CXXX. THE OCCURRENCE AND CHEMICAL NATURE OF VITAMIN K.

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(Received March 27th, 1936.)

It has been shown that the antihaemorrhagic factor of the chick is a fat-soluble and rather thermostable vitamin which can be distinguished from vitamins A, D and E [Dam, 1935, 1, 2; Schönheyder, 1935; Almquist and Stokstad, 1935, 1, 2]. One of the sources of this vitamin—for which the letter K has been proposed [Dam, 1935, 1, 2]—is hog liver fat, where it may be found in the easily soluble non-sterol fraction of the non-saponifiable matter, when cold saponification is used. On partitioning between light petroleum and 90 % methyl alcohol the vitamin was found in the light petroleum layer [Dam, 1935, 1, 2]. Its occurrence in hog liver fat, hemp seed *etc.* was established by means of the preventive method.

Since then a curative and more convenient method has been published by Schönheyder [1936] and it has thereby been possible to determine the content of vitamin K in different foodstuffs with some degree of accuracy and to gain further information about the chemical properties, which are to be taken into account during attempts to isolate the substance.

The method used in the assay was that described by Schönheyder [1936]. The substances were given on 3 successive days in the form of weighed tablets, either alone or, when fats and oils were to be tested, in mixture with a certain amount of the basal diet, one or two animals being used for each determination.

The unit is that amount of the vitamin which is required per g. of the animal on 3 successive days in order to render the clotting power of the blood normal (the whole treatment of an animal weighing 333 g. will require 1000 units).

The simple chemical treatments used in connection with the work will appear from the tables and description.

Table I.

	Units per g.
Dried cabbage, white*	230
Dried cabbage, red*	60
Dried cabbage, "Spitzkohl"*	240
Dried kale*	260
Dried spinach*	270
Dried spinach, "selected quality"†	540
Dried alfalfa, Producer I†	136
Dried alfalfa, Producer II†	250
Fresh carrot, calculated on dry matter	190
Fresh potato, calculated on dry matter	20
Barley	15
Dried hog liver‡	100
Dried dog liver§	67
Dried chicken liver, normal§	< 11
Dried chicken liver, K-avitaminous§	< 11
Dried cod liver§	10

* Dried in air 60°.

† Dried commercially in air of high temperature for a very short time.

‡ Dried in air 40°.

§ Dried in a vacuum desiccator at room temperature.

1. Occurrence of vitamin K in different plant materials and livers.

The figures in Table I do not pretend to represent mean values but are merely obtained by means of 1 or 2 samples of each substance. They do, however, give a very valuable orientation.

The high activity of vegetables is obvious, and the statement of Almquist and Stockstad that alfalfa is an active material is confirmed by these as well as by our previous findings.

It is perhaps somewhat surprising that the liver of normal chicks is a rather poor source of vitamin K.

2. General chemical properties of vitamin K in hog liver fat.

Hog liver fat was, as mentioned before, one of the first sources used in our early investigations on vitamin K. Table II shows the result of different chemical treatments of this substance.

Table II.

	Units calculated per g. liver fat	Loss by saponifi- cation %
Hog liver fat procured 4. vi. 35:		
Without any treatment	266	288
Without any treatment	309	
Non-saponifiable matter (cold saponification 5 min. with the calculated amount of KOH in methyl alcohol)	107	63
(a) Fatty acid fraction	0	—
Fatty acid fraction	0	—
Water-soluble fraction obtained by the saponification	0	—
Liver fat after removal of the free fatty acids	307	—
Hog liver fat procured 16. iii. 35:		
Without any treatment	490	—
(b) Non-saponifiable matter freed from cholesterol by crystallisation from light petroleum (cold saponification 6 hours with 100 % excess of KOH in methyl alcohol)	139	129
The same	119	
Non-saponifiable matter (b) again treated with the same amount of the saponification agent at room temperature for 45 hours	93	28
Non-saponifiable matter (b) treated with the saponification agent on the boiling water-bath for 6 hours	0	100
Non-saponifiable matter (b) + fatty acids (a)	370	—
Non-saponifiable matter (b) + fatty acids (a)	400	—
Non-saponifiable matter (b) heated at 100°, 13 hours	143	—
Non-saponifiable matter (b) + ether containing peroxide, 23 hours	101	—
Liver fat after standing in light and air 147 days	143	—

During work with the preventive method we noted that the process of cold saponification involved a loss in vitamin K activity. This observation was fully confirmed by the curative technique, not only for hog liver fat but also for plant extracts (the saponification experiments with the latter are not reported in this paper in order to avoid unnecessary repetition). It appears from the table that cold saponification lessens the activity about 60–70 %, the duration of the treatment and the excess of KOH being of relatively small influence, while hot saponification destroys the activity completely. The fatty acid fraction (obtained by ether extraction at acid reaction after complete removal of the non-saponifiable matter at alkaline reaction) was found to be inactive in accordance with previous observations by means of the preventive method. The water-soluble

fraction (containing glycerol, glycerophosphoric acid *etc.* and eventually unknown compounds) was also, after neutralisation and precipitation of inorganic salts by alcohol, found to be inactive.

When the fatty acids were added to the non-saponifiable matter, in the same proportion as in the liver fat, the activity was to a large extent restored. The significance of this interesting result is being investigated further. Removal of the free fatty acids of the liver fat had no influence (hog liver fat contains much free acid, the sample from 4. vi. 35 had a saponification value of 160.5 and an acid value of 79.5).

Heating at 100° for 13 hours did not diminish the activity of the non-saponifiable matter. This finding is in accordance with our previous results with liver fat and with the results of Almquist and Stokstad.

Ether containing peroxide (giving a black precipitate with metallic mercury) did not, within 23 hours, effect any large destruction of the vitamin in the non-saponifiable matter, but keeping the liver fat in light and air in the laboratory for 147 days diminished the activity of the liver fat to 29 % of the original value. Whether this is due to the direct action of the light or to rancidity has not yet been elucidated.

Channon *et al.* [1934] have isolated a new hydrocarbon from hog liver fat ($C_{45}H_{76}$ or $C_{50}H_{84}$) which has about the same solubility properties with regard to light petroleum and methyl alcohol as vitamin K. A sample of this substance, courteously furnished by Prof. Channon, was tested for vitamin K; 17 mg. were completely inactive when given to a chick weighing 400 g.

3. Extraction of the vitamin from vegetables.

Previous experiments with hemp seed disclosed the fact that it is difficult to extract the vitamin from this material completely with ether. We have therefore now tested the effect of different solvents on a green vegetable, *viz.* a sample of dried alfalfa. The extraction was carried out in a Soxhlet apparatus surrounded with a vapour jacket whereby the solvent was kept at the boiling point while in contact with the substance to be extracted (see Table III).

Table III. *Extraction of dried alfalfa (138 units per g.) at the boiling point of the solvent.*

Solvent	Duration of extraction hours	Extract % of dried alfalfa	Units per g. extract	Units in the extract of 1 g. dried alfalfa	Apparent output %
Ether	50	3.5	3900	136	100
Alcohol 99 %	10	5.37*	5000*	268*	197
Acetone	3.5	7.15	4160	296	218
CCl_4	13	4.35	4160	174	128

* Calculated on the ether-soluble portion of the alcoholic extract.

It is obvious that a prolonged treatment with ether would appear to extract all the vitamin of the sample (138 units per g.). But the results with the other solvents show that it is possible to obtain a somewhat larger quantity of the vitamin in the extract than that which was found by direct measurement of the dried green vegetable. This effect, which has been established in a series of experiments (not described in this paper) is most probably to be explained by assuming that the animal alimentary tract cannot completely extract vitamin K from dried green vegetables while a much better extraction may be obtained by means of suitable solvents. Acetone appears to be particularly suited for the purpose.

When referring to the content of vitamin K in a sample of dried green vegetable it is, therefore, necessary to distinguish between the effective value, found by feeding the vegetable directly, and the absolute content found after suitable extraction.

4. *Further concentration of the vitamin.*

During the work on liver fat with the preventive technique, it was found that the vitamin remains in the light petroleum layer when the petroleum solution is shaken repeatedly with 90 % methyl alcohol. Almquist and Stokstad, however, state that they found the light petroleum and the methyl alcohol fractions equally active. By means of the present method we have found that when the light petroleum-soluble portion of an acetone extract from alfalfa containing 11,000 units per g. was shaken with 2/3 of its volume of 90 % methyl alcohol three times, the petroleum fraction, representing 39 % of the whole, had 25,000 units per g. while the methyl alcohol fraction had less than 250 units per g. After separation of a less active fraction from absolute alcohol by leaving it in the ice box for several days, and repetition of the partitioning between light petroleum and 90 % methyl alcohol it was possible to obtain a preparation containing 190,000 units per g.

By adsorption on Al_2O_3 and subsequent elution with benzene-alcohol it was possible to recover 87 % of the dry matter from this material, and this fraction (filtrate + eluate) could be shown to be practically inactive. Al_2O_3 , apparently, holds the vitamin (and certain of the chlorophyll decomposition products) so firmly that elution is practically impossible in the ordinary way. This experiment shows that, assuming that the vitamin is not destroyed by the adsorption on Al_2O_3 , the pure vitamin must contain more than $2 \cdot 10^6$ units per g.

When working with small quantities, it was possible to adsorb the vitamin on CaCO_3 or cane sugar from light petroleum. When the pigments including chlorophyll had passed through the column, the vitamin could be eluted by methyl alcohol (or by dissolving the sugar in water and shaking with light petroleum). In this way about the half of the vitamin could be obtained, from CaCO_3 , in a concentration of 600,000 units per g. while the treatment with cane sugar yielded about one-third in a concentration of 10^6 units per g. This latter preparation is a viscid oil. It is the strongest concentrate hitherto prepared. 1 mg. is sufficient to render the clotting of the blood of a 333 g. chick normal.

The concentration and adsorption methods are at present being elaborated with a view to large scale operation and final isolation of the substance.

The concentration process.

2.15 kg. of dried alfalfa (effective content 250 units per g.) were extracted with acetone in a Soxhlet apparatus for 21 hours. The acetone was distilled off and the residue was repeatedly agitated with light petroleum and filtered. Weight of light petroleum extract 70 g. with 11,000 units per g.

The light petroleum solution, 1270 ml., was treated with 810 ml. methyl alcohol and 90 ml. water 3 times in a tap-funnel.

Light petroleum fraction 27 g. with 25,000 units per g.

Methyl alcohol fraction 43 g. with < 250 units per g.

The light petroleum fraction was taken to dryness, dissolved in absolute alcohol and placed in the ice-box for several days. After filtration the dissolved portion contained about 50,000 units per g. After repeated shaking with 90 % methyl alcohol, the light petroleum-soluble fraction was brought up to a strength of 190,000 units per g.

Adsorption on Al_2O_3 (Merck puriss). 2.3 g. substance (440,000 units) in 50 ml. light petroleum were passed through a 5×30 cm. column of the adsorbent, washed with light petroleum and a mixture of this and benzene (30 + 70), elution with benzene-ethyl alcohol (50 + 50):

Filtrate: 0.2 g. (crystals resembling hentriacontane).

Combined eluate: 1.8 g. (including carotene zones and a green-brown zone with subdivisions).

Filtrate and combined eluate contained <20,000 units per g. <40,000 units in all.

Adsorption on finely powdered cane sugar. 1 ml. of a solution of the concentrate in light petroleum containing 56 mg. = 10,600 units, was passed through a 20×20 mm. column of the adsorbent and washed with light petroleum until the pigment zones had just passed through the column. The adsorbed substance was then eluted by dissolving the sugar in water and shaking the solution with light petroleum several times.

Eluate: 3.6 mg. with 10^6 units per g. = 3600 units. Filtrate: 50.4 mg. with 60,000 units per g. = 3000 units.

Adsorption on $CaCO_3$ (Merck pro analysi). 1 ml. of the same solution was passed through a 15×22 mm. column of the adsorbent and treated in the same way. The elution was effected by methyl alcohol-acetone (equal parts):

Eluate: 8.8 mg. with 600,000 units per g. = 5300 units. Filtrate: 50.8 mg. with 60,000 units per g. = 3000 units.

SUMMARY.

1. A series of substances has been tested quantitatively with respect to their content of vitamin K. Green vegetables are particularly rich sources of the vitamin.

2. Cold saponification of hog liver fat lessens the activity to about one-third of the original value and hot saponification destroys it completely. The fatty acid fraction is inactive *per se* but appears to enhance the activity of the non-saponifiable matter obtained by cold saponification. The thermostability of the vitamin is confirmed.

3. The efficiency of different solvents in extracting vitamin K from dried alfalfa has been studied. Acetone has been found to be particularly suited for the purpose. Certain solvents (alcohol, acetone) extract more vitamin K from dried alfalfa than could be found by directly feeding the vegetable. This is explained by assuming that the extraction in the alimentary tract is not complete.

4. Attempts to concentrate the vitamin from dried alfalfa have been made. The method of removing inactive material from a light petroleum solution of the extract by means of 90% methyl alcohol has given good results. Al_2O_3 adsorbs the vitamin so firmly that it cannot be eluted by ethyl alcohol-benzene, but it is possible to obtain a concentration up to 600,000–1,000,000 units per g. by means of $CaCO_3$ or cane sugar.

Part of this work was aided by a grant from P. Carl Petersens Fond.

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