CXXIX. THE QUANTITATIVE DETERMINATION OF VITAMIN K. I.

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IN a recent paper [Dam and Schönheyder, 1934] a deficiency disease in chicks was described, the main symptoms of which are a tendency to large haemorrhages, certain pathological changes in the gizzard and anaemia. The blood of chicks suffering from this disease has a considerably prolonged clotting time. The cause of the disease was ascribed by Dam [1935, 1, 2] to lack of a specific fat-soluble vitamin which is called vitamin K. Almquist and Stokstad [1935], working independently of Dam, have already confirmed this suggestion. The delayed clotting was occasionally observed by Dam [1930] and by McFarlane *et al.* [1931] and has recently been subjected to more intensive study by Schönheyder [1935; 1936].

Out of a total of 323 animals presenting symptoms of vitamin K deficiency, prolonged coagulation time (over 10 min.) was found in 86.5%. The clotting time was determined in the following manner. The brachial vein was opened by a slight cut and 2–3 ml. of blood were allowed to drop into a small porcelain bowl. 30–45 sec. were usually required to obtain this amount of blood. The period of time from the venous puncture until complete clotting is called the clotting time. This is between 1 and 10 min. in normal chicks, while it may be several hours for diseased animals. This simple procedure enables one to differentiate between normal and diseased animals, and the results are in accordance with those obtained by a more exact method of measuring the clotting time (see later).

Previous research [Schönheyder, 1936] on the subject has shown that the prolonged clotting time cannot be due to insufficiency of thrombokinase in the tissue or thrombocytes, or to insufficiency of fibrinogen in the plasma or an accumulation of anticoagulating agents, especially antiprothrombin, in the plasma. The serum calcium values were usually found low and the inorganic phosphorus values constantly elevated in the diseased animals. The addition of Ca^{++} to the plasma of the diseased animals did not shorten the clotting time, and no change in p_{H} or salt content of plasma could be demonstrated.

It was shown, however, that per unit of time and under identical conditions far less thrombin was formed, after addition of thrombokinase, in abnormal plasma than in normal. Normal plasma was further found to contain a surplus of a certain ingredient which accelerates the coagulation process in chickens having lack of vitamin K. The most probable explanation of the prolonged clotting time in the diseased animals is that it is due to reduced concentration of prothrombin.

As with other vitamins, two methods of biological assay, preventive and curative, may also be used for vitamin K. By means of the preventive method we have found that a portion of dried pig's liver equivalent to 20% of the diet gave complete protection while 10% afforded only partial protection. All portions of pig's liver are not of equal potency.

The curative method.

It appeared, that by giving the animals with a considerably prolonged clotting time a vitamin K test substance, it was possible to make the clotting time normal. The animals suffering from vitamin K deficiency became normal with reference to clotting time in 3 days when given sufficient food containing vitamin K. The administration of 11.3 mg. of dried pig's liver per g. chick daily for 3 days was found sufficient to restore the coagulation time to normal values. The curative method of assaying vitamin K is based on this fact. Meanwhile it appeared desirable to find a more exact method of measuring the clotting time. The method of Fischer [1930] appeared to be most suitable as it is especially founded on experiments made with plasma from hens. It is well known that blood from hens does not clot when it is shed through a cannula which is inserted in a blood vessel. In this manner it is possible to obtain blood under identical circumstances. The plasma thus obtained does not clot until a clotting agent is added. Normal and diseased plasmata show an enormous difference in clotting time in the presence of the same clotting agent. Instead of using a clotting agent of constant strength, the concentration of the clotting agent is increased until the plasma of the K-avitaminous animal is made to clot as quickly as normal plasma. The relation between the concentrations of the clotting agent which clot a plasma from a sick animal and normal plasma in the same time indicates the degree of vitamin K deficiency. For practical reasons we have chosen to multiply this relation by 10. This figure, which expresses the animal's condition, is called the *S* figure of the animal (*S*=degree of sickness). If *S* is equal to or less than 10 the animal is normal as regards vitamin K. S_1 and S_2 are taken to be the numerical expression of the animal's condition previous to the vitamin assaying and after the test substance has been given for 3 days respectively. One unit of vitamin K is defined as the least amount of a test substance per day per g. of chick which is able to reduce the animal's $S_1=1500-2000$ to $S_2=10$ when given for 3 days. This unit is small but when it is multiplied by 1000 it equals the amount of substance which is necessary to cure an animal with $S_1=1500-2000$ and a weight of 333 g. in the course of 3 days, the animal receiving one-third every day.

The basal diet is a modification of diet 124 of Dam [1935, 2].

Caseinogen	...	...	...	...	...	10
Dried pig's liver extracted with ether	...	...	...	...	...	10
Dried yeast	...	...	...	...	...	15
Sucrose	...	...	...	...	...	62.3
Salts No. 2	...	...	...	...	...	2.7
						<hr/> 100
Cod liver oil	...	...	...	...	...	4

The caseinogen preparation used was free from vitamin A. It was identical with the preparation used at Statens Vitamin-Laboratorium. According to the declaration of the manufacturer (Dansk Mejeri Industri and Exportkompagni, Copenhagen), it consists of lactic acid caseinogen, which has been subjected to drying in air at room temperature for 24 hours. The liver is spread on thin trays and dried in a current of air not over 40°. The dried liver is scraped from the trays and ground to a fine powder. This is first extracted with light petroleum, which dissolves most of the fat. The rest dissolves when extracted with ether in Soxhlet's apparatus, leaving a fat-free residue. The yeast, which is first autolysed for a day at 40° and then spread out on trays to dry at 40°, is ground to a fine powder. Ordinary grade sugar is used. Salts No. 2, CaCO_3 2.00, MgCO_3 0.10; ferric citrate 0.16; NaCl 0.44; KI 0.000025. When these ingredients have been mixed 4% fresh cod liver oil (from Peder Møller, Oslo) is added.

The animals all became equally ill whether they had received this food from the time of hatching or from the age of 2 weeks. During the first 2 weeks the animals therefore were given Oluf Nielsen's chicken food. The diet was then changed. The animals must be kept on the basal diet mentioned for at least 3 weeks, *i.e.* until 35 days old. Experience has taught us the important fact that when chicks of the same breed are given identically prepared diets there is very little variation in the severity of the attack (S_1) and that this is independent of the age (30–60 days) and the weight during this period. The decisive factor when determining S is the animal's weight. Not until this is 200 g. is it possible to obtain blood under standard conditions. Since haemorrhages occur more frequently the more animals there are in a cage because of mechanical injuries it is advisable to have as few as possible together.

The test substance should be given as tablets. If a small quantity of a substance is to be measured the substance is dissolved in a few ml. of an indifferent oil, for example olive oil. The olive oil is then mixed with a suitable quantity (25 g.) of the basal diet and the whole mixture is made into tablets.

Blood samples. Carel's method [1922] for adult hens is used.

The blood is not taken aseptically. An assistant holds the chick so that it lies on its back on a low table, the animal's neck being allowed to hang over the edge and its head being bent so far back that the front of the throat forms a convex surface. Anaesthesia is not necessary since the animal apparently goes into a hypnotic state in this position. An incision 1.5 cm. long is now made in the mid-line between the base of the head and the upper breast aperture. The skin and subcutis are drawn aside and the muscles and the anterior surface of the columna are now seen, covered with a thin fascia, which is incised. It is easy to find the two carotid arteries which lie close together apparently in the same sheath; they are separated from one another with forceps and one of the arteries is exposed in the field of operation by means of a retractor, and the vessel is held in place by means of a ligature with long ends. A small artery clamp is applied 1 cm. proximal to the ligature on the vessel. The whole operative area is rinsed with Ringer's solution, followed by mineral oil. With a sharp pair of scissors a small incision is now made between the ligature and the artery clamp and the cannula is inserted into the lumen of the blood vessel. The clamp is taken off and the blood allowed to flow through the cannula and collected in an ice-cooled paraffin-lined glass. From 3 to 4 ml. are taken. After centrifuging the plasma is pipetted off and is now ready for use in the clotting experiment.

Determination of clotting time. Since plasma, when clotting agent of constant strength is added, coagulates more and more slowly for each day of standing, it is necessary to undertake the clotting determination on the day of operation. The plasma is always diluted with an equal volume of Ringer's solution, thereby making several determinations possible. One drop of clotting agent is invariably added to 5 drops of diluted plasma. The method of Fischer [1930] was used without modification for determination of the clotting time.

The measurement takes place in a water thermostat at 40°. 5 drops of diluted plasma are dropped into a Fischer's miniature test-tube (30 × 8 mm.; thickness 0.9 mm.). The test-tube is placed in a metal stand, *i.e.* a holder especially constructed to accommodate 15 miniature test-tubes in a row. It can be opened and closed as the upper wall is on a hinge at one end and can be tightened at the other with a screwing device. The underside of the movable wall is covered with a thin layer of rubber which fits tightly over the opening of the tubes. A thin layer of parchment paper is always placed over the rubber, forming a covering above the test-tubes. This must be changed between every determination in order to prevent impurities being conveyed from experiment to experiment.

The holder is closed at once after the clotting agent has been added to the plasma in the glasses and is then transferred to the thermostat. It is inverted once, to mix the contents of the tubes,

and at this moment a stopwatch is started. At the moment the plasma is no longer liquid the time is noticed. The determinations are made in duplicate. We generally work with clotting times between 120 and 600 sec.

A watery extract of hens' lungs was used as clotting agent. Ethyl green was added as antiseptic. The lung extract must be kept in a refrigerator and retains its properties for some time. It is constantly necessary to test the clotting agent on normal animals. No difference whatsoever could be shown in the clotting times in normal adult hens and normal chicks, measuring with the same clotting agent. When the clotting agent is to be used for a determination we make several dilutions with Ringer's solution. The lung extract must be shaken before use. It is necessary to wait 15 min. before using the various dilutions, as they require this period to obtain a constant strength which then remains unchanged for 3-4 hours. The plasma to be examined should be kept on ice. The pipettes are placed in distilled water.

Calculation of the S-values. Fischer [1935] has recently shown that the following equation applies to the relation between the clotting time and the concentration of the clotting agent: $\frac{1}{t} = KC^a$, where t = clotting time, c = concentration of clotting agent, and K and a are constants. Using logarithms in the above equation we get: $\log \frac{1}{t} = \log K + a \log C = K_1 + a \log C$.

This equation is merely an approximation to the actual relationship, but for practical use it is obvious that interpolation is facilitated by obtaining a straight line. The above-mentioned logarithmic dependence was found to deviate only slightly from a straight line and was therefore suitable for the purpose. If a curve has been plotted for the related values of $\log$ conc. and $\log \frac{1000}{t}$, it is only necessary to interpolate to the $\log$ conc., which corresponds to

$$\log \frac{1000}{t} = \log \frac{1000}{180} = 0.74.$$

The following is an example of a test of the clotting agent and the measurement of S in an arbitrarily chosen plasma.

(1) Plasma from a normal chick.

Lung extract in the added drop %	log conc.	$\log \frac{1000}{t}$
0.125	0.0097	0.469
0.25	0.0398	0.602
0.50	0.0699	0.762
0.75	0.0875	0.854
1.00	0.1	0.886

The points are plotted with the abscissa giving the $\log$ conc. and the ordinate $\log \frac{1000}{t}$. A curve is drawn between the points and its point of intersection with

(2) Plasma from chick No. 1298.

Lung extract in the added drop %	log conc.	$\log \frac{1000}{t}$
1.25	0.097	0.057
2.50	0.398	0.222
5.00	0.699	0.392
10.00	1.000	0.619
15.00	1.176	0.762
20.00	1.301	0.824
33.33	1.522	0.903
50.00	1.699	0.921
75.00	1.875	0.947

a line drawn parallel with the abscissa through 0.74 on the ordinate axis is determined. The point of intersection corresponds to 0.066 on the abscissa. This means that 0.46 % lung extract can bring about clotting of normal plasma in 180 sec.

The data are treated as before. The point of intersection corresponds to 1.16 on the abscissa. Antilog $1.16 = 14.46$. According to this the S value of the chick will be $\frac{14.46 \times 10}{0.46} = 314$.

A provisional curve for the relationship between the concentration of the substance and its effect. During the whole experiment the same portion of dried pig's liver was used. It was given in tablets of 2.5 g. each. Before receiving the tablets all animals are assumed to have had $S_1 = 1500$.

Chicken No.	mg. dried liver per g. chick per day for 3 days	S_2	Chicken No.	mg. dried liver per g. chick per day for 3 days	S_2
1260	1.6	500	1312	6.8	40
1275	2.2	162	1184	7.2	21
1292	2.8	178	1265	8.2	19
1293	3.0	136	1286	9.9	12
1195	3.7	78	1214	10.2	10
1268	4.4	47	1194	11.3	9
1297	4.8	62	1217	14.8	8
1289	6.1	39	1279	17.8	9

Fig. 1.

We see that about 10 mg. of dried pig's liver per g. of chick per day are the least quantity which is able to reduce $S_1 = ca. 1500$ to $S_2 = 10$. About 10 mg. dried liver contain one vitamin K unit.

The curve shows that as far as exact results are concerned it is not very important to have a specific S_1 at the beginning of the measurement, provided it is more than 1500. For example we see that in order to reduce $S_1 = 500$ to $S_2 = 10$, 8.5 mg. of dried liver per g. per day are necessary. To reduce $S_1 = 1000$ to $S_2 = 10$, 9.3 mg. are required, *i.e.* a difference of 10 %. Therefore if it is certain, at the beginning of the experiment, that S is greater than 1000 the percentage error cannot exceed 10 % in this respect, if enough test substance is given almost to cure the animals.

As seen above, the curve resembles a hyperbola. The author is inclined to believe that the relationship between the S -values and the amount of the test substance in mg. may be expressed as follows:

$$S_2 \cdot \text{mg.}^2 = 1100.$$

The dotted curve is plotted from this equation. The curve is used as follows. The dose of test substance must be so chosen that S_2 falls to the left of the point of intersection between the curve and the horizontal line through $S_2 = 10$ on the ordinate parallel with the axis of abscissae. Thus it is seen that if a certain amount of a substance per g. of chick per day for 3 days reduces $S_1 = 1500$ to $S_2 = 50$, we may conclude that the quantity of substance in question contains $1/2$ vitamin unit. Twice the amount would be the least quantity which is able to make $S_2 = 10$.

The number of animals required for an exact determination of the curve depends upon the exactitude demanded when determining a vitamin K unit. It might be desirable to determine the curve with other sources of vitamin K than the pig's liver used here. At present we believe that the curve represents general conditions, even though a number of supplementary studies should be made in the future. A more exact evaluation of the technique as well as a mathematical application of the curve based on more extensive data will be reported later.

SUMMARY.

Vitamin K is best estimated by the curative method. An animal suffering from vitamin K deficiency becomes normal with reference to clotting time in 3 days, when food containing sufficient vitamin K is given. It was decided to characterise a plasma by the concentration of clotting agent which upon the addition of 1 drop to 5 drops of 50 % plasma would cause the latter to clot in 180 sec. at 40°.

The relation between the concentration which is necessary to clot a diseased plasma in 180 sec. and that which is necessary to clot a normal plasma presents a quantitative expression of the degree of sickness of the animal. The relation multiplied by 10 is called the animal's S value. A vitamin K unit is defined as the smallest daily dose of a test substance per g. of chicken given for 3 days which is able to reduce the S value from over 1500 to 10. The substance to be tested for vitamin K should be given in tablet form.

The methods of drawing blood from the carotid artery, of determining the coagulation time and of calculating the S value are described. A provisional curve of standardisation is given.

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