VITAMIN K AND THE BIOSYNTHESIS OF THE GLYCOPROTEIN PROTHROMBIN

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SUMMARY

Vitamin K_1 administration to vitamin K deficient rats did not increase the incorporation of labeled amino acids into electrophoretically pure active prothrombin, but did increase markedly the incorporation of labeled glucosamine and labeled mannose into prothrombin. Another protein absorbable on $BaSO_4$, but which increases in vitamin K deficiency (or with warfarin treatment), showed very much less effect of vitamin K_1 treatment on carbohydrate incorporation.

It appears that the site of action of vitamin K in prothrombin formation is at the point of carbohydrate attachment to the waiting polypeptide chain.

The site of action of vitamin K in the formation of the vitamin K dependent clotting proteins has been shown not to be at a transcription step (1,2) nor probably at a peptide bond forming step (2,3,4,5,6), although this is disputed (7,8).

We have reported the occurrence of a protein which, opposite to prothrombin, increases in the blood in vitamin K deficiency (9), and which may be similar to the "modified thrombin zymogen" reported by Tishkoff et al. (10).

Several recent reports have appeared on the existance of isoprenoid glycolipids (11,12,13), which may be carbohydrate donors. Since prothrombin contains approximately 11% carbohydrate (14), a potential site of vitamin K function is in the attachment of carbohydrate (15).

In this communication we would like to report evidence for the existance of the vitamin K deficiency (or warfarin) related blood protein, and on the vitamin K dependence of glucosamine and mannose incorporation into the prothrombin molecule.

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MATERIALS AND METHODS

Male Sprague-Dawley rats were made severely hypoprothrombinemic as previously described (2). Control, normal rats were given the same diet supplemented with 50 μg of water soluble vitamin K (menadione sodium bisulfite) per 100 grams diet.

L-amino acid-^{3}H-(G) mixture, 1 mCi/0.173 mg, was purchased from New England Nuclear Corp., Boston, Mass., D-mannose-UL-^{14}C(180 mCi/mM) and D-glucosamine-^{3}H-GL(1.9 Ci/mM) from Tracerlab, Irvine, Calif. Vitamin K_1 (Nutritional Biochemical Co., Cleveland, Ohio) was solubilized before use with Tween 80 as previously described (2).

Amino acid and carbohydrate incorporation. All experimental rats (deficient and normal) were given intravenously (femoral vein) 20 μCi of labeled compound per 200-250 g rat 90 min before blood was taken by heart puncture. Half of the deficient rats were given additionally 1 mg vitamin K_1 (in 0.9% NaCl) per rat intracardially, immediately after administration of the radioactive compound.

Oxalated blood was centrifuged and plasma from each experimental group of rats was collected separately. Prothrombin and protein X were quantitatively adsorbed from the plasma on $BaSO_4$. Both proteins were eluted from the washed $BaSO_4$ adsorbates with 0.17 M sodium citrate and resolved electrophoretically on large polyacrylamide gels, 12 cm long by 1.4 cm in diameter.

Disc gel electrophoresis was performed with a Canalco apparatus, using the instructions for the tris-glycine buffer system of Davis (16). Sections of the gel corresponding to prothrombin and protein X, as visualized in stained reference gels, were cut out of the gels previously quickly frozen in dry-ice acetone and the proteins were then eluted electrophoretically from each section. The eluates were collected, centrifuged and dialyzed against water. Protein content of each solution was determined by the Lowry procedure (17), purity by analytical disc gel electrophoresis and prothrombin activity by the two-stage assay (18), modified to permit the use of a Fibrometer

(B-D Laboratories Inc., Baltimore, Md.) for electronic determination of the instant of clotting. The eluates from $BaSO_4$ adsorbates of plasma proteins give somewhat higher prothrombin values than are found in the original blood plasma, due to elimination of anti-thrombin by the adsorption-elution procedure. Radioactivity measurements were made in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Fig. 1 shows a typical protein pattern for the $BaSO_4$ eluates derived from plasma of vitamin K deficient animals, vitamin K normal animals and warfarin treated animals. One can readily observe the marked decrease in the amount of prothrombin with vitamin K deficiency or warfarin treatment, and the concomi-

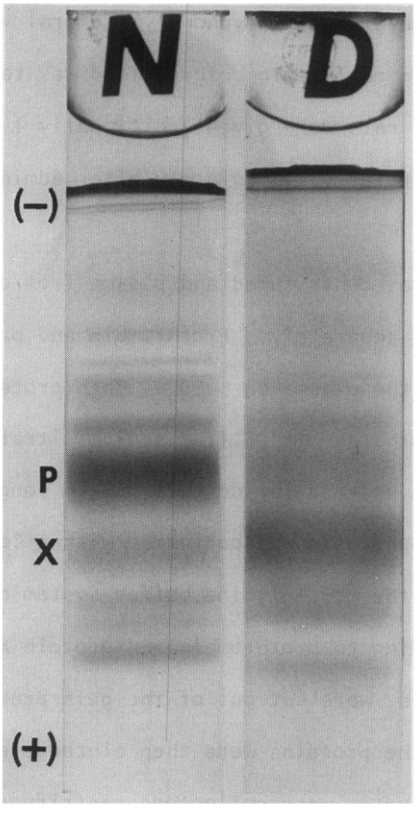

FIG. 1
Representative gels of protein eluates from $BaSO_4$ adsorbates obtained from plasma of normal (N) and nutritionally vitamin K deficient (D) rats. The proteins were resolved electrophoretically on large (12 cm long by 1.4 cm in diameter) polyacrylamide gels. The ratio of prothrombin (P) to protein X (X) changes inversely with vitamin K status of the animals.

tant increase in another protein (protein X) which migrates just ahead of prothrombin on the gels.

Since in earlier work (6) prothrombin and protein X appeared as one band on the gels and were counted together for the amino acids incorporation and because of the contradictory reports of Olson and coworkers (7,8) indicating that vitamin K functions in peptide bond formation, it was particularly important to repeat the amino acid incorporation experiments. These data reported in Table 1 show that the specific radioactivity incorporated into prothrombin isolated from the plasma of vitamin K normal animals, vitamin K deficient animals and vitamin K deficient animals treated with vitamin K_1 90 min before blood was withdrawn, are the same.

In contrast, both the amount of amino acid incorporation into protein X and its specific radioactivity were markedly increased in vitamin K deficiency.

In Table 2 are given the data for glucosamine and mannose incorporation into prothrombin and protein X of normal animals, deficient animals and deficient animals which received vitamin K_1. A great increase in both glucosamine (5 fold) and mannose (9 fold) incorporation into deficient prothrombin following treatment with vitamin K_1 is evident. In the case of mannose a marked decrease in specific radioactivity of prothrombin derived from vitamin K deficient animals, as compared to normal prothrombin, can be seen.

On the other hand, the specific radioactivity of protein X increases in the deficient animals as compared to normal animals in the case of both glucosamine and mannose incorporation. While the specific radioactivity appears to have doubled in the case of glucosamine incorporation into protein X between the deficient and the deficient treated with vitamin K_1, there was no increase in the case of mannose incorporation under the similar experimental conditions.

DISCUSSION

From the disc gel electrophoretic patterns of $BaSO_4$ adsorbable plasma proteins, shown in Figs. 1 and 2, it is obvious that vitamin K deficiency or warfarin treatment, both very drastically reduced the amount of prothrombin in

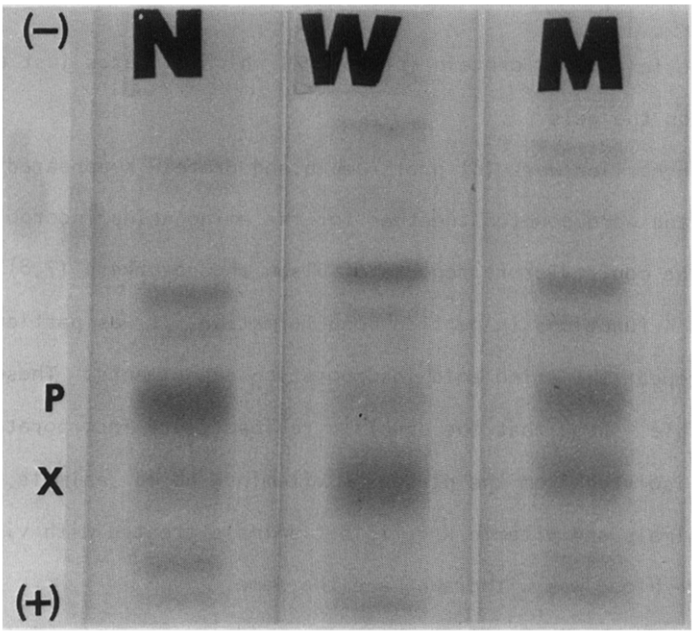

FIG. 2

Representative gels of protein eluates from $BaSO_4$ adsorbates obtained from plasma of normal rats (N) and warfarin treated rats (W), and a gel of a mixture (M) of half normal eluate and half warfarin eluate. Warfarin treated rats were given intraperitoneally 1 mg of sodium warfarin per rat (250 g body weight) 20 hours before blood was taken by heart puncture. The proteins were resolved electrophoretically on small (7.5 cm long by 0.6 cm in diameter) analytical gels. The protein pattern of gel W is similar to that of gel D, Fig. 1.

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the plasma, thus the effect of the deficiency is not only a reduction in prothrombin activity but also a reduction in level of this specific glycoprotein.

From these figures it is also apparent that there is another vitamin K related protein (band X), which migrates on the gel just ahead of prothrombin. This protein, however, increases in vitamin K deficiency or after warfarin treatment. The inverse relationship of this protein to prothrombin makes it interesting to speculate, that this may be an "unfinished" - a "precursor" protein of prothrombin, which spills over into the blood when the vitamin K dependent step in prothrombin formation is blocked. Perhaps this is the "modified zymogen" protein of Tishoff et al. (10).

From Table 1 it can be seen that this protein X turns over at an increased rate, leading to a higher specific activity, as well as a greater amount in the plasma with vitamin K deficiency. In contrast, the labeling of proth-

TABLE I

^{3}H-Amino Acids Incorporation

Vitamin K status	Specific Radioactivity							
	Prothrombin				Protein X			
	cpm/mg protein		average	ratio	cpm/mg protein		average	ratio
Normal	526	665	596	1	954	1186	1070	1
Deficient	628	730	679	1.1	2077	1646	1861	1.7
Deficient given Vitamin K	538	748	643	1.1	1017	1286	1151	1.1

Legend for Table 1.

The underlined data are the averages of two experiments. In each experiment 10 nutritionally vitamin K deficient rats and 4 control normal rats were used. All the rats were given intravenously a mixture of ^{3}H-amino acids (20 μCi per rat) 90 min before blood was taken by heart puncture. Five of the deficient rats received additionally vitamin K_1 (1 mg per rat intracardially) immediately after administration of the labeled amino acids. Prothrombin and protein X were purified electrophoretically, as described under "methods", from the combined plasma of each experimental group of rats.

TABLE II

CHO Incorporation

Labeled carbohydrate used	Vitamin K status	Specific Radioactivity			
		Prothrombin		Protein X	
		cpm/mg protein	ratio	cpm/mg protein	ratio
^{3}H-Glucosamine	Normal	1892	1	7712	1
	Deficient[2]	1713	0.9	12144	1.6
	Deficient given Vitamin K	8833	4.7	25624	3.3
^{14}C-Mannose	Normal[2]	1698	1	3470	1
	Deficient[2]	630	0.37	6786	2.0
	Deficient given Vitamin K	5414	3.2	7000	2.0

[2] Averages of two experiments are given.

Legend for Table 2.

Eight nutritionally vitamin K deficient rats and four control normal rats were given intravenously ^{3}H-glucosamine or ^{14}C-mannose (20 μCi per rat) 90 min before blood was taken by heart puncture. Four of the deficient rats received additionally vitamin K_1 (1 mg/per rat intracardially) immediately after injection of the labeled carbohydrate. Prothrombin and protein X were purified electrophoretically, as described under "methods from combined plasma of each experimental group of rats. In the repeated glucosamine experiment, eight vitamin K deficient rats received ^{3}H-glucosamine. In the repeated mannose experiment four normal and four vitamin K deficient rats were given ^{14}C-mannose as described previously.

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rombin by amino acids essentially is unaffected by either vitamin K deficiency, or upon recovery of prothrombin following vitamin K treatment. This indicates clearly that vitamin K is not involved in the biosynthesis of the polypeptide chain of prothrombin.

On the other hand, Table II shows the great increase in glucosamine and in mannose incorporation into prothrombin of deficient animals following vitamin K treatment, which indicates that the site of function of vitamin K is the step in the addition of the first carbohydrate molecule to the polypeptide chain. Magnusson (14) has shown that sequence of the carbohydrate side chains of prothrombin is glucosamine-mannose, etc.

It has been reported (19) that ribosomal attachment is required for the addition of the first sugar of the carbohydrate side chains, i.e. the glucosamine moiety. Our data on the effects of high level of puromycin and cycloheximide (2), as well as the statement of Suttie (15), indicate that disruption of ribosomes blocks the site of action of vitamin K. Previous amino acids incorporation data (6), and more critically the present data, indicate that these results were not due to the blocking of amino acid incorporation, but to blocking of carbohydrate attachment. Bernacki and Bosmann (20) have reported that both, protein synthesis and glycoprotein synthesis, are accelerated in isolated rat liver mitochondria and certain others subcellular fractions by both vitamin K_1 and by warfarin. Since warfarin blocks prothrombin formation, while vitamin K is required for it, the relationship between this report and the present data is questionable.

We have found, however, and will report later, that as in the cases of retinol (12) and dolicol (13), a glycolipid can be formed enzymatically from vitamin K_1 and GDP-mannose. The finding that at least 20 min *in vivo* pretreatment of vitamin K deficient rats with vitamin K_1 was required for subsequent *in vitro* factor VII synthesis in isolated liver cells (4) suggest the probability of the formation of a functional derivative of vitamin K, which may be the vitamin K_1 glycolipid.

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