CXXXVII. DETERMINATION OF VITAMIN K BY THE CURATIVE BLOOD-CLOTTING METHOD

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IN this laboratory the determination of vitamin K (the fat-soluble anti-haemorrhagic vitamin) has been carried out during the last 2 years by means of the curative blood-clotting method. This method was originally described by Schönheyder [1936, 1, 2] and has since been subjected to a thorough revision.

The method is based on the procedure and apparatus used by Fischer [1930] in his research on the coagulability of bird's plasma.

Blood is taken from the carotid artery of the chick and centrifuged, and the plasma is diluted with an equal part of Ringer's solution. Four droplets (4×0.05 ml.) of the diluted plasma are introduced into the micro test tube of Fischer, and one droplet of tissue extract (0.05 ml.) is added. The test tube is held by a metal frame provided with a detachable cover which closes the tube. The frame is placed in a constant temperature water bath (39°) and rotated in order to mix the contents. By rotating the frame four times a minute, the clotting time is easily measured by a stop-watch.

By using a series of different concentrations of the tissue extract it is possible to find the concentration (K) which produces clotting in 3 min. If the concentration of extract which clots a normal plasma in 3 min. is K_n , the quotient $\frac{K}{K_n}$ ($=R$) will characterize the degree of reduction of the clotting power while the reciprocal value will be a direct measure of the clotting power. Normal chicks have the value $R=1$, with a certain individual variation in both directions.

When the R -values (R_1) of a series of K -avitaminous chicks have been determined the animals receive on three successive days a weighed amount of a test substance in the form of tablets. On the 4th day the R -value is determined again (R_2). The animals are weighed every day during the 3 days' period and the mean weight is calculated.

From the results of this experiment a diagram can be constructed showing the variation of the R_2 -values with the amount of test substance ingested (mg. test substance per g. body wt. per day).

From the amount ingested per g. body wt. per day and the R_2 value it is possible to calculate, by extrapolation, how much of the substance should be given in order to render the clotting power normal ($R_2=1$), provided that the R_2 -value is found to be above 1. This amount of the substance is said to contain 1 curative unit.

Our experience with the method has shown us the necessity for introducing certain improvements.

It is necessary to have a constant and uniform tissue extract. The watery extracts of chick's embryo or hen's lung used by Fischer [1930] and by Schönheyder [1936, 1, 2] exert a strong coagulating effect, but are heterogeneous suspensions liable to undergo sedimentation and putrefaction when stored in a flask in the ice box in a liquid state, even when antiseptics are added. These preparations

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act as thrombokinase, but it has been found that different preparations may yield different *R*-values with the same chicken plasma, owing, in all probability, to contamination with thrombin, even if their power of clotting oxalated plasma may not be demonstrable.

A fairly constant extract may be prepared in the following way.

White muscle (200 g.) from the breast of a hen killed by bleeding is minced and ground with an equal weight of pure sand and 200 ml. Ringer's solution (or a similar solution without phosphate and bicarbonate). After suitable centrifuging and filtering through paper a milky liquid is obtained. This liquid (about 200 ml.) is cooled on ice and, under continuous stirring, is put into ampoules, 0.5 ml. being carefully measured into each by means of the syringe pipette of Krogh. The ampoules are closed, frozen in a freezing mixture and stored in the inner box of the frigidaire at approx. 3°. When determinations are to be made the contents of 1 ampoule are drawn up in a syringe and transferred to a 5 ml. measuring flask; the syringe and ampoule are rinsed with Ringer's solution (or the corresponding solution without phosphate and bicarbonate), the contents of the flask being brought up to the mark with the wash liquid. The solution now represents a 10% concentration of the extract, from which lower concentrations 5, 2.5, 1, 0.5, 0.25, and 0.1% are prepared.

The calculation of *K* from the clotting time obtained with different concentrations is facilitated by plotting the clotting times in seconds against the corresponding concentrations on paper ruled with logarithmic axes.

Determinations of *K* for the plasma from a normal chick, made by a series of dilutions from 10 ampoules, gave the following values:

0.156, 0.153, 0.160, 0.185, 0.188, 0.164, 0.180, 0.180, 0.182, 0.182.

The mean value was 0.173 and the maximum deviation from the mean value was 12%.

The values of *K* for another normal plasma, determined at intervals of 30 min. during 4 hr., ranged from 0.22 to 0.25, the highest values being found at the end of the period. This variation was due to the fact that the weakest concentrations—0.05%—had deteriorated somewhat. The dilutions should therefore preferably be kept at 0° when they are to be used over a period of several hours.

The mean normal value of *K* was determined for 10 normal chicks by one and the same series of dilutions made from the mixed contents of 3 ampoules. The diet used for the normal chicks was a commercial chicken ration in which the principal source of vitamin K was 4% of dried alfalfa containing about 300 units of vitamin per g. The following individual values were obtained:

0.175, 0.100, 0.100, 0.124, 0.158, 0.200, 0.170, 0.100, 0.085, 0.157.

The mean value was 0.137 and the maximum deviation from the mean value was 46%.

This mean value of K_n was used in the calculation of *R*-values, but it is possible that a somewhat higher or lower K_n value might have been found if a much larger number of animals had been used.

The Ringer's solution must always have exactly the same composition; the modified Ringer's solution may consist of NaCl 0.95%, KCl 0.02%, CaCl_2 0.02%. It is advisable to determine the K_n value at intervals.

After storage in the frozen state for several months the tissue extract is rendered flocculent by dilution with Ringer's solution, but the mean value of K_n is not altered.

The diet used to produce the haemorrhagic disease may vary within wide limits. It is difficult to obtain a source of protein which is either free from vitamin K or has a constant low vitamin K content.

The following are some of the diets which have been used:

Diet 124c

Ether-extracted dried hog liver	15
Dried yeast	12
Salts no. 2*	2
Sucrose	71
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	100
Cod liver oil	2

* Salts no. 2 consist of CaCO_3 , 400; MgCO_3 , 20; NaCl , 88; ferric citrate, 32; KI , trace.

Diet 417

Ether extracted dried fish meal	17.5
Dried yeast	7.5
Sucrose	73
Salts*	1
Cod liver oil	1
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	100

* Salts consist of NaCl , 85; ferric citrate, 14; CuSO_4 , 1.

Diet 451

Casein	15
Dried yeast	10
Salts no. 2	2
Sucrose	73
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	100
Cod liver oil	1

Diet 124c enables chickens to thrive fairly well, even if it does not contain all the factors necessary for the chick except vitamin K. It is, however, rather expensive.

Diet 417 resembles the diet of Almquist & Stokstad [1936]. Experience has shown that it may be difficult to obtain a fish meal of constantly low vitamin K content, a circumstance which is probably explained by the possibility of K-vitamin formation by putrefaction of the product before drying.

Diet 451 has often given good results, but in some cases the same difficulty of obtaining protein free from vitamin K has been encountered.

Experience shows further that some of the birds receiving these diets develop the encephalomalacia described by Goettsch & Pappenheimer [1936].

It is absolutely necessary to keep the animals clean, i.e. to change and clean the wire screen bottoms once or twice a day and the food and water containers twice. Animals kept in dirty cages are to a large extent protected from the disease and often have practically normal R -values even after months on the diet. On the other hand, cleanliness will not ensure that all animals have sufficiently high R -values after 3-4 weeks.

In determining his curve Schönheyder [1936, 1, 2] made the supposition that chicks which had received the vitamin K-free diet for at least 3 weeks had a constant R_1 value of about 150-200 and he therefore did not determine the R_1 value before the ingestion of the test substance. Our own experience, which extends over several years, has shown however that the R_1 values may vary widely in different individuals, even when precautions are taken to secure uniformity of the food, cleanliness and a constant tissue extract.

Examples of individual variations are given in the following table:

Chick no	Diet no.	No. of days on the diet	R_1
2749	124 c	23	750
2734	124 c	28	7.4
2738	124 c	36	96
2775	124 c	25	20
2792	124 c	25	280
2802	417	22	280
2814	417	24	13
2819	417	24	600
2829	124 c	23	29
2834	124 c	24	1.3

A standard source of vitamin K has been chosen, consisting of a selected and very uniform dried spinach which has been finely powdered and formed into tablets each weighing 0.25 g. The preparation is stored in completely filled containers at $+3^\circ$. This standard was found by the construction of the curve to contain 500 units per g.

Construction of the curve (Fig. 1)

The animals were given the vitamin K-free diet when they weighed 100–130 g. R_1 was determined when they showed haemorrhages. The ingestion of the standard substance began on the day when the R_1 value was determined, and the determination of the R_2 value followed on the day after the third ingestion.

Animals having R_1 values between 60 and 140 were used for small quantities of the standard substance, while those with lower R_1 values were used for the larger quantities. It is advisable to utilize animals having R_1 values from 20 to 60 in order to save cost. Animals having such R values generally survive better than those having the highest values. The curve is drawn on paper with linear abscissae and logarithmic ordinates.

The points corresponding to animals with high R_1 values are first plotted and the left-hand part of the curve—corresponding to small amounts of the standard substance and R_2 values above 4—is drawn. For those points of the right-hand part of the curve (below $R_2=3$) which have been obtained from animals having R_1 values lying between 60 and 20 a correction is added to the abscissa. This correction is taken from the left-hand part of the curve, which does not need any correction, and is equal to that quantity of the standard substance which is required to depress the R value from between 60 to 140 to the lower R_1 value in question.

The table on p. 1022 gives a survey of the results.

The diagram shows that about 2 mg. of the standard substance per g. body weight per day are the smallest quantity which will make the clotting power normal ($R=1$). This means that 2 mg. of the standard spinach contain about 1 unit. 1 g. therefore contains 500 units.

Future determinations can now be based on this standard substance; this means that the vitamin content of a substance which has produced a decrease of the R value from R_1 to R_2 can be calculated from the amount of the standard spinach which on the diagram produces the same decrease of the R value, the spinach being assumed to contain 500 units per g.

The diagram shows that the limits of error are considerable. An exact calculation of the mean error has not been attempted, owing to the unequal distribution of the individual determinations.

Chick no.	Mean wt. during the 3-day period	Mg. standard substance per g. body wt. per day	R_1	R_2	Corrected abscissa
3521	241	2.9	117	1.35	2.9
3557	367	6.8	280	1.0	6.8
3583	296	12.8	100	1.0	12.8
3576	405	3.16	130	0.76	3.16
3572	413	1.82	42	1.1	1.86
3670	225	1.11	39	1.83	1.15
3580	257	4.28	23	0.66	4.37
3666	300	0.25	150	10.0	0.25
3695	442	1.36	55	0.79	1.38
3718	393	4.07	31	0.73	4.13
3672	390	0.77	62	2.15	0.77
3701	390	0.205	100	12.8	0.205
3713	250	4.00	22	1.8	4.1
3738	266	0.43	73	4.66	0.43
3671	480	0.104	86	16.0	0.104
3727	360	0.97	62	1.67	0.97
3698	423	0.59	88	1.5	0.59
3722	375	1.33	40	1.63	1.37
3885	333	0.192	100	7.3	0.192
3577	394	1.90	39	1.34	1.94
3610	290	0.516	145	2.00	0.516

Fig. 1. Abscissa: mg. standard substance per g. body weight per day. Ordinate: R -value.

Inspection of the area containing the different points shows that the maximum horizontal deviation from the curve is about $\pm 40\%$ for R_2 values between 20 and 2. For animals having high R_1 values (60–140) the error of one single determination appears to be of that order, but if the R_1 value is low, the error is

correspondingly increased. R_2 values lying below 1.8 may only give minimum values for the strength of the preparations. It is therefore necessary to make more than one determination when an accurate estimation is to be made.

The error may be enormous if the R_1 values are not determined even if R_2 is greater than 2.

It is further noted that the variation of the ordinates corresponding to a given abscissa is not due to the variation of the R_1 values. Animals with a high R_1 value may show R_2 values lying below the curve and vice versa.

The diagram shows further the important fact that it is not possible to render the coagulability of the plasma "supernormal" by ingesting large doses of the vitamin. The R values obtained by giving more than 1 unit per g. are distributed about the line $R=1$.

SUMMARY

The determination of vitamin K by the curative blood-clotting method has been subjected to a thorough revision.

A tissue extract of constant activity was made by putting up an extract of hen's muscle in ampoules and storing it in a frozen condition.

The activity of the tissue extract was measured against the plasma of a series of normal chicks.

A selected dried spinach powder formed into tablets and stored under precautions securing stability was chosen as a standard of vitamin K. This standard was used for the construction of a curve of reference; it was found to contain 500 units per g.

The limits of error are discussed.

It was found to be impossible to render the coagulability of the blood plasma "supernormal" by administering large doses of the standard substance.

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