ON THE MECHANISM OF ACTION OF VITAMIN K IN VERTEBRATES AND BACTERIA

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1. Introduction

Recently several publications [1, 2] have appeared in which it was shown that the formation or incorporation of the carbohydrate chain of the glycoprotein prothrombin is dependent upon vitamin K. These investigations have induced us to report about our own experiments which have been carried out in the same field for quite some time, however, based on a somewhat different conjecture. As is well known, the general belief about the mechanism of action of this vitamin is that it is only engaged with the formation of the blood clotting protein(s). This is also the theoretical basis of the above cited publications. In accordance with the supposition of one of us that the effect of a vitamin K deficiency on the biosynthesis of prothombin constitutes only part of a much wider influence of the K vitamins [3], we have concentrated our research mainly on the influence of vitamin K and of some vitamin K-antagonists upon the incorporation of various sugars into the cell substance of different organs of chickens and rats as well as into the K-heterotrophic bacteria *Fusiformis nigrescens*.

As we realized that sugars like galactose and mannose which were tritium labelled in position 1 were converted to a considerable extent into fatty acids without exchange of tritium and that labelled sialic acid was not incorporated at all, most of our experiments were carried out with 1-^{3}H-glucosamine.

Another difficulty arose from the fact that animals which were fed a K-free diet for a somewhat longer period in order to produce a sufficient degree of K-avitaminosis showed alterations of the metabolism. This was probably, at least partly, due to severe bleedings. More reliable results were obtained by the use of vitamin K-antagonists, especially 2-chloro-3-phytyl-1,4-naphtoquinone [4], which when applied to animals for 3–4 days produces a prolongation of the blood clotting time from approx. 15 sec to 4 or more min. This substance has the advantage that it has no influence on the rate of oxydative phosphorylation as is the case with the better known and widely used anticoagulants of the dicoumarol type which are potent uncouplers.

In all experiments ^{14}C-leucine was given together with the tritium-labelled glucosamine in order to measure the ratio of the incorporation of the carbohydrate and the amino acid in the tissues.

2. Materials and methods

2.1. *Experiments with rats and chickens*

Two rats (Wistar, 200 g) were given 4 × 5 mg 2-chloro-3-phytyl-1,4-naphtoquinone (abbrev: "Chloro-K_1") subcutaneously during 3 days. 2 hr after they had received the last dose these rats and 2 control animals were given 19 μCi 1-^{3}H-glucosamine HCl (specific activity: 2.6 Ci/mmole and 6 μCi 1-^{14}C-DL-leucine (55 mCi/mmole) subcutaneously. 4 hr later the rats were sacrificed, the different organs lyophylized, powdered and aliquots dissolved in toluene (Packard, Chicago) for counting.

The results (table 1), which show a higher ^{3}H/^{14}C ratio in the organs of the control animals than in the

Table 1
Incorporation of ^{14}C-leucine and ^{3}H-glucosamine into the organs of rats.

Organ	Control rats						Rats treated with "Chloro-K_1"					
	dpm/mg dry weight						dpm/mg dry weight					
	^{14}C		^{3}H		^{3}H/^{14}C		^{14}C		^{3}H		^{3}H/^{14}C	
	1	2	1	2	1	2	1	2	1	2	1	2
Liver	271	251	2,672	2,331	9.9	9.3	295	361	1,921	1,969	6.5	5.5
Kidneys	327	335	1,758	1,588	5.4	4.7	333	316	1,518	631	4.6	2.0
Heart	200	196	853	1,134	4.3	5.8	179	202	858	896	4.8	4.4
Serum*	603	578	12,731	11,600	21.1	20.1	653	800	12,179	14,697	18.7	18.4

* dpm/mg protein. The serum was dialized against water and the proteins precipitated with sodium tungstate.

Table 2
Incorporation of ^{14}C-leucine and ^{3}H-glucosamine into the organs of chicken.

	^{3}H/^{14}C ratios			^{3}H/^{14}C ratios after extraction		
	control animals*	animals treated with "Chloro K_1"**	warfarin treated animals**	control animals*	animals treated with "Chloro K_1"**	warfarin treated animals**
Liver	2.25	1.71	2.38	1.85	1.67	1.51
Heart	1.42	1.08	1.25	1.10	1.02	0.95
Small intestine	1.8	1.9	1.3	1.98	1.81	1.49
Plasma	5.62	5.12	3.60	–	–	–

* mean values of 4 animals
** mean values of 3 animals

animals which had received the K antagonist, thus indicate a diminished incorporation of glucosamine in the case of a vitamin K deficiency.

To investigate the incorporation of labelled leucine and glucosamine into different cell fractions, parts of the livers were homogenized and 4 fractions obtained in the usual manner by centrifugation. The results showed rather uniform distribution between nuclei, mitochondria, microsomes and soluble fraction.

Experiments with chickens were carried out essentially in the same manner. The animals – 1 day old chickens – received 2 × 5 mg 2-chloro-3-phytyl-1,4-naphtoquinone or warfarin (200 μg/100 g) by subcutaneous injection during 24 hr. 2 hr after the second dose each animal was given 0.25 μCi 1-^{3}H-glucosamine and 0.75 μCi 1-^{14}C-D,L-leucine (specific activities as above) and sacrificed 4 hr thereafter. Parts of the dried organs were used directly for the estimation of the incorporated isotopes, other parts were first extracted twice with chloroform/methanol 2:1, then suspended in water and precipitated with tungstic acid in order to remove lipids and water-soluble substances. In table 2 only the ^{3}H/^{14}C ratios thus obtained are given. They show that in chickens the incorporation of glucosamine is enhanced by vitamin K in the same manner as in rats, even if all fat and water soluble substances were extracted from the tissues before the measurement of their isotope content.

Table 3
Incorporation of 1-^{3}H-glucosamine and 1-^{14}C-leucine into the cell wall of *F. nigrescens*.

	Time of incubation (min)	Concentration of phylloquinone in the incubation mixture (M)	Number of experiments	dpm/ml			Glucosamine incorporation (%)
				^{14}C	^{3}H	^{3}H/^{14}C	
Exp. I	180	10^{-6}	6	508	1,335	2.63	100
		$\leqslant 10^{-8.5}$	3	493	899	1.82	69
		$\leqslant 10^{-8.5}$ + 100 γ chloramphenicol/ml	3	37	225		
Exp. II	100	10^{-6}	2	365	778	2.12	100
		$\leqslant 10^{-9}$	2	466	491	1.09	51.5

All values are corrected for a small incorporation (or absorption) observed with heat denatured cells.

2.2. *Experiments with bacteria*

" Vitamin K depleted" cells of a K-heterotrophic strain of *Fusiformis nigrescens* were grown essentially according to Lev [6] in the presence of 3×10^{-9} molar phylloquinon, harvested, washed twice and suspended in Eagle's medium [5] containing 10^{-9} M 1-^{3}H-glucosamine (68,000 dpm/ml medium) and 2×10^{-4} M 1-^{14}C-D,L-leucine (214,000 dpm/ml medium).

This suspension (20 ml, 2.2 mg protein/ml) was divided into 2 parts, to one of which was added phylloquinon at a final concentration of 10^{-6} M, to the other the corresponding amount of emulsifier (Cremophor EL. BASF) which was used to bring the vitamin K into solution. After anaerobic incubation for 180 or 100 min at 37° the bacteria were centrifuged. In order to remove all loosely adsorbed isotopic material they were then suspended in 8.5 ml of a solution containing unlabelled glucosamine (0.05 M) and D,L-leucine (0.1 M) and this suspension was kept for 1½ hr at 0°. This procedure was repeated once and thereafter the cells disrupted by sonification with a Branson sonifier model S 110 for 75 min at position 6. The cell walls were obtained by centrifugation (10 min, 25,000 *g*) and subjected once more to the washing procedure described above. The results of these experiments (table 3) clearly show that the presence during the incubation period of a somewhat higher concentration of vitamin K (10^{-6} M) than that present in the "K depleted" bacteria (about 10^{-9} M or less) brings about a markedly higher incorporation of glucosamine into the bacterial cell walls.

3. Discussion

These experiments seem us to indicate a common role for vitamin K in the cells of all organisms which contain or need this substance. Vitamin K apparently is concerned, in a way so far unknown, with the incorporation of carbohydrates into compounds of a more complex nature, among them prothrombin. The question whether other carbohydrates besides glucosamine behave in a similar manner cannot be answered as yet. Preliminary experiments with galactose labelled in the 3,4-position showed similar results to those reported here.

As our results show, we have found the effect of the K vitamin to be more pronounced in bacteria than in vertebrate tissues. It is likely that several reasons account for this effect. There is experimental evidence (unpublished data) that the mode of action of the K vitamin is more complex in vertebrate tissues than in bacterial cells. This will be discussed in a future paper.

References

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