CLV. STUDIES ON THE MODE OF ACTION OF VITAMIN K.

By HENRIK DAM, FRITZ SCHØNHEYDER and ERIK TAGE-HANSEN.

From the Biochemical Institute, University of Copenhagen.

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SCHØNHEYDER [1935; 1936, 1, 2] has shown that the most probable explanation of the low clotting power of blood from K-avitaminous chicks is a reduced content of prothrombin in the plasma.

The present brief investigation lends definite support to this view, because it has been possible to precipitate, by the acetone method of Howell [1911] and the acetic acid method of Mellanby [1930], the prothrombin from the plasma of normal chicks, whilst a corresponding precipitate from the plasma of K-avitaminous chicks could be shown to be inactive as prothrombin.

The fact that a fat-soluble vitamin is involved in the maintenance of the necessary amount of prothrombin in the blood of chicks would be explained if prothrombin contained a lipoid component. Prothrombin is, as commonly known, water-soluble and follows the globulin fraction in many precipitation reactions.

When the prothrombin precipitate was washed several times with acetone and ether, whereby lipoids, including carotene pigments, are removed, the washed precipitate was still found to be active, and the lipoids which passed into solution did not show any activity as prothrombin. These facts do not, of course, exclude the possibility that vitamin K, or a derivative thereof, might be present in the prothrombin as a prosthetic group which is held in firm combination with the rest of the molecule.

The washed prothrombin precipitate was, by a single test, found to contain vitamin K.

Further investigation with more elaborate methods of concentrating the prothrombin must decide whether vitamin K is an essential constituent of the proenzyme.

A direct test of vitamin K for prothrombin activity was carried out and gave a negative result.

EXPERIMENTAL.

Precipitation of prothrombin from the plasma of normal and K-avitaminous chicks.

1. *Acetone method* [Howell, 1911].

(*a*) *Normal.* 3 ml. plasma were mixed with an equal volume of acetone at 0° and immediately filtered on a suction filter. After washing with ether the precipitate was dried on the filter-paper in a vacuum desiccator and dissolved in 3 ml. Ringer's solution; undissolved particles were removed by centrifuging.

(*b*) *K-avitaminous.* 3 ml. plasma were treated as above and the precipitate was dissolved in 3 ml. Ringer's solution.

2. *Modified acetone method.*

(*a*) *Normal.* 4 ml. plasma were mixed with 4 ml. acetone at 0°, centrifuged and the precipitate washed with acetone and ether, all these operations being

performed at 0°; after drying in a vacuum desiccator, the precipitate was dissolved in 4 ml. Ringer's solution.

3. *Acetic acid method* [Mellanby, 1930].

(*a*) *Normal.* 30 ml. plasma were diluted with 300 ml. water at 0° and brought to p_H 5·3 by means of 1 % acetic acid. After 1 hour the precipitate was separated by centrifuging, washed with acetone and ether and dried in a vacuum desiccator. All operations up to the centrifuging were performed in paraffin-coated vessels at 0°; weight of the precipitate, 239 mg. 7·9 mg. were dissolved in 1 ml. Ringer's solution.

(*b*) *K-avitaminous.* 5 ml. plasma were treated in the same way as (3 *a*); weight of the precipitate, 30 mg. The whole was dissolved in 5 ml. Ringer's solution.

Preparation of an emulsion of the lipoids.

4. 9 ml. plasma were allowed to stand with 10 ml. alcohol (96 %) for 24 hours, centrifuged and the precipitate washed twice with alcohol and three times with ether. The combined liquids were taken to dryness *in vacuo* and extracted with ether. The evaporated ether extract was dissolved in acetone and allowed to flow in small droplets into 9 ml. water with constant shaking; after evaporation of the acetone *in vacuo* the solution was made up to 9 ml.

Preparation of an emulsion of the lipoids from the prothrombin preparation (2 a).

5. The combined acetone and ether washings were dried *in vacuo*, dissolved in acetone and emulsified as above. The volume of the emulsion was made up to 2·5 ml. after evaporation of the acetone.

Emulsion of a concentrate of vitamin K.

6. 8·8 mg. of a concentrate of vitamin K from alfalfa, containing 650,000 units per g., were dissolved in light petroleum (10 ml.). 1 ml. of this solution was taken to dryness *in vacuo*, dissolved in acetone and mixed with 1 ml. of distilled water. After evaporation of the acetone *in vacuo* the volume was adjusted to 1 ml. 1 ml. of the emulsion contains 570 units.

Dried plasma.

7. Plasma from 2 normal hens was taken to dryness in a vacuum ($CaCl_2$) desiccator, spread in a thin layer on porcelain discs, and made into tablets. This plasma was practically colourless.

Prothrombin precipitate for vitamin K test.

8. 940 mg. precipitate were prepared from 99 ml. normal plasma (intense yellow) from 4 normal hens, the acetic acid method described under (3 *a*) being used. The precipitate was tested for prothrombin and made into tablets.

Lipoids from normal plasma for vitamin K test.

9. 60 ml. normal plasma (intense yellow) were stirred with 120 ml. 96 % ethyl alcohol, placed in ice for 30 min., centrifuged and the precipitate washed with alcohol and ether until the ether remained colourless. The liquids were evaporated and the residue extracted with ether. The combined extract weighed 748 mg.

Tests for prothrombin activity. As test substrate plasma from K-avitaminous chicks with an *S* value above 2000 was used [*cf.* Schønheyder, 1936, 2]. This plasma was diluted with Ringer's solution—equal parts—and a certain amount (measured by means of a dropping pipette giving 20 droplets per ml.) of the

preparation to be tested as well as a thrombokinase solution were added. Thrombokinase from hen's muscle is available in the form of "Thrombisol" a watery solution prepared by the firm "Leo" Copenhagen. Corresponding experiments without thrombokinase served as controls for thrombin activity of the preparations. The apparatus used for determining the clotting time was that of Fischer [1930; see Schønheyder, 1936, 2]. The "thrombisol" must be so concentrated that it gives a suitable clotting time, *e.g.* 5–7 min., with the plasma from K-avitaminous liver when no prothrombin is added (*cf.* the experiments in which Ringer's solution was used instead of thrombokinase). If necessary, the thrombisol must be diluted with Ringer's solution before use. The activity of the prothrombin preparations is demonstrated by the difference in clotting time between the experiments with Ringer's solution and those with "prothrombin". The clotting times in the experiments with lipoid emulsions are to be compared with the corresponding experiments with distilled water instead of emulsion.

The results appear in Table I, the plasma and thrombokinase being the same in each experiment.

Table I.

Exp. no.	Preparation	Droplets	Diluted "K-avitaminous" plasma droplets	Thrombokinase droplets	Clotting time (sec.)
1	Ringer's solution	1	5	1	285, 300, 315, 300
	Prothrombin, normal 1*a*	1	5	1	130, 130
	Normal hen's plasma	1	5	1	85, 85, 85, 85
	Prothrombin, normal 1 *a*	1	5	0	>600, >600
	Prothrombin, sick 1 *b*	1	5	1	315, 315
	Prothrombin, sick 1 *b*	1	5	0	>600, >600
2	Ringer's solution	1	5	1	390, 390, 405, 435 450, 435, 450, 465
	Prothrombin, normal 3 *a*	1	5	1	135, 135
	Prothrombin, normal 3 *a*	1	5	0	>900, >900
	Prothrombin, sick 3 *b*	1	5	1	375, 390, 375, 390
	Prothrombin, sick 3 *b*	1	5	0	>720, >720
3	Lipoids of normal plasma 4	3	3	1	210, 210
	Distilled water	3	3	1	195, 210, 210, 210
4	Ringer's solution	1	5	1	195, 225, 210, 210 195, 180
	Prothrombin, normal 2*a*	1	5	1	90, 90, 90
	Prothrombin, normal 2*a*	1	5	0	>1800, >1800
	Distilled water	1	5	1	165, 165
	Lipoids from prothrombin 5	1	5	1	170, 170, 180, 180
	Equal parts of 2*a* and 5	1	5	1	105, 105, 105, 105 105, 90
	Equal parts of 2*a* and 5	1	5	0	>1800, >1800
5	Distilled water	2	4	1	240, 285
	Vitamin K concentrate 6	2	4	1	270, 300
6	Distilled water	1	4	1	345, 345
	Vitamin K concentrate 6	1	4	1	345, 345

Tests for vitamin K. The method of Schønheyder [1936, 2] was used.

1. 3×1 g. dried plasma were given to a chick weighing 233 g. The S value was found to be 160, corresponding to a vitamin K content of 46 units per g. dry matter.

2. 3×310 mg. prothrombin preparation were given to a chick weighing 192 g. $S = 180$ corresponding to 142 units per g.

3. 3×222 mg. lipoid extract were given to a chick weighing 233 g. $S = 143$, corresponding to 323 units per g. lipoid.

Discussion.

Exp. 1 shows that the prothrombin precipitate (1*a*) from a normal chick (Howell's acetone method) accelerates the clotting time from about 300 sec. to 130 sec., whilst the corresponding precipitate (1*b*) from a K-avitaminous chick has no influence. The normal plasma itself has a somewhat stronger action than the prothrombin preparation (1*a*) (acceleration to 85 sec.). The effect of the prothrombin preparation (1*a*) is not due to thrombin, since the preparation without kinase does not clot the plasma within the time of observation (600 sec.).

Exp. 2 shows corresponding results for prothrombin precipitated by acetic acid according to Mellanby (3*a* and 3*b*).

Exp. 3 shows that the lipoids of normal plasma do not accelerate clotting.

Exp. 4 shows that the prothrombin precipitated from normal plasma by acetone (2*a*) in a modified way which secures a better removal of lipoids than the original Howell method, also accelerates the clotting, and that the lipoids (5) which are removed from the precipitate during washing with acetone and ether do not have any accelerating effect. A mixture of the prothrombin preparation (2*a*) and the lipoids (5) is no better than the prothrombin itself.

Exps. 5 and 6 show that vitamin K does not act as prothrombin *in vitro*. The number of K-units which have been added in Exp. 5 is 57 in 7 droplets of liquid = 0·35 ml., corresponding to 163 units per ml. This is a very large amount as compared with the content of normal plasma. The pale normal plasma (7) contained 46 units of vitamin K per g. of dry matter, which is about 3 units per ml.

The lipoid fraction and the prothrombin fraction of intensely yellow plasmata were found to contain 323 and 142 units per g. respectively. It is thus possible that vitamin K circulates in the plasma in two different forms, one of which is easily extractable with acetone and ether whilst the other is more firmly combined with the proteins. Further investigation is, however, required to settle this question and to ascertain whether vitamin K is also a constituent of more concentrated prothrombin preparations.

Certain authors [Nolf, 1905; 1908; Gratia, 1914] have suggested that prothrombin is formed in the liver. It might therefore be expected that vitamin K would act through the liver of the chick and be stored there. It is, in this connection, interesting to note that Dam and Schønheyder [1936] found very little vitamin K in the liver of a normal chick—less than 11 units per g. dry matter, a figure which is much smaller than that found in the plasma.

Summary.

1. It is possible to precipitate prothrombin from the plasma of normal chicks by means of the acetone method of Howell and the acetic acid method of Mellanby, whilst the corresponding precipitates from the plasma of K-avitaminous chicks are inactive as prothrombin.

2. The prothrombin precipitate from normal chicken plasma is still active after removal of lipoids by acetone and ether, and the lipoid obtained in this way has no prothrombin activity. This also holds good for the lipoid which can be extracted from the plasma itself by alcohol and ether.

3. A concentrate of vitamin K was found not to accelerate clotting *in vitro*, when tested against plasma + thrombokinase.

4. The prothrombin preparation precipitated by acetic acid (Mellanby) and washed with acetone and ether was found to contain vitamin K. The interpretation of this result, however, requires further investigation.

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