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EFFECT OF WARFARIN ON FACTOR VII FORMATION BY A CELL-FREE SYSTEM FROM RAT LIVER*

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Abstract—A study was conducted on the effect of warfarin on factor VII formation by a cell-free system from rat liver. Administration of warfarin to the rat from which the liver is obtained results in a marked decrease in factor VII formation by the liver homogenate. The rate of fall of factor VII formation was considerably more rapid than the rate of disappearance of factor VII from warfarin-treated rats.

Restoration of factor VII formation upon the administration of vitamin K to warfarinized rats was also investigated. Factor VII was not made by homogenates prepared from livers removed from the rat immediately after administration of vitamin K, although slices prepared from such livers were able to make the clotting factor. However, homogenates prepared from livers taken a half hour or longer after giving vitamin K were able to make factor VII. These results suggest that vitamin K must be modified before it can participate in clotting factor formation, and that under the condition of these experiments this modification can take place in slices but not in homogenates.

SEVERAL clotting factors made in the liver require vitamin K for their formation.[1] The vitamin appears to play a role in the conversion of a polypeptide precursor to the active clotting factor.[2–7] To facilitate the study of this process, a cell-free system was developed in which the vitamin K dependent formation of factor VII takes place.[8] In the present paper, studies are reported on the inhibition of this factor VII forming system by warfarin.

MATERIALS AND METHODS

Tissue culture medium (MEM Eagles) was obtained from Grand Island Biologicals. Alhydrox for the absorption of factors II, VII, IX and X was purchased from Cutter Laboratories (Berkeley). Warfarin (Coumadin) was obtained from Endo Laboratories, Garden City, N.Y. Vitamin K_1 (Aquamephyton) was purchased from Merck. Other reagents were the best grade commercially available and were used without further purification.

Livers were obtained from 150–200 g male Sprague–Dawley rats purchased from the Charles River Breeding Laboratories, Wilmington, Mass. The rats were anesthetized and the livers freed of blood by perfusion as previously described.[8] Slices, prepared with a Stadie–Riggs microtome, were incubated according to the

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technique of Babior.[2] Each incubation contained 1 liver slice together with 1·4 ml tissue culture medium and 0·4 ml 0·01 M $CaCl_2$. Incubations were terminated by homogenizing the contents of the incubation vessel (liver slice plus tissue culture medium) at 0° in a glass Potter–Elvehjem homogenizer with a Teflon pestle. The factor VII content of the resulting homogenate was determined by the method previously reported[8] and protein concentration was determined by the biuret method.[9] Preparation, incubation and assay of homogenates were performed according to the method of Babior and Kipnes.[8]

RESULTS

The formation of factor VII by liver homogenates from rats treated with warfarin is shown in Fig. 1. On the X-axis is shown the time interval between the administration of warfarin and the removal of the liver from the animal, while the Y-axis indicates the amount of factor VII made by the homogenate in a half hour. Inhibition of factor VII formation is seen to develop rapidly after the administration of warfarin.

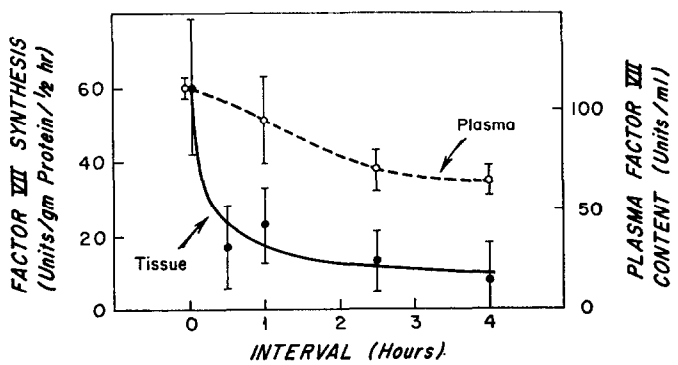

FIG. 1. Inhibition of factor VII formation by warfarin as a function of time. Factor VII formation (●—●). Rats were initially poisoned with warfarin at a dose of 2·5 mg/100 g, given by tail vein. After the time interval noted in the figure, the livers were removed from the rats and homogenates prepared as described in Materials and Methods. From each homogenate, two incubation mixtures were prepared, each containing 0·5 ml homogenate, 0·5 ml tissue culture medium and 0·2 ml 0·01 M $CaCl_2$. One mixture was immediately assayed for factor VII, while the other was incubated for a half hour at 37° prior to factor VII assay. The difference between the factor VII levels of the two incubation mixtures represents factor VII formation by the homogenate. Each point in the figure shows the mean ± 1 S.E. for four homogenates. Factor VII levels in the plasma (○—○). Rats were poisoned with warfarin as described above. At the times noted, 1·8 ml of blood was drawn by cardiac puncture into a syringe containing 0·2 ml of 3·8% sodium citrate. Cells were removed by centrifugation and the plasma was diluted with 9 vol. of 0·154 M NaCl. The diluted plasma was mixed with an equal volume of imidazole buffer,[8] and the factor VII content of 50μl of this mixture was determined as previously described. Each point represents the mean ± S.E. for plasma from at least four animals.

Factor VII levels in the plasma of warfarin-treated rats are also plotted as a function of time. The rate of disappearance of factor VII from the plasma is somewhat slower than the rate previously reported for factor VII in the rat.[10] The prothrombin time, a coagulation assay which tests prothrombin and factors V and X in addition to factor VII, followed a similar course. It is noteworthy that at 1 hr, when the factor VII level of blood had only fallen by 13 per cent, factor VII formation by liver tissue was already inhibited to the extent of 70 per cent.

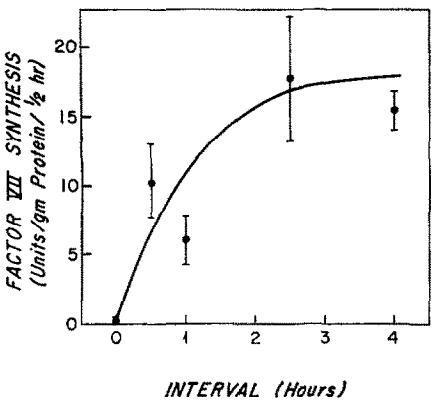

FIG. 2. Restoration of factor VII formation in liver homogenates from warfarin-treated rats after administration of vitamin K. The experiments were conducted as described in the text. The dose of warfarin was 2·5 mg/100 g given intraperitoneally, that of vitamin K was 2 mg, given either by intracardiac injection or by tail vein. Homogenates were prepared as described in Fig. 1.

In Fig. 2 is shown the rate at which factor VII formation by liver homogenates from warfarin-treated rats is restored by vitamin K administration. In these experiments, vitamin K was administered to rats which had received warfarin 16–20 hr previously. The livers were removed from the animals at various time intervals after the administration of vitamin K, and homogenates were prepared and tested for VII-forming activity. The results show that essentially no VII-forming activity is present in homogenates from livers removed immediately after injecting vitamin K (the time between the injection of vitamin K and the perfusion of the liver with saline was about 5 min) but that by a half hour after administration of vitamin K significant activity appears. The activity continues to rise slowly over the next hour or two.

In contrast to this are the results obtained with slices (Fig. 3). In these experiments,

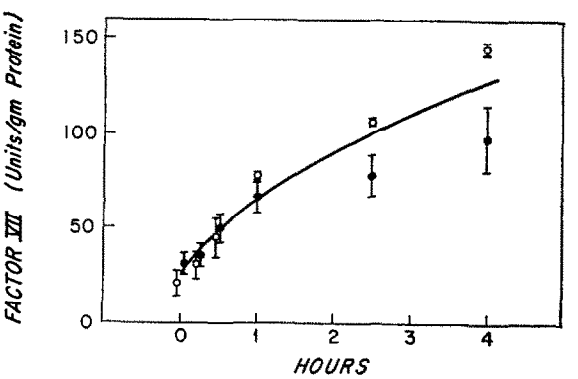

FIG. 3. Factor VII formation by liver slices from warfarin-treated rats after administration of vitamin K. Where indicated, rats were treated with warfarin and vitamin K as described in the text. Doses of warfarin and vitamin K were as given in Fig. 2. Slices prepared from each liver were incubated for 0, 0·25, 0·5, 1, 2·5 and 4 hr. Incubations were then terminated and the incubation mixtures assayed for protein and factor VII as described in Materials and Methods. ●—●, rats treated with warfarin and vitamin K; ○—○, untreated rats.

too, vitamin K was administered to rats which had received warfarin 16–20 hr previously. However, all livers were removed "immediately" after giving vitamin K (Fig. 2). When formation of factor VII by slices from these livers was compared with factor VII formation by liver slices from untreated rats, essentially no difference was observed.* This result may be compared with the results of Fig. 2, in which homogenates from livers obtained immediately after administration of vitamin K are unable to make factor VII.

DISCUSSION

The determination of factor VII depends upon a shortening of the coagulation time of a factor VII deficient system by the material to be assayed. In principle, a shortening of the coagulation time of the test system by the incubation mixture containing the liver homogenate could be due either to the appearance of new factor VII in the incubation mixture, or to the activation of factor VII or other clotting factors already present in the homogenate prior to incubation. Evidence has been presented previously indicating that the former interpretation is correct—that is, that new factor VII appears during the incubation.[8] This conclusion is supported by the present observation that factor VII formation is inhibited by warfarin to the extent of 70 per cent at a time when the vitamin K dependent clotting factors are only depressed to the extent of 13 per cent. If the shortening of the coagulation time was due to the activation of pre-existing clotting factors, it would be surprising to find such an extensive inhibition of "factor VII formation" as measured by this assay system in the presence of these relatively high levels of clotting factors.

The experiments dealing with the restoration of factor VII formation by the administration of vitamin K to warfarin-treated rats are of interest in connection with the possibility that vitamin K has to be modified in some way before it can participate in clotting factor formation. There are several lines of evidence bearing on this possibility. In 1966, Lev and Milford[11] isolated from pig liver a compound not identical with any known vitamin K which was able to support the growth of a vitamin K-requiring microorganism. More recently, it was found that over half the label recovered from the factor VII synthesizing fraction[8] of liver homogenates prepared from rats treated with radioactive vitamin K was present in a form other than the vitamin.[12] In addition, Ranhotra and Johnson[6] have demonstrated that puromycin given *in vivo* to K-defined rats blocks the effect of vitamin K on clotting factor synthesis in these animals even though it has no effect *in vitro*. This result suggests that before it can function in clotting factor formation vitamin K must be modified by a process which is dependent on protein synthesis. The lag observed in the vitamin K dependent restoration of factor VII formation by liver homogenates from warfarin-treated rats (Fig. 2) provides further support for this possibility. That this lag is not merely due to a failure of uptake of vitamin K by the liver at the earliest time point is indicated by the results observed with liver slices obtained from similarly treated rats. The fact that these slices were fully capable of factor VII formation even though they were prepared from livers taken "immediately" after vitamin K administration implies that vitamin K was taken up by the liver in the interval between the administration of the vitamin and the removal of the organ from the animal. There would appear then to be some modification of

* Liver slices from animals treated with warfarin alone fail to make factor VII.[2]

vitamin K which can take place in the whole liver or the liver slice, but not in the cell free homogenate, and which is necessary for the participation of the vitamin in factor VII formation.

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