CCXLIV. ESTIMATION OF THE ANTI-HAEMORRHAGIC VITAMIN

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In a former paper [Almquist & Stokstad, 1937] it was stated that chicks fed on diets containing low levels of vitamin K tended to show a maximum blood clotting time at 2 weeks of age and a decreasing blood clotting time after this age. The method of preventive assay and the use of a reference standard, as well as a negative control, were briefly illustrated. Further studies of this assay procedure have been made and may now be reported.

Methods and results

The feeding and housing of the chicks and the determination of blood clotting times were identical with the procedures already described [Almquist & Stokstad, 1937]. In withdrawing blood, incisions about 1 mm. long were made with a fine pointed scalpel in a wing vein which was exposed near the junction of the ulna, radius and humerus.

The sole source of supplementary vitamin K used in these experiments was a hexane extract of alfalfa which has been adopted as a reference standard in our vitamin K assays. 1 ml. represented 1 g. dried alfalfa.

Chicks from hens on diets containing approximately the same vitamin K content were divided into groups and given the basal diet plus various levels of the standard vitamin K sources. The results of one such experiment are given in Table I. The marked tendency toward a maximum blood clotting time at 2 weeks of age is a most striking feature of these results.

Table I. *Average blood clotting times in relation to dietary vitamin K level and age of chicks. Exp. I*

Standard vitamin K soln. per kg. diet (ml.)	No. of chicks	Average blood clotting times in min. at age of			
		1 week	2 weeks	3 weeks	4 weeks
2·0	10	20·2±5·6	Greatly prolonged	10·1±1·2	8·9±0·6
3·0	10	10·3±2·3	19·5±3·0	7·1±0·8	7·0±1·0
4·0	10	6·8±1·0	7·6±0·7	5·8±0·9	4·8±0·6
5·0	10	5·7±0·6	6·3±0·9	5·2±0·6	4·2±0·4
10·0	10	—	Greatly prolonged*	4·2±0·8	4·0±0·5
50·0	10	3·4±0·4	3·5±0·4	3·4±0·4	2·7±0·3

* This group had received only the basal diet for 2 weeks; all individuals were markedly deficient. They received the vitamin K supplement at an age of 2 to 4 weeks.

In a second experiment of this nature we obtained chicks from hens receiving diets rich in vitamin K. Day-old chicks from this source had an average blood clotting time of 1·9 min. as compared with 3·2 min. for chicks from the sources used in the first experiment. These chicks with a high reserve of vitamin K did

not show such a marked increase in blood clotting time when they received less vitamin K in the diet as those of the first experiment, although there was a definite increase during the second week. Apparently, the high vitamin K reserve of these chicks was sufficient to compensate for the low vitamin K intake through the critical period up to 2 weeks of age. The results are given in Table II.

Table II. *Average blood clotting times in relation to vitamin K level and age of chicks. Exp. II*

Standard vitamin K soln. per kg. diet (ml.)	No. of chicks	Average blood clotting time in min. at age of			
		1 week	2 weeks	3 weeks	4 weeks
2·0	16	5·2±0·8	9·6±1·9	10·9±1·7	8·0±1·1
2·5	16	3·5±0·6	8·3±1·2	9·4±1·2	—
3·0	15	3·4±0·2	7·2±1·1	7·8±1·4	—
4·0	15	2·4±0·2	6·6±1·2	6·9±0·9	6·5±0·3
5·0	15	2·6±0·2	5·1±0·5	4·0±0·3	—
10·0	15	2·7±0·3	3·0±0·3	3·0±0·2	—
50·0	15	2·4±0·3	3·9±0·3	3·2±0·2	—
0·0	6	—	Greatly prolonged	—	—

In a third experiment chicks from the same source as in Exp. I were used with a repetition of the results of Exp. I. There was again the well-defined tendency toward a maximum blood clotting time at 2 weeks of age, with many individuals of the lowest vitamin K intake having clotting times that were greatly prolonged. About 15 chicks per group were used in the last two experiments.

The blood clotting times of chicks at 3 weeks of age in these experiments are given in Table III. It is apparent that, although certain groups of chicks in

Table III. *Average blood clotting times at 3 weeks of age of chicks in Exps. I, II and III*

Standard vitamin K soln. per kg. diet (ml.)	Average blood clotting time in min.		
	Exp. I	Exp. II	Exp. III
2·0	10·1±1·2	10·9±1·7	12·5±1·4
2·5	—	9·4±1·2	8·6±0·8
3·0	7·1±0·8	7·8±1·4	7·0±0·8
4·0	5·8±0·9	6·9±0·9	5·7±0·5
5·0	5·2±0·6	4·0±0·3	4·8±0·6
10·0	4·2±0·8	3·0±0·2	4·1±0·2
50·0	3·4±0·4	3·2±0·2	—

Exps. I and III had passed through a period of markedly prolonged blood clotting time and those of Exp. II had not done so, all groups at 3 weeks of age had approximately the same blood clotting time for the same vitamin K level in the diet. It appears that at this age the chicks had achieved a balance with respect to the vitamin K content of the diet.

During Exp. III, three groups of chicks, all with greatly prolonged individual clotting times, were given at 2 weeks of age diets containing 3 ml., 5 ml. and 10 ml. of the standard solution per kg. The average clotting times for these groups at 3 weeks of age were, respectively, 6·9, 5·2 and 3·8 min., while the average clotting times of contemporary groups which had received these vitamin K levels continuously from 1 day of age, were, respectively, 7·0, 4·8 and 4·1 min. These results show that, in spite of a previous condition of severe depletion, the chicks were able, within 1 week or less, to attain an average blood clotting time which depended principally upon the vitamin K level in the diet.

In order to make certain that the actual consumptions of vitamin K were comparable from group to group, careful records of food consumption and growth were kept. These data may be effectively summarized by the statement that growth and food consumption were not appreciably different within the groups of any one experiment and, in fact, did not vary to any significant extent in the separate experiments. This, of course, is consistent with the fact that vitamin K is not a growth factor.

It was stated in a former paper [Almquist & Stokstad, 1937] that there was no evident relation between vitamin K deficiency and haemoglobin level in non-haemorrhagic individuals. Exception to this statement has been taken by Thayer *et al.* [1937] who have reported restoration of both blood clotting power and haemoglobin level after the administration of vitamin K to deficient chicks, but who did not distinguish in their report between haemorrhagic and non-haemorrhagic individuals. It is well known that haemorrhagic chicks suffer from anaemia induced by loss of blood into the tissues and that within 2 or 3 days after administration of adequate doses of vitamin K the blood may be reabsorbed and the haemoglobin level restored toward normal.

During the course of these and other studies, haemoglobin measurements were made on the blood of individual chicks with prolonged clotting time. Measurements were made in these cases with the New Dare Haemoglobinometer, the mean of 5 or more closely agreeing readings being taken as the final value. Eleven chicks, 2 weeks old, with individual clotting times over 60 min. in each case, were found to have haemoglobin levels ranging from 8·10 to 9·60 g. per 100 ml. with a mean of 8·56. None of these chicks were afflicted with visible haemorrhages. The mean value compares favourably with one of 7·97 obtained by Harmon [1936] with the identical instrument in the case of 2-week old normal chicks fed on a practical ration. Four additional non-haemorrhagic chicks with individual clotting times greater than 60 min. were tested at an age of 3 weeks. The haemoglobin levels found were 7·8, 8·4, 9·0 and 9·7 g. per 100 ml. These values are far above what may be considered an indication of anaemia.

One of the most serious obstacles to accuracy in vitamin K assay by any procedure is the large variability of blood clotting time encountered, although source of chicks, level of vitamin K intake and reserve of the chicks are made as nearly uniform as possible. This variability is not appreciably lowered by previous severe depletion of the chick. It remains high even in chicks that have all received the same amount of the vitamin in the diet for as long as 5 weeks and that have grown equally well.

In a search for other factors influencing the blood clotting time, we measured the clotting times of chicks maintained on the same low dietary vitamin K level, in certain individuals only after complete anaesthesia induced by subcutaneous injection of sodium pentabarbital (Nembutal), and in others in the usual manner. A reduction in blood clotting time due to a condition of fright seemed possible. Such an effect would probably be minimized in birds that had been under the influence of a powerful anaesthetic for ½ hr. or more. However, over a range of 6–19 min., no appreciable differences in average clotting time were found.

Second samples of blood taken immediately after the first samples and from the same incision usually clotted in slightly less time than the first samples. This was also found to be the case for first and second samples taken from different incisions, one on each wing of the chick. In no case was more than approximately 0·2 ml. taken per sample. Since there was no appreciable loss of blood while these samples were being taken, the quantity of blood lost could scarcely have exerted any influence on the blood clotting time. It does not

appear that this reduction in clotting time was due to contamination of the second blood sample by fluids from the cut tissues, since the same slight shortening of the clotting time was observed whether the samples were taken from one cut or from different cuts in the same bird. Furthermore, the incisions were made in such a manner as to avoid cutting of tissue other than the vein.

In a further study of this shortening of the clotting time it was found that the differences in the average clotting times of the first and second blood samples lacked significance in the statistical sense, although there was a consistently shorter average time for the second samples.

None of the above findings aid in explaining the large variability in blood clotting times in chicks of the same group. It is logical to expect that a large part of such variability may be caused by individual differences in the chicks in regard to their vitamin K metabolism. In an attempt to secure a measure of such individual source of variability, we have obtained at intervals of a few days the blood clotting times of 57 identified chicks maintained on the same diet low in vitamin K. Data illustrating the characteristically different blood clotting times of certain chicks reared together upon the same diet and vitamin K level are given in Table IV.

Table IV. *Blood clotting times in minutes of individual chicks fed on the same diet low in vitamin K*

	Age in days			
Chick no.	21	24	27	29
5370	>30	>30	>30	>30
5389	24·4	>30	>30	26·0
5392	>60	>30	21·5	>30
5360	7·0	7·1	4·7	5·4
5378	2·9	5·7	2·8	3·6
5386	13·4	9·7	8·6	10·5

This group of chicks was of random selection. Individuals in the group grew at rates which varied over a twofold range. A small positive correlation between chick weight and blood clotting time was indicated by the coefficient, $0{\cdot}276 \pm 0{\cdot}086$, which is barely significant. This tendency for blood clotting time to be influenced by rate of growth can be almost entirely obliterated by selecting chicks of uniform size at 1 week of age.

Since it has been shown by Greaves & Schmidt [1937] that lack of bile interferes with the absorption of vitamin K in the rat, it seemed possible that chicks in a state of chronic deficiency might be cured by addition of bile acids to their diet, if one cause of their deficient condition were poor absorption of vitamin K. Experiments were conducted using chicks of prolonged blood clotting times; however, the addition of the bile acids, cholic acid, deoxycholic acid, dehydrocholic acid and taurocholic acid to the diet at a level of 0·5% had no detectable effect on the average blood clotting time after 1 week of such feeding, although a uniform, nearly adequate level of vitamin K was present in the diet. Control groups also showed no appreciable or general changes. The characteristic clotting time differences, as illustrated in Table IV, were evidently not due to a lack of bile acids in the digestive tract.

As a result of the large individual variability of chicks in regard to blood clotting time, it becomes evident that a reduction of the probable errors in average blood clotting time requires primarily an increase in the number of chicks per test group. This means that any procedure requiring complicated and lengthy

treatment of each animal and of each blood sample will be extremely laborious as well as more susceptible to experimental errors. For these reasons we have sought to perfect the most simple method of conducting a vitamin K assay.

In the procedure now adopted by us 10 chicks (more when greater accuracy is desired) that have been maintained on the basal diet for 1 week since hatching are placed in each test group and fed on a supplemented basal diet from 7 days to 14 or 21 days. The purposes served by maintaining the chicks for 1 week on the basal ration are many, e.g. depletion of the reserve, detection of weak, defective and slow-growing chicks and elimination from the test pens of most of the usual early chick mortality. Included with the test groups receiving the supplement intimately mixed with the diet are a negative control group having no supplementary vitamin K in the diet and several groups on diets containing various levels of the reference standard.

The reference standard could, of course, be assigned a certain "unit" value, but we have preferred not to follow the common tendency to define "units" and possibly lead vitamin K into the confusion of meanings and definitions that has been troublesome in the case of certain other vitamins. It would seem better to defer such definitions until requirements can be based upon a weight of a pure or standard preparation.

As previously shown, the age of 14 days is one of maximum susceptibility to inadequate vitamin K intake. An assay can therefore be terminated at this time, after 7 days of supplementary feeding, provided that a series of suitable reference standard control groups are available and all chicks are of a common origin and have received the same treatment.

For more precise assay it is preferable to continue the supplementary and control feeding to 3 weeks of age. As indicated in Table III, chicks at this age seem to attain a final adjustment of blood clotting time to the vitamin K level of the diet. The results of Table III have been used in constructing Fig. 1, which expresses the relation of the logarithm of the vitamin K concentration in the diet to the reciprocal of the average blood clotting time. The relation is evidently linear, which means that the clotting power of the blood, as expressed by the reciprocal of the clotting time, is a simple function of the logarithm of the vitamin K concentration in the diet. The line relating the reciprocal of the clotting time to the logarithm of the dietary vitamin K concentration appears to extrapolate toward the origin. This implies that, as the vitamin K level is decreased, blood clotting time tends to become extremely prolonged.

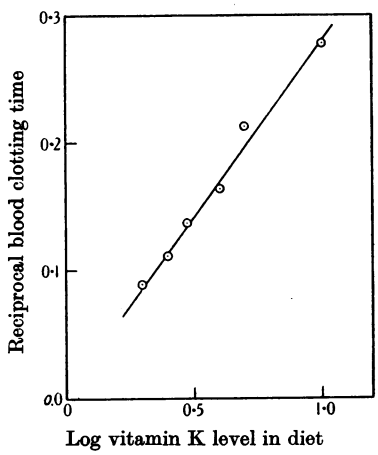

Fig. 1. Relation of the reciprocal blood clotting time to the logarithm of the vitamin K level in the diet (ml. reference standard sol. per kg. diet).

For the curve given it was found that

$$\log \text{vitamin K concentration} = \frac{3{\cdot}6}{\text{blood clotting time}}.$$

It is probable that the constant in this equation will not hold in all cases for chicks of this age and that the interpolation of the results of any assay should be made separately for each series. Although the same equation was obtained from the results at 4 weeks, the constant was definitely lower, approximately 2·8.

This constant would be expected to decrease with increasing age of the chicks.

The linear relation of the logarithm of the dietary vitamin K level to the reciprocal of the blood clotting time affords a very useful means of interpreting average blood clotting times of test groups in terms of the potency of the reference standard. Thus, for interpolating the results of an assay it is necessary to have only two accurately determined points from reference standard groups.

It appears inadvisable at present to specify certain values for blood clotting times to serve as a basis for defining units or activity of vitamin K concentrates, as Thayer *et al.* [1938] have proposed. These workers have defined a unit of vitamin K as that quantity of material required to reduce the clotting time of the blood of 50% of the chicks to 10 min. or less. A clotting time of 10 min. or less was considered normal. It is only necessary to recall the variability of blood clotting time with age (Tables I and II) to realize that highly erroneous results can be obtained by such method of assay unless the ages of chicks and the vitamin K reserves of chicks are closely standardized.

The fact that on low dietary levels of vitamin K chicks with only a moderate K reserve tend to show at 2 weeks a maximum blood clotting time, which later decreases, while chicks with a greater reserve may not show such marked changes, has several interesting implications. The day-old chick weighs 30–40 g. and has a corresponding, definite and increasing requirement for essential food factors. However, the intake of such factors from the diet starts at zero from which it increases as the chick consumes food. The chick must soon obtain entirely from the diet the essential factors which it may have carried in reserve at the time of hatching. During the first days of the chick's life, therefore, the reserve is being depleted pending the acquisition of a sufficient quantity of essential food factors from the diet. When one of these food factors is limited by a low intake in the diet, the possibility of deficiency is greatly increased and, when the reserve of the chick is also low, a seriously deficient condition is almost certain.

During the period from 1 to 2 weeks the chick makes the maximum gains per unit of food consumed. It is during this period of most rapid gain in relation to food consumed that the chick is most affected by an inadequate dietary supply of vitamin K. Even in Exp. II, where the chicks had a high reserve of vitamin K, it was found that the greatest increase in blood clotting time took place during the second week. It is significant, in this respect, to note that Harmon [1936] has shown that at an age of 2 weeks the haemoglobin levels of normal chicks pass through a minimum value which is elevated again at 3 weeks.

After an age of 2 weeks the gain in wt. per unit of food consumed decreases and it is found that the blood clotting time also drops, indicating that the requirement of vitamin K per unit of diet has decreased. This decrease is probably analogous to the lowering of the vitamin G dietary levels required by chicks [Heuser *et al.*, 1938].

Summary

1. The blood clotting time of the chick varies with the age and the vitamin K reserve in the chick, and the vitamin K level in the ration, and tends to reach a maximum at 2 weeks of age.

2. At 3 weeks of age the chick achieves a balance of blood clotting power with respect to the vitamin K level in the ration.

3. The reciprocal of the blood clotting time is a simple linear function of the logarithm of the vitamin K level in the ration, over a practical range of values.

4. Chicks free from haemorrhage but with greatly prolonged blood clotting times had normal haemoglobin levels.

5. An improved assay procedure for vitamin K is suggested.

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