III. THE CHEMICAL CONCENTRATION OF VITAMIN K

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THE chemical concentration of vitamin K, the blood-clotting vitamin (fat-soluble antihæmorrhagic vitamin), has been dealt with in a series of papers by Dam [1935, 1, 2; 1936], Dam & Schönheyder [1936, 1, 2], Almquist & Stokstad [1935, 1, 2], and Almquist [1936, 1, 2].

We have now worked up a large quantity of alfalfa and carefully controlled each step of the preparation by means of the method of Schönheyder [1936] which is based upon the specific action of the vitamin to sustain the clotting power of the blood. The aim was to prepare a large amount of a concentrate to be used as starting material for the final isolation and to find out the behaviour of the vitamin towards various methods and reagents, some of which are being used in the isolation process.

BIOLOGICAL TECHNIQUE

The writers have an extensive experience of the test method of Schönheyder [1936]. The animals were procured from the dealer when weighing about 100 g. and then placed on diet 60. They were operated on before and after ingestion of the substance (which took place on 3 consecutive days) and the S -value was determined. The clotting agent, aqueous preparation of hen's lungs, was tested against normal chicken plasma many times during the period (up to more than 1 month) in which it was used, and the mean value was found graphically by plotting the concentrations against the dates. Only chicks with an S_1 -value of above 300 (in most cases above 1000) were used for the tests.

The chemical concentration has been carried out at the same time as the biological researches of different workers in this laboratory. It has turned out that in some groups of animals the full development of the high S_1 -value, corresponding to the enormous fall in clotting power, takes a much longer time than in others (in spite of the occurrence of hæmorrhages), apparently independently of the diet. This phenomenon, which is being investigated, has at times retarded the work, and this is the reason why the concentration process, which was started in January, has required about 5 months. This phenomenon, which resembles refection in vitamin B experiments, may in its most extreme form, even lead to suppression of hæmorrhages and is probably the reason why certain authors have denied the existence of the alimentary hæmorrhagic disease.²

Certain other workers use the preventive method and calculate the percentage of the animals which show hæmorrhages within 14 days. We consider this procedure to be inaccurate because hæmorrhages may occur in the broad range of S -values from 70 to 30,000. The S -value 70 corresponds to an amount of vitamin K about 40% of that which renders the clotting power normal (compare the diagram in the paper of Schönheyder [1936]). Dam (up to 1935) and Dam & Schönheyder [1934] have previously used the preventive method but

¹ With the collaboration of Mrs I. Svendsen.

² Almquist & Stokstad [1936] have recently described the same condition and set forth an explanation which is in general agreement with our experience.

have always kept the animals under inspection during at least 1 month. In their experiments the haemorrhages most frequently came at the age of about 20 days.

THE CONCENTRATION PROCESS

The concentration process is summarized in the following scheme:

The extraction was carried out in a continuous vapour extraction apparatus in batches of about 10 kg., each batch being extracted for 22 hours. After cooling a green precipitate formed, which has been previously found to be practically inactive. All evaporations were carried out in a vacuum still. The partitioning between light petroleum and 90 % methanol was carried out in portions of about 9 l., the methanol being regenerated and used again after determining how much water it was necessary to add in order to get the same volume of upper phase as when working with pure methanol.

Almquist [1936, 2] has claimed that the vitamin passes into the methanol, but this statement could not be confirmed in our experiments. It is possible that this discrepancy may to some extent be explained by the fact that Almquist does not use acetone for the extraction whereby less methanol-soluble material is extracted.

The treatment with solid CO_2 was as described by Karrer *et al.* [1931]. According to our experience, it seems possible to simplify the concentration process by extracting directly with light petroleum and to omit the treatment with acetic acid, whereby only a small part of the mixture is removed. The separation of the green material may be effected by the treatment with solid CO_2 .

Small portions of the oil marked X with 200,000 units per g. were subjected to further treatments in order to estimate the value of the different methods which offer themselves for the further concentration, and at the same time to get some information about the chemical properties of the substance.

As already mentioned by Dam & Schönheyder [1936, 1] vitamin K can be sublimed in a high vacuum. 54 mg. of the oil were now subjected to a sublimation process at 0.6×10^3 mm. Hg.

The sublimation vessel was of the usual type which has been described by Breusch [1934] but with unit ground joint instead of a rubber stopper. The pump was a mercury diffusion pump, Modell IV, Gaede, and the pressure was controlled both by a v. Reden and a mol-vacuummeter (Gaede). All connexions were of glass and the apparatus was furnished with the necessary cooling devices to stop vaporization from the substance and from the mercury. The grease for the ground joints was "apiezon". The whole process required about 3 hours,¹ and gave the following result:

Fraction 1: 130–155° light yellow oil,	11 mg.,	700,000 units per g.,	7700 units in all.
„ 2: 155–200° yellow oil,	7.5 mg.,	90,000 units per g.,	700 units in all.
„ 3: 200–250° orange oil,	23 mg.,	0 units per g.,	0 units in all.
Residue: green-brown oil,	13.5 mg.,	0 units per g.,	0 units in all.
Recovered: 8400 units in all (75%).			

A repetition of the experiment with a larger quantity, 621 mg., 124,000 units in all, in the same apparatus; pressure 2×10^{-3} mm. Hg:

Fraction 1: up to 160° light yellow oil,	82 mg.,	360,000 units per g.,	29,500 units in all.
„ 2: 160–180° yellow oil,	61 mg.,	590,000 units per g.,	36,000 units in all.
Residue: green-brown oil,	479 mg.,	33,000 units per g.,	16,000 units in all.
Recovered: 81,500 units (66%).			

That the most potent fraction in this experiment sublimes at a somewhat higher temperature than in Exp. 1 is most probably explained by the fact that the thickness of the layer of oil in the vessel was greater and the liquid was not boiling.

Fraction 2 contained 20% sterol, which could be removed by digitonin. It contained no nitrogen (micro-Dumas carried out with 7.490 mg.).

The ultraviolet absorption spectra of all the preparations were photographed by Prof. Schou, to whom the authors are indebted. No characteristic absorption was found. The spectrum in the visible region shows absorption in the blue and violet.

Distillation at a higher pressure, 0.1 mm. Hg (and up to 250°), of the oil Y led to destruction of the greater part of the vitamin: only 25% was recovered.

The authors have studied the availability of different adsorption media for the chromatographic separation of the oil X and similar preparations. It has previously been reported [Dam & Schönheyder, 1936, 2] that aluminium oxide

¹ A full account for this experiment was given by one of us (H. D.) to the 19th Congress of Scandinavian Natural Scientists, 11 August 1936, in Helsinki.

Since then a paper by Almquist [1936, 2] has appeared in which a similar result of a vacuum distillation at a lower pressure is described.

was unsuitable, and that the eluate (with methanol) was practically inactive in spite of the fact that it contained 90 % of the substance by weight. We have since then, with the same result, tried a long series of elution media including extraction with boiling alcohol in a Soxhlet apparatus. MgO behaves like Al_2O_3 , and even the solution of the adsorbent in the calculated amount of dilute HCl and extraction in a tap funnel with ether did not yield an active eluate. It is therefore concluded that these strong adsorption media destroy the vitamin.

Silicic acid also destroyed a large portion of the vitamin.

CaCO_3 and cane sugar have a very weak adsorptive power. The separation with these adsorption media could not be reproduced.

Burned gypsum appears to be one of the mildest adsorption media by which separation from a trace of chlorophyll derivatives and carotenoid pigment can be effected. It destroys up to 50 % of the activity depending on the duration of the treatment.

Oil X is completely resistant to treatment with acetic anhydride at 100° for $\frac{1}{2}$ hour: 10 mg. of the oil (2000 units in all) + 0.2 ml. acetic anhydride; evaporation of every trace of the reagent *in vacuo* at 50° after the treatment; recovered 2000 units = 100 %.

Addition of excess of bromine in ether to the oil led to the formation of a green and thereafter a white precipitate; after standing with excess bromine in ice-water for half an hour and removal of the free bromine with sodium sulphite, the activity was lost. Addition of bromine to the distillation fraction 2 (distillation no. 2), after removal of sterol by means of digitonin, did not lead to the formation of a precipitate.

It is interesting to note that after treatment with trimethylacethydrazide-ammonium chloride [Girard & Sandulesco, 1934; 1936] the vitamin is found in the non-ketone fraction: 288 mg. of oil Y (with 280,000 units per g.) were boiled in 5 ml. absolute alcohol with 0.5 ml. acetic acid and 250 mg. of the reagent (*T*) for 37 min., and then treated exactly as indicated by Girard & Sandulesco [1936]. As extraction media freshly distilled ether and light petroleum were used. The extracts were washed with water to neutrality before evaporation: non-ketone fraction: 240 mg. with more than 200,000 units per g.; ketone fraction: 10 mg. (possibly containing traces of the oil which were not removed by the first extraction of the non-ketone fraction), activity about 60,000 units per g.

SUMMARY

1. The concentration of vitamin K from alfalfa on the large scale using the Schönheyder curative test as control for the different steps of the preparation has been described.

2. Small portions of the concentrate (an oil with 200,000 units per g.) have been subjected to various treatments whereby it was established that:

(a) The oil may be concentrated 3 to $3\frac{1}{2}$ times by one sublimation in a high vacuum (10^{-3} mm. Hg and about 160°). The sublimate is a yellow oil which does not contain nitrogen.

(b) The vitamin in the concentrate is resistant to treatment with acetic anhydride at 100° for $\frac{1}{2}$ hour.

(c) Treatment with Girard's reagent (Trimethylacethydrazide-ammonium chloride) leaves the vitamin in the non-ketone fraction.

(d) Treatment with bromine in excess and removal of free bromine with sodium sulphite.

(e) Adsorption of the vitamin on strong adsorption media (Al_2O_3 , MgO, etc.) results in destruction.

3. The ultraviolet absorption of the different concentrates was examined by Prof. S. A. Schou. No characteristic absorption could be detected.

4. Further concentration of the oil is being attempted by means of repeated vacuum distillation and other methods, on the preliminary supposition that the vitamin has the chemical character of an ester.

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