VITAMIN K DEFICIENCY IN GERMFREE RATS*

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The intestinal synthesis of vitamins has recently been reviewed by Mickelsen (1956), and there is ample evidence that, under ordinary conditions, intestinal microorganisms are able to supply a considerable amount if not all of the required amount of vitamin K. It is, then, astonishing that studies of vitamin K deficiency in germfree animals have given conflicting results. Gustafsson (1948) reported the deaths of weanling germfree rats, with bleeding tendency and impaired clotting of the blood. Signs of disturbances in the blood-clotting mechanism in germfree rats were also observed in studies on liver necrosis by Luckey *et al.* (1954). These signs, however, could be partially prevented by vitamin E. When germfree chicks were maintained by Luckey *et al.* (1955) on a diet deficient in vitamin K, the blood-clotting times increased for one week, but then returned to normal for the remainder of the experimental period of 24 days. Moreover, Luckey *et al.* (1955) were unable to produce prolongation of the blood clotting time in germfree rats on a vitamin K-deficient diet for 400 days.

In view of these conflicting observations the purpose of the present investigation has been to study the effect on germfree animals of the withdrawal of vitamin K from the diet.

Methods

Rats of the germfree colony of the Long-Evans strain reared according to Gustafsson (1948, 1958) were given a vitamin K-deficient diet (TABLE 1). This diet is basically the same as is generally used for rearing the germfree colony with vitamin K omitted, however, and the casein replaced by a commercial brand of vitamin-free casein. In some series the casein and starch were extracted with petroleum ether. The study comprised 44 germfree rats and 29 conventional rats of both sexes. The two groups of rats were kept on raised screens in the germfree apparatus and in the animal room, respectively. The diet, sterilized by autoclaving for 30 min., and water were consumed *ad libitum*.

In some series the diet was given to the pregnant females that later delivered the experimental animals. In two other series the animals received the diet at 1 and 2 months of age, respectively, after having been weaned to the standard vitamin K_1-containing diet.

Prothrombin levels were determined as the prothrombin time according to Quick's method as modified by Lehmann (1941), using human brain thromboplastin. The values were calculated as percentages of the normal content corresponding to a mean prothrombin time of 20 seconds in stock rats, using the same human brain thromboplastin preparation.

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TABLE 1
VITAMIN K-DEFICIENT DIET

Casein, "vitamin free"*	22%	
Wheat starch*	63%	
Peanut oil	10%	
Salt mixture HMW†	4%	
Vitamin mixtures	1%	
Vitamins added per 100 gm. diet:		
Vitamin A	2100	I.U.
Vitamin D	450	I.U.
Vitamin E	50	mg.
Thiamine	5	mg.
Riboflavin	2	mg.
Pyridoxine	2	mg.
Calcium pantothenate	10	mg.
Nicotinamide	20	mg.
Choline	200	mg.
Inositol	100	mg.
p-Aminobenzoic acid	30	mg.
Biotin	0.1	mg.
Folic acid	2	mg.
Vitamin B_{12}	0.002	mg.
Ascorbic acid	100	mg.

* Extracted with petroleum ether.
† According to Hubbell *et al.* (1937).

Results

TABLE 2 shows that all of the 44 germfree rats developed hypoprothrombinemia, 42 rats having severe deficiency symptoms either with bleeding tendency or prothrombin values below 10 per cent (prothrombin times above 200 seconds). In some of these animals the blood did not coagulate even after several hours. At autopsy 35 of the germfree rats had hemorrhages, very severe in some cases, and 12 animals succumbed during the experiments.

The 29 control rats on the sterilized vitamin K-deficient diet had prothrombin times that were the same as, or shorter than, those of the stock rats. None of these animals showed hemorrhages, and there were no fatalities.

The first hemorrhages were encountered at 26 days of age in 18 animals born

TABLE 2
FREQUENCY OF DEFICIENCY SYMPTOMS IN GERMFREE AND CONTROL ANIMALS ON A VITAMIN K-DEFICIENT DIET

Number of animals	Germfree	Control
Total on diet	44	29
With hypoprothrombinemia	44	0
With bleeding tendency or prothrombin below 10 per cent	42	0
With bleeding tendency	35	0
Dying	12	0

to mothers on the deficient diet, that is, 5 days after weaning. In 12 animals turned over from a vitamin K_1-containing diet to the deficient diet at 30 and 60 days, respectively, a bleeding tendency occurred after 7 to 9 days. In these three series, in each of which the animals showed symptoms within 9 days, the casein and starch of the diet were extracted. When this operation was not performed, a bleeding tendency was not noticed until after 22 days in the group of 14 animals given the diet at the age of 30 days. In this latter group there was also 1 rat with a prothrombin value above 50 per cent.

As soon as the first signs of bleeding tendency were encountered the animals were used for special studies (see below) or sacrificed to procure material for histological examinations. The corresponding control animals of the series were sacrificed at the same time.

In some animals the hemorrhages were extensive. The distribution is interesting, as 19 of the 35 rats with bleeding tendency had hemorrhages in the thymus (FIGURE 1), which was sometimes so greatly distended that it filled more than one half of the thoracic cavity, dislocating the heart and lungs. This was probably the reason for the labored breathing seen in the animals having an enlarged thymus.

Cephalic hematomas of a clinical and pathological pattern very similar to those in newborn children occurred in 13 of the 35 animals with hemorrhages (FIGURE 2). In the subcutaneous connective tissue hemorrhages were seen outside the parietal region in 9 animals. Muscles, testes, and intestines were the sites of bleedings in 7, 5, and 2 animals, respectively.

With the exception of the labored breathing described above and the swellings due to subcutaneous hemorrhages, the animals were without symptoms,

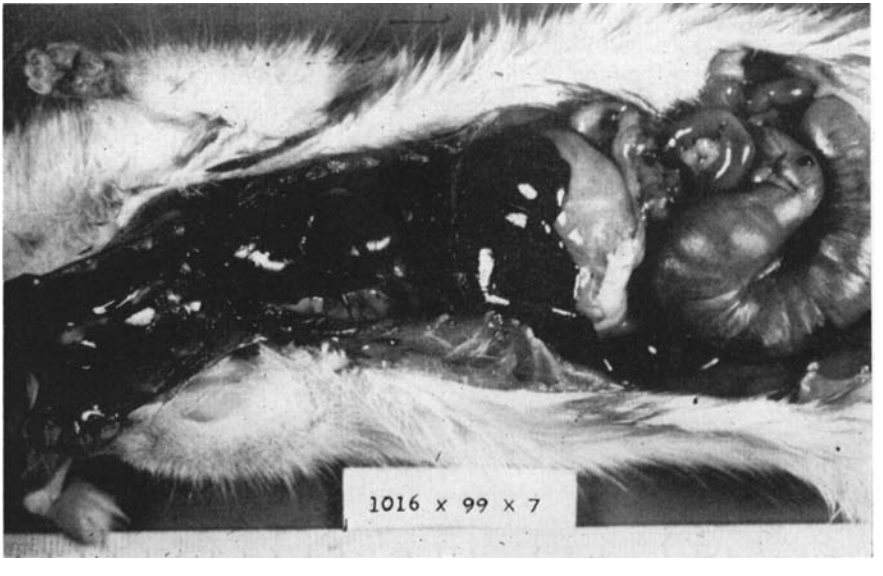

FIGURE 1. Hemorrhage of the thymus in a germfree rat with vitamin K deficiency.

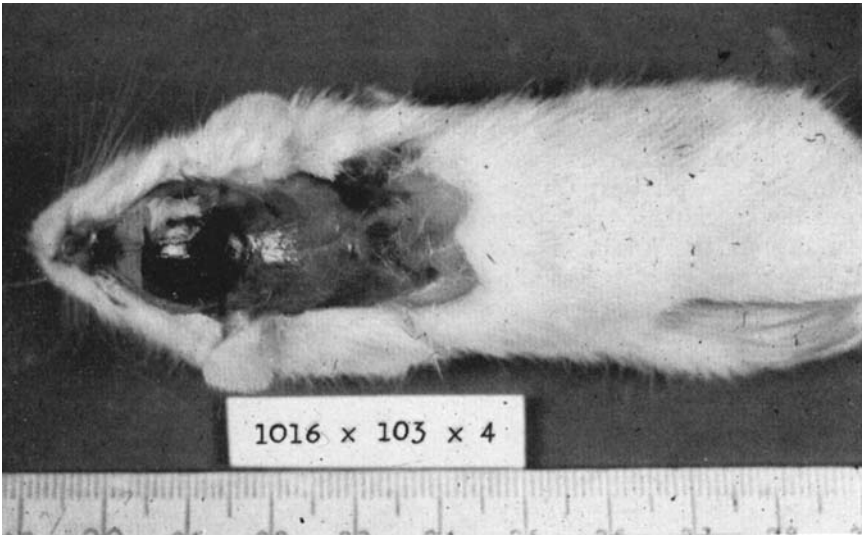

FIGURE 2. Cephalic hematoma in a germfree rat with vitamin K deficiency.

appeared remarkably healthy, and seemed lively. In many cases, the rats remained so until a very short time before death.

Treatment Experiments

To ensure that the germfree state was the reason for the difference between the germfree and control groups, animals with prothrombin values at or below 10 per cent were transferred from the tanks to the laboratory without changing the diet. To demonstrate the influence of the flora, some of these vitamin K-deficient animals were placed in heavily infected surroundings, that is, in cages that had been occupied just previously by the control animals. As the cages were not washed, there was a possibility that the animals were able to consume small amounts of fecal material by licking the screen. A second group of germfree, vitamin K-deficient animals was moved to cleaned metabolism cages in the animal room, that is, to a moderately infected area. A third group of germfree K-deficient rats was transferred to a laboratory well away from the animal room and kept in glass jars or stainless steel boxes that had been washed with a germicide. The prothrombin times were determined on blood samples from the tails every 24 hours. Each of the 3 experiments showed the same primary result. FIGURE 3 gives the results for 1 of the experiments. The animals in the heavily infected surroundings had normal prothrombin times within 48 hours, whereas those in contact with surroundings with presumably less bacteria and, possibly, a flora of quite another type showed only a slight change within 72 hours. Rats in the moderately infected area formed an intermediate group.

The administration of drugs and the repeated taking of blood samples were rather difficult in the smaller types of tanks used in these studies. The fol-

lowing experiments, in which we attempted to remedy the vitamin deficiency, were therefore performed on germfree, vitamin K-deficient animals from groups in which a bleeding tendency had developed. These animals were then transferred from the germfree tank to the glass jars and stainless steel boxes described, where the probability of recovery due to an established flora might be slight within the first 24 hours.

In the first experiment 2 animals of the same litter and with almost the same prothrombin times were transferred to the laboratory. The animal with the lowest prothrombin value was given a dose of water-soluble vitamin K_1 equivalent to 1 mg./kg. body weight by stomach tube immediately after the transfer. The prothrombin time was determined every hour in each animal. FIGURE 4 demonstrates that there was a very rapid increase of the prothrombin

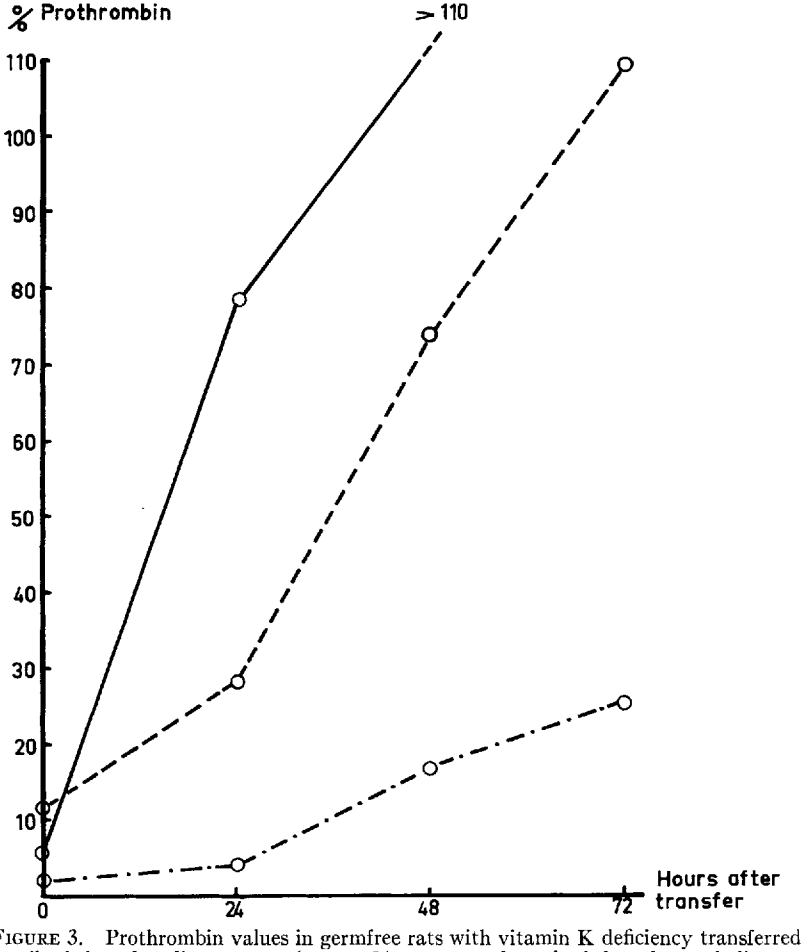

FIGURE 3. Prothrombin values in germfree rats with vitamin K deficiency transferred to a heavily infected ordinary cage (O——O); to a moderately infected metabolism cage (O------O); and isolated in glass jars in the laboratory (O·····O).

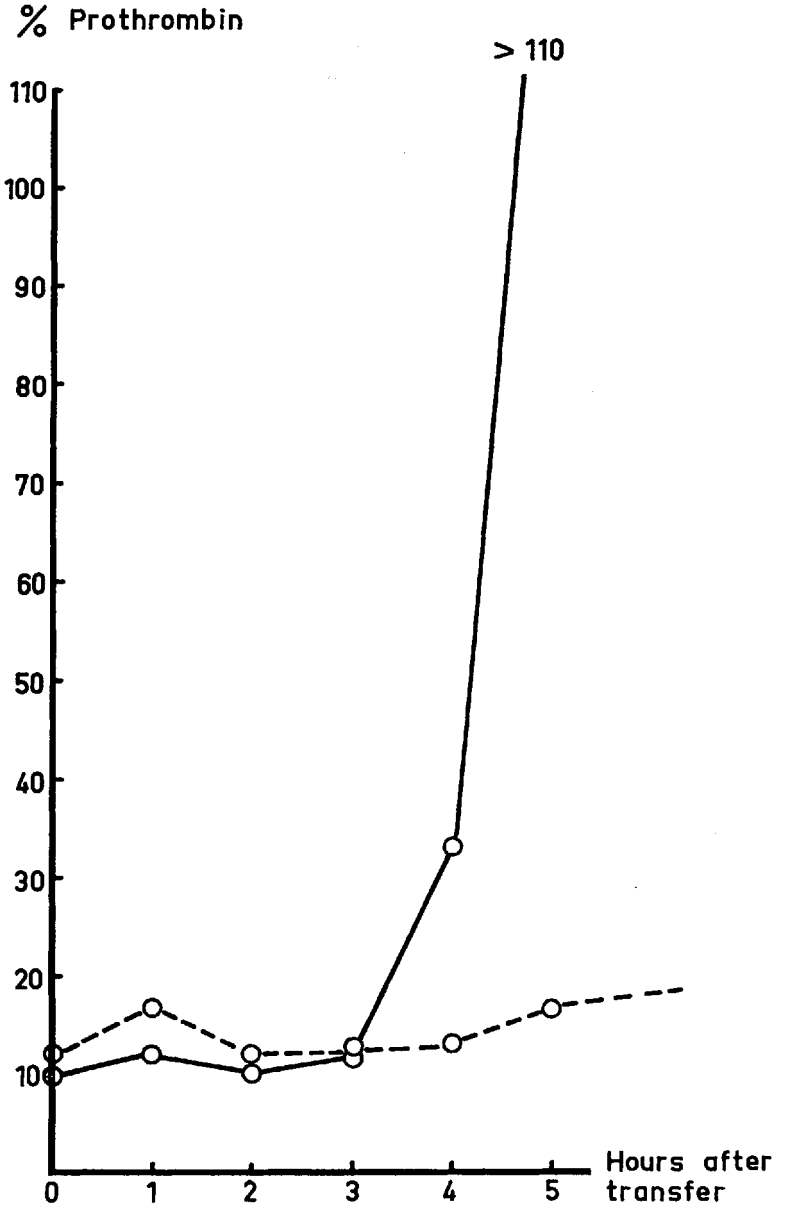

FIGURE 4. Prothrombin values after a single oral dose of 200 μg. vitamin K in a germfree rat with vitamin K deficiency (O——O) and of a littermate given sodium chloride solution (O------O) at 0 hours.

value after 3 hours, and that the value was normal within 5 hours. The other animal, which received the same amount of fluid as the littermate, was given physiological saline by means of a stomach tube and showed only small fluctuations within the first 5-hour period.

In similar experiments on a limited number of animals with a prothrombin value below 10 per cent the efficiencies of sodium menadione sulfate, sodium menadione phosphate, and fat-soluble 2,3-methyl-1,4-napthohydroquinone were compared with that of vitamin K_1. The compounds were given by stomach tube in amounts equivalent to 1 mg./kg. body weight. After 8 hours no change was found in the prothrombin levels of the 6 rats that had received physiological saline, sodium menadione sulfate, or 2,3-methyl-1,4-napthohydroquinone. The two rats given sodium menadione phosphate had respective values of 57 and 71 per cent after 8 hours, whereas at the same time the 3 rats given vitamin K_1 all had normal prothrombin times. At 16 hours the rats given the menadione phosphate still had somewhat subnormal values (75 and 90 per cent). The control rats and those given menadione sulphate and the fat-soluble menadione had not increased their prothrombin times above the 10 per cent level at 16 hours after transfer from the germfree tanks.

Discussion

The germfree animals on the vitamin K-deficient diet showed greatly prolonged prothrombin times and a bleeding tendency within the rather short time of 5 to 9 days, whereas the control conventional animals on the same diet still had normal prothrombin values after 24 days and no tendency to bleed. This difference is probably due to the absence of bacteria in the germfree animal. This and the facts that the deficient animals were cured by transfer to an infected area and also cured very rapidly by the administration of vitamin K_1 form strong evidence for the long postulated concept that vitamin K is synthesized by the microorganisms normally present in the intestines of mammals. This synthesis is capable of supplying the total need of the animals under ordinary conditions. Whether the vitamin is absorbed directly or after coprophagy is still debatable. Barnes and Fiala (1958) have recently demonstrated that by complete prevention of coprophagy in weaned rats receiving a vitamin K-deficient diet, 100 per cent of the animals showed prothrombin values lower than 17 per cent within 28 days. Many of them showed bleeding tendencies.

In some of our series the vitamin K-deficient diet was prepared with casein and starch that had not been extracted with petroleum ether. The onset of symptoms of bleeding tendency was much delayed, that is, until the twenty-first day. Discrepancies in the preparation of the diets or profound differences among species might explain the controversial finding by Luckey and his collaborators that germfree chicks and rats do not develop vitamin K deficiency when vitamin K is omitted from the diet.

The results on the curative efficiency of menadione compounds must be considered to be only preliminary. These experiments are fraught with the difficulty that the effect could not be followed for more than 16 hours. Later the developing bacterial flora might have influence under the experimental

conditions used. The negative result with sodium menadione sulfate is, however, in accordance with the findings in earlier series of our germfree rats reared to work out the semisynthetic diet. A large number of both suckling and adult rats showed prolonged clotting times and died with hemorrhages, although 2 mg. sodium menadione sulfate was supplied per 100 gm. of diet.

Summary

On a semisynthetic diet containing vitamin-free casein and starch and with all the known vitamins except vitamin K_1 added, 42 of 44 germfree rats developed signs of severe hypoprothrombinemia. Thirty-five rats had bleeding tendency, and 12 died. The disturbances of the blood-clotting mechanism could be alleviated by transferring the animals from the germfree tanks to more or less heavily contaminated surroundings. After oral administration of vitamin K_1 the prothrombin values below 10 per cent also returned to normal within 5 hours.

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Discussion of the Paper

QUESTION: There is a theory that vitamin K must be converted to a natural form of the vitamin before it can be utilized in normal blood-clotting mechanisms with respect to the prothrombin level. Vitamin K_3 cannot be utilized in this manner. Have you any explanation for the fact that menadione sulfate gave a negative result while menadione phosphate gave a positive result?

GUSTAFSSON: I can offer no suggestion other than that there must be some difference in the rate or manner of absorption?

QUESTION: Then we must still assume that menadione phosphate as such acted by being converted to natural vitamin K?

GUSTAFSSON: On the basis of the present data I believe this to be the case. In this connection I point to the well known fact that vitamin K_1 was much more effective than menadione in relieving the bleeding tendency of dogs intoxicated by Dicumarol. In some of these dogs menadione had no effect at

all. The theory has been proposed that Dicumarol acts by preventing a supposed conversion by intestinal bacteria of menadione to a natural form of vitamin K. Although this possibility is rather remote, we administered Dicumarol to a limited number of germfree and conventional rats. The germfree rats developed a bleeding tendency at the same time and to the same degree as the conventional rats.