CXXVIII. DETERMINATION OF THE ANTI-HAEMORRHAGIC VITAMIN

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In an earlier paper [Almquist *et al.* 1938] we presented a procedure for vitamin K assay. This method has been improved and is now capable of very satisfactory accuracy. We wish to give here the details of our improved method. For the convenience of those interested in using this method we have repeated some details already reported.

METHODS

Single-comb White Leghorn chicks are placed at hatching in metal, wire floored, electrically heated battery brooders. For the first week they are fed only the basal ration which consists of:

Sardine meal, ether-extracted	...	...	...	...	17.5
Dried brewer's yeast,* ether-extracted	...	...	...	...	7.5
Salt†	...	...	...	...	1.0
Cod liver oil	...	...	...	...	1.0
Calcium carbonate	...	...	...	...	0.5
Ground polished rice	...	...	...	...	72.5
					100.0

* Obtained from Northwestern Yeast Co., Chicago, Illinois.

† Contains 0.5% manganese in the form of manganous sulphate.

The diet contains approximately 18.5% protein, 1.1% Ca and 0.5% P. It permits satisfactory growth and freedom from nutritional disturbances other than vitamin K deficiency, which becomes severe within 7–12 days. The level of yeast used in this diet cannot be lowered much without danger of other nutritional deficiencies.

Maintenance of chicks on the basal diet for 1 week aids in eliminating weak or defective animals and reduces the vitamin K reserves of the chicks before the start of the actual assay.

Supplements of vitamin K are then included in the diets by mixing them in thoroughly either as a dilute solution or a fine powder. The chicks are maintained on these diets for 2 weeks, i.e. until 3 weeks old. As shown in a former paper [Almquist *et al.* 1938], chicks do not attain a close adjustment of average blood clotting times to the vitamin K level in the diet until about 3 weeks of age. Attempts to shorten the assay time have usually given more variable and less satisfactory results.

For the determination of the clotting ability of the blood we have now adapted to vitamin K assay the "prothrombin time" method of Quick [1937]. Blood is rapidly and conveniently obtained by cutting off the head of the chick with scissors. The bird may be held in the left hand with neck between thumb and forefinger in such a way as to direct the blood into a tube calibrated to 2 ml. and containing 0.2 ml. of 0.1 *M* sodium oxalate solution. When blood has been drawn to the 2 ml. mark, the tube is thoroughly shaken. Individual oxalated

blood samples are obtained in this way from all chicks in a group. Slight contamination of the blood with tissue juice is of little importance in this method, since an excess of tissue juice is added to the sample later. This convenience may be contrasted with the extreme precaution against admixture with tissue juice necessary in the assay method given by Dam & Glavind [1938].

For a clotting agent, an extract of chicken breast muscle is prepared [Dam & Glavind, 1938]. Approximately 10 g. of breast muscle from a chicken killed by bleeding are ground with sand and 10 ml. 0.85% NaCl solution. The mixture is centrifuged and filtered on coarse paper. The resulting liquid is stored in a refrigerator. It is preferable to make a fresh tissue extract for each group of tests. When used, the extract is diluted with the salt solution to a concentration representing 5% of muscle. This concentration has proved ample in our experiments. The diluted clotting agent (thrombokinas) at a concentration of 5% is then mixed with an equal volume of 0.025 *M* CaCl₂ solution. The mixture should clot the blood of a normal chicken in 25 to 30 sec. when tested as in the present procedure.

Samples of 0.1 ml. of the oxalated blood are pipetted into small cylindrical vials, 15 × 50 mm., with a flat bottom. A 0.2 ml. portion of the mixed thrombokinas-CaCl₂ solution is run into a vial and a stop-watch started simultaneously. The vial is placed at once in a thermostat adjusted to 38.5–39.0°. A device is employed to tilt the vial to an angle of approximately 45° and back to a vertical position once per second. The end-point, i.e. clot formation, occurs very sharply. The watch is stopped when the bottom of the vial remains covered by a definite gelatinous film. The procedure is repeated to obtain a check measurement. Almost invariably the duplicate time measurements agree very closely, often to one second.

In this work, blood clotting times were also obtained by our former method of puncturing a wing vein, previous to taking the larger sample of blood for prothrombin time measurements. This was done to permit further study of the linear relation between the reciprocal of the blood clotting time and the logarithm of the vitamin K level in the diet [Almquist *et al.* 1938], with a view to employing this relation in an accurate assay procedure. In some cases the prothrombin times of plasma were also obtained after measurements of the whole blood prothrombin time by centrifuging the remaining oxalated blood and repeating the procedure on the clear plasma. This was done to furnish a basis for deciding whether to employ whole blood or plasma in later work. Finally, we also wished to decide whether the reciprocal prothrombin times bear the same relation to the logarithm of the dietary vitamin K level that is shown by the simple clotting times.

The source of vitamin K used in this work was our reference standard solution, 1 ml. of which represents 1 g. of dried alfalfa.

RESULTS AND DISCUSSION

In Table I are given results of our first comparative study of blood clotting times and whole blood prothrombin times as related to the relative vitamin K level in the ration. The chicks, fed according to the usual schedule, received the supplemented diets from the end of the first to the end of the third week, at which time the measurements were made.

It was apparent that the whole blood prothrombin times were subject to far less variability than were the simple blood clotting times, which led us to believe that the former is a more consistent measurement of the prothrombin content of

Table I. *Means, standard errors and coefficients of variability of simple blood clotting times and of whole blood prothrombin times. Exp. A*

Ref. standard of vitamin K per kg. diet ml.	No. of chicks	Blood clotting time		Prothrombin time	
		Mean and standard error (min.)	Coefficient of variability	Mean and standard error (sec.)	Coefficient of variability
4	10	7.97±1.78	66.9	43.6±3.5	24.4
8	9	4.38±0.23	15.1	33.6±1.4	11.6
10	11	3.50±0.86	73.4	31.2±2.1	20.0
16	10	2.63±0.55	65.8	30.1±0.5	5.2

blood owing, probably, to effective elimination of thrombokinase and Ca ion variations. The speed and reproducibility of the prothrombin time measurements appeared as further advantages.

The relation of reciprocal simple clotting time to the logarithm of the relative vitamin K dietary level (Fig. 1, curve A) showed the same linear character as reported formerly. Similarly, the reciprocal average prothrombin times exhibited

Fig. 1.

Fig. 2.

a closely linear relation to the dietary vitamin K level for the three lower level (Fig. 2, curve A) but dropped below the expected value in the case of the highest vitamin K level. The results suggested that a maximum level of 10 or 12 ml./kg. of diet should not be exceeded if the linear relation be expected to hold.

In a second experiment we obtained individual prothrombin time measurements on the plasma also. Results are given in Table II.

The same consistent relation between the reciprocal times and the logarithms of the vitamin K levels is shown by this experiment (Fig. 1, curve B and Fig. 2, curve B). As in the first experiment, the variability of the prothrombin times was much less than that of the simple clotting times.

The mean plasma prothrombin time was greater than that for whole blood. It was noted in this and in subsequent studies that centrifuging caused a small and very irregular prolongation of the prothrombin time. In very deficient

Table II. *Means, standard errors and coefficients of variability of simple blood clotting times, whole blood prothrombin times and plasma prothrombin times. Exp. B*

Ref. standard of vitamin K per kg. diet ml.	No. of chicks	Blood clotting time		Whole blood prothrombin time		Plasma prothrombin time	
		Mean and standard error (min.)	Coefficient of variability	Mean and standard error (sec.)	Coefficient of variability	Mean and standard error (sec.)	Coefficient of variability
3	15	10.07±2.31	61.1	42.0±1.9	17.1	51.2±1.8	9.1
6	15	3.42±0.55	60.2	37.3±1.0	9.6	40.2±1.0	9.3
12	15	1.97±0.37	72.1	30.5±0.5	6.1	—	—
5*	14	3.71±0.64	62.2	40.6±2.9	25.9	49.3±3.0	21.7

* A separate group included here for additional comparison.

blood the prothrombin time of plasma approached that of the whole blood. It appears that centrifuging the blood added no further accuracy to the method and perhaps caused, in cases of short prothrombin times, a distinct, variable loss of prothrombin (or thrombin). Examples illustrating these statements are given in Table III.

Table III. *Comparison of individual whole blood and plasma prothrombin times from the same blood samples*

Whole blood prothrombin time sec.	Plasma prothrombin time sec.
25	27
29	37
30	41
31	37
40	50
40	57
40	47
40	42
47	51
50	54
59	64
67	72
76	79
235	267
315	343
355	353

Notwithstanding the irregular differences introduced by centrifuging, it was found in several experiments that the correlation between individual whole blood prothrombin time and plasma prothrombin time was high, coefficients of 0.813, 0.975 and 0.930 being obtained. It was decided to continue with the use of whole blood rather than plasma since the former seemed to yield very consistent results and to offer a simpler procedure through the elimination of centrifuging. An additional advantage is that the formation of a clot is much more easily seen in whole blood than in the nearly colourless plasma.

In a further experiment we attempted to get longer whole blood prothrombin times in at least one group in order to extend the lower range of the observations. Results obtained in this experiment are given in Table IV.

The relation of the reciprocal mean prothrombin time to the logarithm of the vitamin K dietary level is given by curve *C* of Fig. 2. The results of this experiment are a little more variable than might have been expected, owing to the fact

Table IV. *Means, standard errors and coefficients of variability of whole blood prothrombin times in relation to relative vitamin K level in the diet. Exp. C.*

Ref. standard of vitamin K per kg. diet ml.	No. of chicks	Mean prothrombin time (sec.)	Standard error	Coefficient of variability
2	16	94.4	± 6.8	27.8
4	16	60.2	± 3.4	21.9
8	12	35.1	± 2.8	26.1
12	16	31.3	± 1.1	13.0

that blood samples were taken at 2½ weeks rather than at 3 weeks. It is obvious, however, that the same linear relation holds as noted in former cases.

By the use of this line we were able to evaluate in terms of the reference standard one of our preparations of the molecular compound of vitamin K with deoxycholic acid [Almquist & Klose, 1939]. This preparation was fed at levels of 25 and 50 mg./kg. diet to other chicks of the same lot as used in Exp. C and over the same interval of time. Average prothrombin times obtained were, respectively, 83 and 51 sec. By graphical interpolation we obtained equivalent volumes of the reference standard solution of 2.34 and 4.47 ml. respectively. The activity of this preparation is, therefore, 0.094 ml. of reference standard per mg. in one determination and 0.089 ml./mg. in the other, values which are in very fair agreement. In each series of assays it is necessary to include at least two groups of chicks treated with the reference standard solution at levels, usually, of 2 ml. and 10 ml./kg. of diet, in order to establish the line for interpolating the activities of supplements administered simultaneously. This procedure is now being employed by us with very satisfactory results.

On several occasions calculations were made of the coefficients of correlation between individual simple blood clotting times and prothrombin times within groups of chicks. Values obtained were 0.651, 0.363 and 0.610, showing definite but not high correlation. On the other hand, the correlation between the mean simple blood clotting times and the mean prothrombin times in a series of groups receiving different amounts of vitamin K was found to be expressed by the coefficient 0.89 in one case and 0.99 in another, indicating that the two mean times may serve with approximately equal accuracy when groups of sufficient size are used. The much lower variability of the prothrombin times together with the fact that conveniently measurable values for prothrombin time can almost invariably be obtained, even when simple clotting times are greatly prolonged, has led us to adopt the prothrombin time measurement in our assay procedure. We occasionally employ the simple clotting times when it is desired to obtain an advance indication of the effect of a vitamin K supplement.

It may be remarked that the range of clotting times within which our assay procedure is conducted is almost entirely below the clotting time of 10 min. which some workers have regarded as "normal" [Thayer *et al.* 1938]. It is obvious that there is a vast difference in the amount of vitamin K required to maintain a clotting time of 10 min. as compared with one of 2 min., for example. Preparations of vitamin K which are stated to produce "normal" clotting time can be compared only on the basis of the relation we have demonstrated.

In a former paper we presented individual blood clotting times of chicks reared upon the same diet. These times showed a large range of values which were fairly constant for an individual. We have since obtained further data showing that chicks of the same age and size and on the same diet can exhibit large consistent differences in simple blood clotting time. It is almost impossible

to account for the consistent individual values by assuming, in the case of the prolonged clotting times, a uniform lack of contamination with tissue juice or, in the case of the short clotting times, a uniform degree of such contamination. The causes of the large variability in simple blood clotting times undoubtedly lie within the blood itself, and it is evident that the prothrombin time measurement disposes of these to a large extent. It is also evident that simple blood clotting times obtained from a few chicks tested only a few hours or days after receiving a vitamin K supplement are likely to be extremely misleading.

SUMMARY

An improved method for conducting a vitamin K assay is described.

The reciprocal of the mean blood clotting time and of the mean prothrombin time bears a linear relation to the logarithm of the vitamin K level in the diet over a practical range of levels.

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