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Plasma Abnormal Prothrombin and Microsomal Prothrombin Precursor in Various Species[1] (38492)

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The only generally accepted function of vitamin K in higher animals is that of regulating the synthesis of prothrombin (factor II) and three other plasma clotting factors (VII, IX, and X). There is evidence to suggest (1) that the vitamin is involved in the conversion of a liver precursor protein to prothrombin. An abnormal, biologically inactive, form of prothrombin appears in the plasma of cows treated with the vitamin K antagonist dicoumarol (2). This protein may represent the excretion of an incompletely modified precursor, and it has been shown (3, 4) that the major difference between this protein and prothrombin is in its failure to adsorb to insoluble barium salts, or to bind calcium ions in solution. This appears to be due (5) to the lack of a specific calcium binding prosthetic group on the abnormal protein. This protein will however yield thrombin when treated with specific snake venoms.

We have shown (6) that a protein with similar properties increases in the liver of rats given coumarin anticoagulants and have postulated that this protein represents the liver precursor to prothrombin. Some of the properties of this protein have been described (7, 8). As substantial amounts of an abnormal plasma prothrombin have been identified only in bovine and human (9–11) plasma, we have surveyed a number of species for both plasma abnormal prothrombin and liver precursor activity.

Methods. Young adult male animals from the following sources were used: rats, Holtzman & Co., Madison, WI; mice, Rolfsmayer & Co., Madison, WI; guinea pigs, O'Brien, Oregon, WI; rabbits, Sand Valley Farms, Spring Green, WI; hamsters, A. R. Schmidt, Madison, WI; calves, healthy mongrel dogs and Ancona chickens were obtained from the University of Wisconsin Animal Sciences or laboratory animal facilities. Sodium warfarin was administered intraperitoneally in 0.9% NaCl and 2-chloro-3-phytyl-1,4-naphthoquinone (Chloro-K) was emulsified in Tween 80 in 0.9% NaCl for intracardial injections.

All animals were fasted for 18 hr before sacrifice. Blood obtained by cardiac or venipuncture was drawn into a syringe containing 0.15 *M* potassium oxalate (10% of final volume) and centrifuged for 20 min at 2000 *g* to obtain plasma. Livers were removed, washed twice, minced, and homogenized in 5 vol of 0.25 *M* sucrose in a glass-Teflon homogenizer at 1500 rpm for 30 sec. The homogenate was centrifuged for 15 min at 12,800 *g*, and the resulting supernatant was centrifuged for 60 min at 105,000 *g*. The microsomal pellet was surface washed twice, suspended with a Dounce homogenizer in 1.5 vol of nine parts calcium-free Krebs–Ringer bicarbonate buffer, pH 7.2, and one part of 0.15 *M* potassium oxalate, and 2/7 vol of 1% Triton X-100 in the same buffer was added. This suspension was stirred for 15 min and centrifuged for 45 min at 105,000 *g* to yield the microsomal extract which was assayed. All steps were carried out at 0–4°.

Plasma prothrombin concentrations were measured by the two-stage method of Ware and Seegers as modified by Shapiro and Waugh (12). A standard dilution curve for each species was prepared using a one to 30 diluted pooled control plasma and the prothrombin concentrations following anticoagulant treatment were expressed as a percent of this control plasma. *Echis carinatus* venom (Sigma Chemical Co.) was used (13) to generate thrombin-like activity in plasma samples as follows: To 0.1 ml of 5% acacia (0.9% NaCl, 7.5 m*M* in $CaCl_2$, 42

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TABLE I. PLASMA ABNORMAL PROTHROMBIN AND LIVER PROTHROMBIN PRECURSOR IN ANTICOAGULANT TREATED ANIMALS.

Species	Plasma clotting activity (% of control)[a]			Microsomal precursor[d] (NIH units/g liver)
	Two-stage prothrombin[b]	Venom generated activity		
		Before $BaSO_4$	After $BaSO_4$	
Rat (6)	11 ± 1	23 ± 2	5 ± 1	14.5 ± 0.6
Cow (2)	24	96	84	<3[e]
Mouse (6)	20 ± 1	33 ± 1	17 ± 1	4.8 ± 0.2
Hamster (7)	17 ± 1	23 ± 2	<5	4.2 ± 0.4
Guinea pig (6)	14 ± 1	11 ± 1	<5	4.5 ± 0.3
Rabbit (5)	14 ± 1[c]	12 ± 4	<5	15.6 ± 2.0
Dog (4)	16 ± 2	13 ± 2	<5	5.0 ± 0.2
Chick (6)	14 ± 1	60 ± 4	32 ± 3	11.3 ± 0.9

[a] Assays described in Methods section.

[b] The desired degree of anticoagulation was produced in the various species in the following manner: Rat, 5 mg/kg Na warfarin ip, 18 hr; mouse, Na warfarin 5 mg/kg ip, 20 hr; rabbit, 20 mg/kg Na warfarin ip each day for 5 days; guinea pig, 20 mg/kg Na warfarin ip, 36 hr; dog, 10 mg/kg warfarin in feed on day 1 and 3, killed on day 4; chick, 0.2% Na warfarin in drinking water for 36 hr; cow, 1.85 mg/kg dicoumarol in feed each day for 7 days; hamster, 5 mg/kg Chloro K ic, 24 hr.

[c] The response of rabbits to warfarin is variable, and only those rabbits which had prothrombin levels between 10 and 20% were selected for study.

[d] Precursor activity is defined as thrombin units generated from $BaSO_4$ adsorbed microsomal preparations following a 15 min incubation with *Echis carinatus* venom.

[e] A clotting time of 70 sec was equivalent to 3 thrombin units/g of liver, and values in excess of this were not of sufficient reproducibility to calculate a thrombin concentration. Actual clotting times observed for the cow preparations were in excess of 120 sec. All values are mean ± S.E. for the number of animals in parentheses.

m*M* in pH 7.2 imidazole buffer) in a Fibrometer cup were added 0.1 ml of fibrinogen (1% clottable protein in 25 m*M* pH 7.2 imidazole buffer) and 0.1 ml diluted plasma. Clotting time after the addition of 0.1 ml of 0.25 mg/ml *Echis carinatus* venom was measured with a Fibrometer Coagulation Timer and the result expressed as percent of control values. All reagents were at 37°. Thrombin activity generated in liver microsomal extracts by incubation with *Echis carinatus* venom (precursor activity) was measured as previously described (7). Plasma and microsomal extracts were adsorbed with $BaSO_4$ by carefully mixing the samples with 35 mg/ml of citrate-washed $BaSO_4$ six times during 1 hr at 0°; the $BaSO_4$ was removed by centrifugation.

Results. Plasma prothrombin levels following anticoagulant treatment (Table I) ranged from 10 to 25% of control values. The level of thrombin-like activity generated in plasma by *Echis carinatus* venom markedly exceeded prothrombin levels in the cow; a substantial excess was seen in the chick and a slight excess in the mouse and rat. This increase should be one measure of the presence of an abnormal prothrombin in the plasma, and a second indication should be the amount of thrombin generating material which remains after barium salt adsorption. After adsorption with $BaSO_4$, substantial thrombin was generated by *Echis carinatus* treatment in cow plasma, somewhat less in chick, and a small amount in mouse plasma. Negligible amounts were seen in the other plasmas studied. In all cases, $BaSO_4$ adsorption of control (non-anticoagulant treated) plasmas removed all measurable *Echis carinatus* generated thrombin activity.

The precursor activity in liver microsomal extracts was measured in treated and control animals both before and after $BaSO_4$ adsorption of the extracts. The values for $BaSO_4$-adsorbed extracts are also reported in Table I. In control animals, liver precursor activity was measurable (greater than 3

NIHU/g liver, less than 70 sec clotting time) only in rat liver preparations. With the exception of the cow, treated animals showed elevated levels of liver precursor activity. Rats, rabbits, and chicks had markedly elevated liver precursor concentrations, while the other species had levels near 5 NIHU/g. The microsomal extracts were also assayed with venom prior to $BaSO_4$ adsorption (data not shown) and the percentage of liver microsomal precursor adsorbed to $BaSO_4$ was found to be 30–40% in rabbits, and 0–15% in other species. Two-stage assays of $BaSO_4$ treated microsomal extracts from all species showed less than 1.5 NIHU/g prothrombin present (data not included).

Previous studies (6) indicated that the precursor activity of $BaSO_4$-adsorbed rat liver microsomal extracts developed within minutes of incubation with *Echis carinatus* venom, and remained essentially constant thereafter. This was not found to be the case for the other species studied. Development of thrombin activity in $BaSO_4$-adsorbed microsomal extracts during 3-hr incubations with *Echis carinatus* venom is shown in Fig. 1. Mouse and hamster activity increased during this period to two to three times the activity seen at 15 min; dog activity increased until 2 hr and then decreased; chick and rabbit activity rapidly decayed during the first 0.5 hr, while guinea pig activity rose and then fell. The thrombin inhibitor hirudin (14) was found to block the clotting activity present in microsomal extracts from the various species tested. In each case the preparation was incubated until maximum activity was generated and then the addition of hirudin was shown to completely block the clotting activity.

Postmicrosomal supernates from both treated and control livers were also assayed for precursor activity. No measurable activity was found in the rat, cow, mouse, hamster, or dog. Some activity (10 unit/g liver) was found in postmicrosomal supernates from guinea pigs, and most of this appeared to be due to two-stage assayable prothrombin. Both control and treated chicks showed some activity in this cellular fraction, as did treated rabbits.

Discussion. These data indicate that in

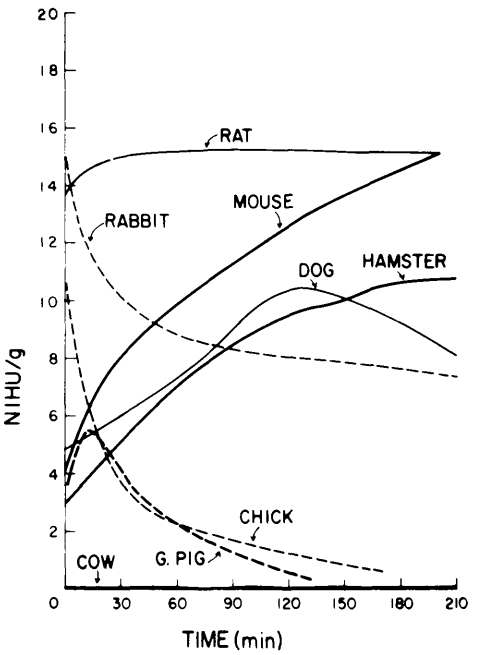

FIG. 1. Effect of period of incubation on apparent microsomal precursor activity. Pooled, $BaSO_4$ adsorbed microsomal preparation from at least three animals for each species were incubated with *Echis carinatus* venom as described. Samples were taken following 1, 5, 10, 15, 30, 45, 60, 90, 120, 150, and 180 min. Activity did not develop in control microsomal preps even after long incubations.

contrast to the bovine and human, most species do not respond to anticoagulant treatment by producing and excreting an abnormal prothrombin into the plasma. In this study only the anticoagulant treated chick and mouse had a significant amount of an abnormal prothrombin when it was measured as the amount of non-barium salt adsorbable protein which would yield thrombin when treated with *Echis carinatus* venom. The amount found was, however, much less than that seen in the bovine. A second indication of the amount of abnormal prothrombin was obtained by comparing the amount of thrombin generated by the two-stage assay (physiological activators) and that generated from venom incubation. There was general agreement between the two methods of measuring this amount of this material. A major exception was in the case of chick plasma where there was a difference of about 45% of normal prothrombin

concentrations between the two-stage and venom assay, but only about 30% when measured by venom incubation after barium salt adsorption. This would imply that in the case of the chick there is a certain amount of barium salt adsorbable prothrombin which cannot be activated by the reagents of the two-stage assay. This would be consistent with some reports (15, 16) of the properties of bovine and human material. The failure to find a significant amount of an abnormal plasma prothrombin in the rat in this study would contradict earlier reports (17, 18) of its existence, but would be consistent with the failure of Morrissey *et al.* (19) to find such a protein.

The assays of microsomal preparations indicated that there was a significant amount of precursor in all species studied except the cow. Although the data in the table show large increases only in the case of the rat, rabbit, and chick, a comparison with the incubation time courses given in Fig. 1 suggests that this may be misleading. The data in Table I are for standard 15 min incubations with venom done for purposes of comparison between species. The time courses of activation shown in Fig. 1 suggest that these values underestimate the precursor activity in all cases but that of rat and guinea pig. Extrapolation of the values in Table I to an incubation time giving maximum activity yields 15–20 units/g liver for chick and rabbit, and 10–15 units/g for mouse, hamster and dog liver. The only species not having significant precursor activity is therefore the bovine. This may be related to the large amount of abnormal prothrombin which appears in this species, and would suggest that in this species the precursor is readily excreted, perhaps in a modified form, as an abnormal prothrombin rather than building up in the liver. Assays of human liver preparations would be of interest to determine if this is a general phenomenon. The basis for the widely differing rate of thrombin generation from the liver preparations upon incubation with *Echis carinatus* venom is not known. The differences may reflect either differing rates of activation by the procoagulant in the venom or they may reflect different stability of the thrombin generated to the conditions of incubation. In any event, the activity was in all cases inhibited by the specific thrombin inhibitor hirudin.

The lack of a consistent detection of a significant amount of precursor activity in the post microsomal supernate does not necessarily mean that some of the precursor does not end up in this fraction during the isolation procedure. The presence of a potent inhibitor of thrombin activity in this fraction (20) may result in a severe underestimation of thrombin activity.

These data are consistent with the general theory of action of the vitamin which has been gained from the study of the rat and the bovine. In species other than the cow, it appears that, as in the rat, a prothrombin precursor protein which cannot be activated by physiological activators and which does not adsorb to barium salts builds up in liver microsomes when anticoagulants are administered. There does not appear to be significantly more of this protein in other species than in the rat, and our efforts (7) to purify and more completely characterize this protein will continue with the rat microsomal material.

Summary. Abnormal, biologically inactive forms of prothrombin have previously been shown to appear in the plasma of cows or humans given coumarin anticoagulants. We have previously shown that a protein with similar properties increases in the liver of rats given these vitamin K antagonists, and have postulated that this protein represents the liver precursor to plasma prothrombin. Eight species, rats, mice, guinea pigs, hamsters, rabbits, calves, dogs, and chickens, have now been surveyed for both plasma abnormal prothrombin and liver precursor activity. Large amounts of plasma abnormal prothrombin were found in the bovine, substantial amounts were seen in the chick, and small amounts in rat and mouse plasma. With the exception of the bovine, all anticoagulant treated animals showed elevated levels of liver precursor activity in microsomal preparations. The relationship of these observations to the mechanism of action of vitamin K is discussed.

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