Vitamin K and the Synthesis of Factors VII–X by Isolated Rat Liver Cells (34248)

G. S. RANHOTRA AND B. CONNOR JOHNSON

University of Oklahoma, School of Medicine, Biochemistry Department and Oklahoma Medical Research Foundation, Biochemistry Section, Oklahoma City, Oklahoma 73104

Vitamin K functions to maintain the plasma level of the clotting proteins—prothrombin and factors VII, IX and X. Factor VII activity is formed by isolated perfused rat liver (1–2), by liver slices (3, 4) and by isolated rat liver cells (5, 6) when adequate vitamin K is present in the animals from which the preparations are made. However, such liver preparations from vitamin K-deficient rats or rats given a vitamin K antagonist, fail to elaborate factor VII activity. These findings, along with the immunofluorescence studies of Barnhart and Anderson (7, 8) have conclusively demonstrated that the production of factor VII and other vitamin K-dependent clotting proteins occurs in response to vitamin K. However, the site in the production of those proteins at which vitamin K functions, still remains elusive. The evidence so far suggests that vitamin K with a regulatory protein, is involved subsequent to the formation of specific messenger RNA (1–3, 9). This paper presents further definition of the probable site of action of vitamin K in the synthesis of factors VII–X using isolated rat liver cells.

Materials and Methods. Male, 120–150 g Sprague-Dawley rats, housed in coprophagy-prevention cages (10) and fed a vitamin K-deficient diet (11) became hypoprothrombinemic (Quick prothrombin time (12), over 80 sec) in about 1 week. Control normal rats (prothrombin time, 15 sec) were housed in regular cages and fed the same diet but containing 50 μg of vitamin K_3/100 g of diet.

Liver cell suspensions were prepared by sacrificing rats and perfusing livers immediately (via the portal vein) with cold 0.027 *M* sodium citrate in calcium-free Locke's solution (13). Pressure during perfusion, which took 3–5 min, was gradually increased till the liver was well blanched and moderately distended. Cells from the excised liver were then dispersed by the method of Jacob and Bhargava (14) using Tris–KCl buffer (15), pH 7.4. Cells were counted on a hemocytometer (100 magnification) and examined for their structural integrity (430 magnification). Respiratory activity of the cells was measured in the Warburg respirometer (16) in an air atmosphere.

Three to four million cells suspended in 1 ml of Tris–KCl buffer (containing 1.25 m*M* $CaCl_2$) were incubated at 37° in a Dubnoff shaker in an atmosphere of 95% O_2 and 5% CO_2. At various intervals during incubation, 0.1 ml of cell suspension was removed, diluted with 0.9 ml of cold dilution fluid (6), centrifuged (1000 *g*/10 min at 4°) and the supernatant was immediately assayed for factor VII–X activities by the method of Pechet (17) using charcoal filtered bovine plasma (18) and commercial thromboplastin preparation (Simplastin, Warner-Chilcott). Results are expressed in units (U). A standard curve for factor VII–X activities was obtained with serially diluted pooled plasma from four normal rats. One ml of citrated plasma was assigned 100 U activity. Cycloheximide (Upjohn), puromycin (Nutritional Biochemical) and warfarin (Endo) when used were added at the start of the incubation, pH being always adjusted to 7.4. One mg of vitamin K_1 (Aquamephyton, Merck) was administered, intracardially, to K-deficient rats 25 min prior to sacrificing them for preparation of liver cells which requires about 15 min. Some K-deficient rats were given puromycin (30 mg/100 g of body wt) intraperitoneally simultaneously with injection of vitamin K_1.

Results. Respiratory activity of liver cell

TABLE I. Respiratory Activity of Rat Liver Cells in Suspension.[a]

Rat no.		Oxygen uptake (μl/10^6 cells)			
	(min):	0–30	30–60	60–90	90–120
Normal rats					
1		7.6	7.2	7.6	5.5
2		6.4	7.7	6.1	5.4
3		9.2	7.9	8.6	5.6
K-deficient rats					
4		6.5	6.7	8.5	3.9
5		7.8	8.5	7.3	3.1

[a] Incubation mixture contained 6.5×10^6 to 11×10^6 cells in 2 ml of Tris–KCl buffer. Reactions were carried out at 37° after equilibrating flasks (110 oscillations/min) for 5 min. Results represent average of duplicate determinations.

suspensions from normal and from K-deficient rats was significant and it stayed so for about 90 min (Table I). From then onwards, some decline in oxygen uptake was observed in both the cell preparations.

In preliminary studies, rat liver cells were found not to release measurable prothrombin activity into the incubation medium in agreement with Pyrdz (6). The studies were thus directed toward formation of factors VII–X. When liver cells from normal rats were incubated, factor VII activity appeared in the medium (Table II), however, liver cells from vitamin K-deficient rats failed to produce such activity and the addition of vitamin K_1 to these cells was without effect. Administration of vitamin K to deficient rats 25 min prior to sacrifice resulted in the formation of factor VII activity in their isolated liver cells and at a rate similar to that of cells from normal rats (Tables II, III). Concomitant with the decline in the respiratory activity of liver cells, factor VII–X activities ceased to increase beyond 1.5-hr incubation.

Cycloheximide and puromycin, known inhibitors of protein synthesis, were added to cell suspensions at levels high enough to block protein synthesis (1, 3, 9, 19). The response of liver cells was then studied in the presence of these inhibitors. Whether or not these inhibitors were present, liver cells from vitamin K-deficient rats failed to elaborate factor VII–X activities. The "activity" in such liver preparations, however, appeared rapidly when vitamin K was administered *in vivo* and neither cycloheximide (1 mg/ml) nor puromycin (5 mg/ml) added *in vitro*, blocked the formation of factor VII–X activities (Table III).

On the contrary, *in vivo* administration of puromycin (30 mg/100 g of rat wt) at a level sufficiently high (9) to block protein synthesis, inhibited the response of isolated K-deficient liver cells to vitamin K administered to rats simultaneously with puromycin (Table IV). Warfarin, a vitamin K antagonist, added to liver cell suspensions from normal rats, greatly inhibited the formation of

TABLE II. Production of Factor VII–X Activities by Rat Liver Cells in Suspension.[a]

Treatment	No. of rats		Factor VII (U/10^6 cells)		
		(min):	0–40	0–80	0–120
1. Normal rats	4		1.9 ± 0.2	3.9 ± 0.3	3.8 ± 0.3
2. K-deficient rats (vitamin K injected)	4		2.9 ± 0.3	5.2 ± 0.8	5.1 ± 0.9
3. K-deficient rats (vitamin K added *in vitro*)	3				
Control (no vitamin)			0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.3
Vitamin added			0.5 ± 0.0	0.4 ± 0.1	0.6 ± 0.1
+ cycloheximide			0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.3
+ puromycin			0.1 ± 0.1	0.4 ± 0.1	0.5 ± 0.3

[a] Incubation mixture contained 3×10^6 to 4×10^6 cells in 1 ml of Tris–KCl buffer and where indicated vitamin K_1, 1 mg; cycloheximide, 1 mg; and puromycin, 5 mg.

TABLE III. Effect of Inhibitors on the Production of Factor VII–X Activities by Rat Liver Cells in Suspension.[a]

Treatment	No. of rats	(min):	Factors VII–X (U/10^6 cells) 0–30	0–60	0–90	0–120
K-deficient rats	4					
Control			1.8 ± 0.1	3.7 ± 0.4	4.2 ± 0.4	3.7 ± 0.4
Cycloheximide			1.8 ± 0.1	3.7 ± 0.4	4.3 ± 0.7	4.4 ± 0.6
Puromycin			2.2 ± 0.2	4.3 ± 0.6	4.7 ± 0.7	4.8 ± 0.6
Normal rats	4					
Control			2.0 ± 0.2	3.7 ± 0.2	5.2 ± 0.5	3.9 ± 0.2
Cycloheximide			1.2 ± 0.3	4.3 ± 0.3	5.0 ± 0.0	4.2 ± 0.2
Puromycin			1.8 ± 0.4	3.8 ± 0.3	4.2 ± 0.1	4.3 ± 0.2

[a] Incubation mixture contained 3×10^6 to 4×10^6 cells in 1 ml of Tris–KCl buffer and where indicated cycloheximide, 1 mg; and puromycin, 5 mg. Vitamin K-deficient rats were given vitamin K_1 intracardially 25 min prior to sacrifice.

factor VII–X activities upon incubation, (Table V) but at the same time also significantly interfered with the uptake of oxygen by such liver preparations (Table VI).

Discussion. Liver cells in suspension appeared structurally intact and maintained a high rate of respiration. They were thus considered functional and were used to gain understanding of the site of vitamin K action.

Vitamin K in the formation of factor VII and other vitamin K-dependent clotting proteins, may intervene at one of many possible sites; at the genetic level, in the formation of nascent peptide precursor, in its release from the ribosomes, in the activation of precursor or even in the transport of active factor out of cells. The last possibility does not seem likely since Barnhart and Anderson (7, 8) using a fluorescent antibody technique observed a very rapid appearance of prothrombin in liver cells of Dicumarol-treated dogs when vitamin K was administered. That vitamin K is also not involved at the genetic level has been demonstrated adequately in studies with intact animals (9), with isolated perfused liver (1), and with liver slices (22) using actinomycin D, a known inhibitor of RNA synthesis. Failure of cycloheximide to block vitamin K-induced factor VII–X synthesis in isolated liver cells (Table III) and prothrombin synthesis in intact animals (9) seems to suggest that vitamin K functions subsequent to the cycloheximide block. Cycloheximide is reported to inhibit the transfer of charged tRNA from site A to site P on the ribosome (20). In contrast to the findings of Suttie (1) and Prydz (6) but in agreement with those of Babior (3), Olson *et al.* (2) and Lowenthal and Birnbaum (21), puromycin did not inhibit the formation of factor VII–X activity by isolated rat liver cells (Table III).

The inhibition of factor VII–X syntheses in isolated liver cells by puromycin given to the intact vitamin K-deficient animal simultaneously with vitamin K indicates that

TABLE IV. Effect of Administration of Puromycin *in Vivo* on Vitamin K-Induced Synthesis of Factors VII–X by Liver Cells in Suspension (K-deficient rats).[a]

Treatment	No. of rats	(min):	Factors VII–X (U/10^6 cells) 0–30	0–60	0–90	0–120
No puromycin	4		1.8 ± 0.1	3.7 ± 0.4	4.2 ± 0.4	3.7 ± 0.4
Puromycin	3		0.7 ± 0.4	1.2 ± 0.3	1.1 ± 0.4	1.1 ± 0.3

[a] Incubation mixture contained 3×10^6 to 4×10^6 cells in 1 ml of Tris–KCl buffer. Puromycin (30 mg/100 g of body wt) was administered simultaneously with vitamin K_1.

TABLE V. Effect of Warfarin on the Production of Factor VII–X Activities by Rat Liver Cells in Suspension (3 normal rats).[a]

Treatment (min):	Factors VII–X (U/10^6 cells)			
	0–30	0–60	0–90	0–120
Warfarin	1.9 ± 0.3	2.7 ± 0.3	3.6 ± 0.8	4.1 ± 0.1
Control	2.0 ± 0.2	4.6 ± 0.4	5.4 ± 0.4	5.8 ± 0.4

[a] Incubation mixture contained 3 × 10^6 to 5 × 10^6 cells in 1 ml of Tris–KCl buffer and where indicated warfarin 0.75 mg/10^6 cells.

TABLE VI. Effect of Warfarin on Respiratory Activity of Rat Liver Cells in Suspension (normal rats).[a]

Rat no.	Treatment (min):	Oxygen uptake (µl/10^6 cells)			
		0–30	30–60	60–90	90–120
1	Warfarin	3.8	3.5	2.1	1.3
	Control	7.4	7.9	7.4	6.4
2	Warfarin	3.7	3.1	2.0	1.1
	Control	6.2	7.7	7.7	3.1
3	Warfarin	1.9	1.8	2.0	1.9
	Control	7.2	8.9	7.9	6.6

[a] Incubation mixture contained 8 × 10^6 to 9 × 10^6 cells in 2 ml of Tris–KCl buffer and where indicated, warfarin 0.75 mg/10^6 cells.

formation of factors VII–X is in some way dependent on synthesis of some protein although continued vitamin K stimulated formation of factors VII–X in the isolated liver cells continues in the presence of puromycin. This requirement for protein synthesis may involve synthesis of a vitamin K-containing enzyme which is unstable in the absence of vitamin K and which with vitamin K functions in the "activation" of factors VII–X. It appears that the formation of factor VII–X activities elaborated by liver cells in suspension is not dependent on protein synthesis. The inhibition of factor VII–X activities by warfarin added to isolated liver cells cannot be attributed to its specific effect on formation of factors VII–X since it also adversely affects the respiratory activity of liver cells and thus blocks all protein synthesis.

Summary. Isolated liver cells from normal rats and from vitamin K-deficient rats injected with vitamin K continued to produce factor VII–X activities *in vitro* for at least 1.5 hr while cells from K-deficient rats showed little formation of factors VII–X. Both types of cells showed a sustained oxygen uptake for at least 1.5 hr before starting to decline. Cycloheximide and puromycin failed to inhibit vitamin K-dependent production of factor VII–X activities in surviving isolated liver cells. However, puromycin administered *in vivo* inhibited production of vitamin K-dependent factors VII–X in such liver preparations. Warfarin added to liver cells inhibited formation of factors VII–X but at the same time inhibited their oxygen uptake. It is suggested that vitamin K functions beyond the site of puromycin block in the formation of active factors VII–X.

This work was supported in part by NIH grant AM 10282.

1. Suttie, J. W., Arch. Biochem. Biophys. **118**, 166 (1967).

2. Olson, J. P., Miller, L. L., and Troup, S. B., J. Clin. Invest. **45**, 690 (1966).

3. Babior, B. M., Biochim. Biophys. Acta **123**, 606 (1966).

4. Pool, J. G. and Robinson, J., Amer. J. Physiol. **196**, 423 (1959).

5. Prydz, H., Scand. J. Clin. Lab. Invest. **16**, 540 (1964).

6. Prydz, H., Scand. J. Clin. Lab. Invest. **17**, 143 (1965).

7. Barnhart, M. I. and Anderson, G. F., Biochem. Pharmacol. **9**, 23 (1962).

8. Anderson, G. F. and Barnhart, M. I., Am. J. Physiol. **206**, 929 (1964).

9. Hill, R. B., Gaetani, S., Paolucci, A. M., RamaRao, P. B., Alden, R., Ranhotra, G. S., Shah, D. V., Shah, V. K., and Johnson, B. C., J. Biol. Chem. **243**, 3930 (1968).

10. Metta, V. C., Nash, L., and Johnson, B. C., J. Nutr. **74**, 473 (1961).

11. Mameesh, M. S. and Johnson, B. C., Proc. Soc. Exptl. Biol. Med. **101**, 467 (1959).

12. Quick, A. J., "Hemorrhagic Diseases," p. 397. Lea and Febiger, Philadelphia, Pennsylvania (1958).

13. Dawson, R. M. C., Elliot, D. C., and Jones, K. M., "Data for Biochemical Research," p. 209. Oxford Univ. Press, London and New York (1959).

14. Jacob, S. T. and Bhargava, P. M., Exptl. Cell. Res. **27**, 453 (1962).

15. Ontko, J. A., Biochim. Biophys. Acta **137**, 13 (1967).

16. Umbriet, W. W., Burris, R. H., and Stanffin, J. F., "Manometric Techniques," 4th ed. p. 1. Burgess, Minneapolis, Minnesota (1964).

17. Pechet, L. *in* "Blood Coagulation, Hemorrhage and Thrombosis" (L. M. Tocantis and L. A. Kazal, eds., p. 213. Grune and Stratton, New York (1964).

18. Adamis, D., Sise, J. S., and Kimball, D. M., J. Lab. Clin. Med. **47**, 320 (1956).

19. Godchaux, W., Adamson, S. D., and Herbert, E., J. Mol. Biol. **27**, 57 (1967).

20. Munro, H. N., Baliga, B. S., and Pronczuk, A. W., Nature **219**, 944 (1968).

21. Lowenthal, J. and Birnbaum, H., Federation Proc. **28**, 385 (1969).

22. Lowenthal, J. and Simmons, E. L., Experientia **23**, 421 (1967).

Received June 3, 1969. P.S.E.B.M., 1969, Vol. 132.