new clotting factor, rather than activation of preexisting clotting factor, since tissue thromboplastin, which is associated with microsomes (Williams, 1964; Nemerson, 1966), is absent from the 105,000g supernatant, and (b) the discrepancy between the factor VII time obtained with calcium plus phospholipid and that obtained with calcium plus thromboplastin indicates that activation of clotting factor accounted for only a small fraction of the factor VII appearing during the course of the incubation. The latter observation also constitutes evidence against the possibility that shortening of the factor VII time was due to nonspecific activation of VII by proteolytic enzymes present in the soluble supernatant (Gordon *et al.*, 1969; Barnhart, 1960).

Although vitamin K was able to restore clotting factor synthesizing ability to liver homogenates from warfarinized rats when it was administered *in vivo*, it could not do so when added directly to the incubation mixture. This result may be due to difficulty in dissolving vitamin K in the aqueous reac-

tion mixture, even though the vitamin was added as an emulsion. On the other hand, it might reflect the possibility that the vitamin is not active as such, but may need to be converted into another species before it can fulfill its role in clotting factor synthesis. The formation of the active species from an inactive precursor is seen with other fat-soluble vitamins, including vitamin A (Olson, 1968) and vitamin D (Blunt *et al.*, 1968). As evidence for the existence of a modified form of vitamin K, Lev and Milford have isolated from pig liver a compound not identical with any known vitamin K which is capable of supporting the growth of a vitamin K requiring microorganism (Lev and Milford, 1966). It is conceivable that the failure of vitamin K to restore clotting factor synthesizing activity when added directly to the homogenate is attributable to failure of conversion of the vitamin into the active form under the conditions of the incubation.

One unexpected result of this investigation was the finding that the clotting factor forming activity resided entirely in the soluble fraction of the homogenate. It might be anticipated that the microsomal fraction would be necessary for the formation of factor VII, since this fraction is rich in lipid and vitamin K is a highly lipid-soluble substance. However, this fraction also contains tissue thromboplastin, and since the entire extrinsic clotting system, including fibrinogen, prothrombin, and factors V, VII, and X is made in the liver, the formation of factor VII at a location remote from the microsomes might be necessary to prevent intracellular fibrin formation from taking place.

Acknowledgment

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