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Vitamin K

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VITAMIN K.

Raymond C. McIllece

Senior Thesis
1940

UNIVERSITY OF NEBRASKA
COLLEGE OF MEDICINE
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INTRODUCTION

Vitamins have entered the lives of the people today with almost bombastic force. We are continually made conscious of their presence by advertisements, in printed form or by radio. These advertising media fling their challenge -- "Have you had your vitamins today?" That a new vitamin should make its appearance at this stage of vitamin popularity is appropos of the present times and their advancement.

The vitamin is K.

A few years ago, a young Scandinavian, Dam, and his associates (34,35), described a severe hemorrhagic disorder associated with plasma prothrombin deficiency. The causative factor was a deficiency of certain fat soluble substances from an otherwise adequate diet. Their experiments were performed upon chicks. (30,27)

The factor deficient from the diet of the chicks and responsible for the hemorrhagic tendencies of the chicks was called KOAGULATIONS VITAMIN, in the German and Scandinavian language. The abbreviated form and the name by which it is most commonly known today, is Vitamin K.

Vitamin K is proving to be of value in the clinical aspect of medicine, especially in surgery and obstetrics.
The aim and purpose of this paper is to point out the chemistry and physiology of the vitamin, as well as its occurrence in nature and the estimation of prothrombin. This being shown adequately, the next purpose of this article is to show the part Vitamin K plays in jaundice, by its action upon prothrombin levels and the clotting time of the blood plasma. This is with reference to jaundice occurring due to biliary fistula and to obstruction of the biliary ducts.

Jaundice has been known since antiquity. The deleterious effect it has upon the clotting time of the blood has been known since the advent of surgery. Vitamin K, I firmly believe, will be a powerful weapon for the surgeon in the treatment of hemorrhage occurring because of the obstructive jaundice and biliary fistula. By its usage, he will be able to operate when the operation is vitally needed.

Finally, the question of therapeutics will be touched upon.
DISTRIBUTION

A search for an anti-hemorrhagic factor, which was precipitated by Dam and Assac's discovery of a hemorrhagic disorder attributable to a dietary deficiency, has been carried on intensively and unceasingly by investigators. The trial and error method was obviously the only method to use in the search of the source and distribution of the anti-hemorrhagic factor.

Dam and Assac ran a series of tests and found the factor to be contained in the following in varying amounts: (29, 30) (35)

<table>
<thead>
<tr>
<th>Alfalfa</th>
<th>Cabbage</th>
<th>Spinach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass</td>
<td>Cauliflower</td>
<td>Nettle</td>
</tr>
<tr>
<td>Chestnut</td>
<td>Pine Needles</td>
<td>Peas</td>
</tr>
<tr>
<td>Sunflowers</td>
<td>Spruce Needles</td>
<td>Tomatoes</td>
</tr>
<tr>
<td>Strawberries</td>
<td>Ripe Hips</td>
<td>Hemp</td>
</tr>
<tr>
<td>Seeds of Sunflowers</td>
<td>Soy Bean  (12)</td>
<td>Roots</td>
</tr>
<tr>
<td>Tuber Bulbs.</td>
<td>Feces  (52)</td>
<td></td>
</tr>
</tbody>
</table>

These were examined and found to possess protective qualities for the hemorrhagic entity. Alfalfa proved (76) to be quite efficacious in raising the prothrombin level in chicks, while sunflower seeds and roots proved to be a poor source for the factor.

The vitamin or anti-hemorrhagic factor was found to be in the photosynthetic portion of the plant, by (6) Almquist. This is proven by the fact that the tops of carrots contained the vitamin, while the rest of the carrot did not. The leafy portion of plants proved to
be a good source of Vitamin K. Almquist, Dam and Stokstad (36, 37) began using an extract of alfalfa with good results. A short time later, fish were found to be rich in the vitamin, if allowed to putrify before extracting the vitamin.

Osterberg (51) and Butt, et al, (26) found the extract of fish meal to be satisfactory, in their experiments on Vitamin K. Almquist, Dam, Stokstad and Glavind used extracts of alfalfa. Next, spinach made its appearance, and was used by Dam, et al, in their experiments. Dam designated 2 mg. of spinach as a unit of potency of the vitamin.

Vitamin K from putrified fish and the photosynthetic portion of plants, remained the principal source, until Almquist and Stokstad found the vitamin in feces of chicks. (3) Greaves (40) arrived at a similar conclusion from his experiments with rats. These findings focused attention upon the possibility of a synthesis of Vitamin K in the intestinal tract.

Greaves (39) (40), by using the extract of droppings of chicks which had been allowed to drop into 1% phenol, pointed out that synthesis of the Vitamin K takes place in vivo.

Snell (67), working on the source of Vitamin K and its possible synthesis in the intestinal tract,
found that certain bacteria contain the vitamin. *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and microorganisms were found to have the vitamin, even when grown upon vitamin free media, indicating it to be a product of bacterial metabolism.

Almqquist and Klose (8) then identified the form of Vitamin K (phthiocol) as the pigment of *Mycobacterium tuberculosis* (human). This pigment had been previously synthesized by Anderson and co-workers. (33) The possibility that Vitamin K, or a substance possessing the anti-hemorrhagic characteristics of Vitamin K, could be synthesized opened a new source for obtaining the vitamin. (37)

Feiser, et al, (38) and other investigators, working on the structure of the vitamin, found the active portion to be due to its naphthoquinone nucleous. Next, Ansbacher (15) observed that Vitamin K and other phytol (natural) derivatives of methyl naphthoquinone are not as active and not as readily utilized by the animal body as 2-methyl-1-4, naphthoquinone, which is not from nature, but synthesized in the laboratory. This man-made product proves to be more potent, more rapidly absorbed and more easily utilized by the body, than the Vitamin K obtained from nature.
In summarizing, we see the principal source of the anti-hemorrhagic factor is the photosynthetic portion of plants, putrified fish, hog liver oil, bacterial and synthetic source.
CHEMISTRY

In 1935, Hendrik Dam (30), while working on the nature and distribution of the anti-hemorrhagic factor, discovered that the factor is a fat soluble vitamin, occurring in hog liver, hemp seed and vegetables. The anti-hemorrhagic factor was localized in the fat soluble, unsaponifiable, non-sterol fraction. It proved to be stable to moderate heating. (7) (77)

In the early stages of the work on the anti-hemorrhagic vitamin, the extracted material was saponified in alcoholic potassium hydroxide to remove the fat and chlorophyll. Alfalfa was the source used. The extraction was performed by using hexane. This hydrocarbon solvent extracts less extraneous material than other types of solvents. It soon became apparent, however, that the non-saponified fraction had up to 50% less potency than the crude extract. Further treatment of a non-saponifiable preparation with alkali resulted in the loss of more than 75% of its activity. This showed that the vitamin was alkali labile.

It remained for Almquist (6) to add the next step to the knowledge of the new vitamin. He (7) had previously obtained concentrates of high potency by adding activated magnesium oxide (in place of the alkalis)
to the hexane extract, causing no loss of potency of the extract. Next, activated carbon was added to remove in part the orange and red pigment remaining, the carotene and xanthophyll. Following this, the step of concentrating the hexane solution and chilling out the fats and sterols. The hexane solution was evaporated to dryness under a vacuum. The solid residue was taken up with methyl alcohol at fifty degrees. This was chilled for twenty-four hours at a negative 1.1 degrees. The solution was then centrifuged, the solid reddish material containing sterols being discarded. The solution was again chilled, and the process repeated. Finally, upon the addition of 10 c.c. of water to the mixture, there ensued a separation of about 2 c.c. of a reddish oil. This was dried under a vacuum and dissolved in hexane, then allowed to evaporate under a vacuum at room temperature. These reddish oils contained the Vitamin K.

Almquist (6), by using these concentrates in a specially constructed retort which had a heating element constructed about the closed end of the retort, was able for the first time to obtain the antihemorrhagic factor in a crystalline form. It (6) was determined, at this time, by Almquist, that the final attainment of a colorless concentrate shows the
vitamin to be colorless. It was during this experiment that he confirmed his previous finding (10), namely, that the anti-hemorrhagic factor, Vitamin K, contained the Benzene Ring or nucleus.

Binkley, et al, (17) by the use of decolso, permutit and dorco as absorbents, have isolated Vitamin K in a practically pure state. Then, crystallization, distillation and recrystallization gave the pure vitamin. Next, they converted the vitamin into the diacetate of dihydro Vitamin K with a potency of 500 units per mg. Then, by means of the Grignard reaction, the diacetate has been converted back to Vitamin K, with 100% enhancement of potency, 1000 units per mg. This is an improvement over Almquist's method, in that he lost 50% of the vitamin and the concentration was unpredictable.

Vitamin K2 was converted to the diacetate of dihydro Vitamin K2 by the same method used for Vitamin K1. Binkley, et al, (19) offered this as proof that Vitamins K1 and K2 have been isolated. The names, Vitamin K1 and Vitamin K2, were given by Binkley, et al (20). In their article on "The Isolation of Vitamin K" (17), they explained their terminology as follows: "Since two different pure substances possessing Vitamin K activity were isolated, the
compound from alfalfa, which has the smaller molecular weight, was called Vitamin K₁. and the compound from nutrified fish meal having the larger molecular weight, Vitamin K₂."

During the past year, observations made by Almquist and Klose (5) (6) (7) (8) (9), have revealed that the naphthoquinone structure constitutes the essential structure of Vitamin K. A variety of naphthoquinones may cure the Vitamin K deficient chicks and other species, some in as low amounts as one mg.

According to Binkley, et al, (20) Vitamin K₁ is, as follows:

1. Obtained from alfalfa;
2. Vitamin K -- light yellow oil at room temperature;
3. Changes upon cooling into a crystalline condition;
4. Potency of about 1000 units per mg. (21)
5. Molecular Weight estimates vary from 443 to 568;
6. Absorption maxima 243, 248, 261, 270 and 323 μm.;
7. Structural formula was found to be 2-Methyl, 3-Phytlyl-1, 4-Naphthoquinone. (10)
8. See following page for structural formula.
Vitamin K2:

1. Obtained from fish meal;

2. Molecular weight estimates vary from 628 to 654; \( @9 \)

3. Potency approximately 600 units per mg. \( @1 \)

4. Absorption maxima 244, 249, 264, 270, 332; \( @7 \)

The chemical structure and formula of Vitamin K2 was recently revealed by Binkley, et al, \( @2 @ \) at the 34th Annual Meeting of the American Society of Biological Chemists, held March 13-15, 1940.

"A model experiment in which the diacetate of dihydro Vitamin K1 gave an excellent yield of 1, 4-diacetoxy-2-methylnaphtholene-3-acetaldhyde, which was further characterized as the semi-carbozone. Ozonization of the diacetate of dihydro vitamin K2 under the
same condition gave 1, 4-diacetoxy-2-methylnaptholene-3 acetaldehyde. Analysis and mixed melting point showed this aldehyde as well as its semicarbozone to be identical with the same compound obtained from Vitamin K. The isolation of this aldehyde shows conclusively that Vitamin K2 is a 2-methyl-1,4-naphthoquinone. From the ozonization reaction, levulin-aldehyde was isolated as the bis-2, 4-dinitrophenyl-hydrozone in 81% yield, based on the assumption that one mole of the vitamin would yield five moles of levulin-aldehyde."

"Acetone was also isolated from the ozonization mixture in 56% yield, assuming that one mole of acetone originates from one mole of Vitamin K2. The acetone was characterized as the 2, 4-dinitrophenyl-hydrozone and by idiometric titration."

C₄₁H₅₆O₂ is the empirical formula proposed as correct for Vitamin K2.
Ansbacher (10) stated that Vitamin K1 and other phytol derivatives are not as active, nor as readily absorbed by the animal system as 2-methyl-1-4-naphthoquinone, which is actually the nucleus of Vitamin K1 and Vitamin K2.

Emmet, et al, (36) have found that of the various compounds prepared in their laboratory 4-amino-2-methyl-1-naphthol and 4-amino-3-methyl-1-naphthol are the most potent and easiest to utilize. These preparations are taken up by the body more readily than the Vitamin K1 and Vitamin K2. These preparations likewise will go into solution, thus rendering them available and suitable for injection. These laboratory preparations owe their solubility in solutions and their greater ease of utilization in the human body to the difference in structure from that of Vitamin K1 and Vitamin K2. The difference lies in the fact that these preparations have the 1 and 4 positions, hydroxy group and the amino group.

The fact that these laboratory prepared compounds will go into solution, are three times as active as Vitamin K1, and are easily utilized by the body, promises to add a new concept to the antihemorrhagic therapy. (36)
New advancement will in all probability take place through these new preparations. The amino group will react with HCl to form the chloride of the substance. In the body, these preparations will be oxidized.

We see, therefore, both Vitamin K1 and Vitamin K2 have the same nucleus, namely, a 2-methyl-1,4-naphthoquinone.

The difference lies in their side chains. Vitamin K1 possesses the following side chain:

\[
\begin{align*}
\text{CH}_2\text{CH} &= \text{C} - \text{(CH}_2\text{)}_3 - \text{CH} - \text{(CH}_2\text{)}_3 - \text{CH} - \text{(CH}_2\text{)}_3 - \text{(CH}_2\text{)}_3 - \text{CH} \\
\text{CH} &= \text{CH}_3
\end{align*}
\]

Vitamin K2 possesses this side chain:

\[
\begin{align*}
\text{CH}_2\text{CH} &= \left[\text{CH}_3 - \text{CH}_2 - \text{CH}_2\right]_5 - \text{CH}_3
\end{align*}
\]
The chief functions of Vitamin K in the body, so far as is known, appears to lie in its utilization by the liver for the maintenance of a normal concentration of prothrombin in the blood plasma. Until the enigma of prothrombin, namely, its chemistry, has been solved, the mechanism by which the vitamin affects prothrombin will probably remain obscure.

"Because of their bearing upon the development of clinical states of Vitamin K deficiency, attention has been focused upon factors which influence the absorption of Vitamin K. Perhaps the most important of these are the bile salts". (27)

Proof for this fact may be seen in the absence of an adequate amount of bile salts from the intestines. This can be observed in obstructive jaundice and bile fistula. A resultant fall in prothrombin of the plasma occurs, while on the other hand, upon administration of bile salts, a resultant rise is seen in the plasma prothrombin.

Rhodes (59) states that sodium deoxycholate is probably the most effective bile salt per unit weight. He further states that the hemorrhagic tendency in jaundice is due to a deficiency in plasma prothrombin, which in turn, is due to a K avitaminosis,
and this results from a lack or absence of bile salts, in the intestinal tract.

Greaves (f) came to the conclusion that bile in the intestine is necessary for absorption. To prove his contention, he ligated the biliary ducts of rats, and maintained them on a stock diet. The rats became icteric, with a loss in prothrombin and also, a loss in the coagulability of the blood. When the flow of bile was again established into the intestinal tract, the prothrombin level increased, as did the coagulability of the blood to normal, within a short period of time.

Greaves kept his experimental rats on a low fat diet for a period of 30 to 35 days. Then a condition was noted, where the animals when inflicted with small cuts, underwent prolonged bleeding. The prothrombin levels were low. Administration of 2 - 3 c.c. of beef bile brought the prothrombin level up markedly. Here the low stock diet contained enough alfalfa to supply the normal demand for Vitamin K, yet the bile fistula rats developed loss of blood coagulability upon maintenance on this diet. Only when bile salts were added, or massive amounts of Vitamin K, did the coagulation time of the blood return to normal. These experiments point towards a
very significant possibility, that bile salts act as a carrying agent for Vitamin K across the intestinal tract.

Brinkhous, et al, (23) have studied the effect of feeding bile or bile salts supplemented with a fat soluble extract of alfalfa on the prothrombin level, in a number of cases of obstructive jaundice with hypoprothrombinemia. They found that the feeding of bile alone gave a rise in prothrombin level, but the rise was very gradual. In contrast, they obtained a rapid rise in the prothrombin level upon administering the bile and alfalfa extracts. In two of their cases, the prothrombin level rose rapidly during periods of increasing jaundice, indicating jaundice per-se is not the essential factor.

Hawkins and Brinkhous (43) fed bile to bile fistula dogs, and kept the plasma prothrombin levels within the limits of normal. The dogs were kept on a diet which contained Vitamin K in small quantities.

Snell (47), another investigator, is in accord with the above findings.

It remained for Greaves (59) to demonstrate that bile is necessary for the absorption of Vitamin K by the rat, and that in animals subsisting on a diet
deficient in Vitamin K, bile has no effect on the hemorrhagic tendencies, whereas, Vitamin K and bile salts are effective immediately.

These investigators, in their experiments and demonstrations in the rat, dog and in man, have shown fairly conclusively that bile acids act as carrying agents for the passage of the fat soluble, Vitamin K, across the intestinal mucosae.

The exact site of absorption of Vitamin K through the intestine has not been definitely proven. However, work performed by Dixon and Clark of Mayo's (25) indicates that the jejunum is the site where the absorption is at its maximum, while it does not seem to be absorbed by the stomach or colon. In substantiation of jejunum as the site of absorption, we might quote Snell's statement to the effect that in cases of Sprue and Regional Ileitis, "deficiency of prothrombin because of an insufficient intestinal absorption of Vitamin K, has been noted". (67)

The answer to the question of prothrombin formation and the role played by Vitamin K in this process is being sought by a number of investigators, at present. "There is considerable evidence that the liver is the chief, if not the only site of formation of prothrombin, and that an adequate amount of normally
functioning liver tissue is essential for the proper utilization of Vitamin K in this process." (27)

Warner (72) offers support to the above statement with his work on albino rats. He found that "extirpation of a large portion of the liver in rats resulted in a marked decrease in the plasma prothrombin". The plasma prothrombin level gradually returns to normal during the period required for restoration of the liver to its normal weight.

Roderick (61) reported in 1929, that cattle that were fed toxic sweet clover suffered from marked hypoprothrombinemia and hemorrhage. Autopsy revealed liver necrosis.

Recently, Smith, Warner and Brinkhous (65) reported a bleeding tendency in dogs that were inflicted with chloroform intoxication. Plasma prothrombin decreased in proportion to the liver injury.

Smith, Hoffman (40) and other investigators on this subject are agreed that the evidence compiled to date points strongly towards the liver as the site of formation of prothrombin. At present, little is known of the chemistry of prothrombin. Undoubtedly, rapid strides in the investigation of prothrombin and its source will take place, as soon as more is known of prothrombin per-se.
Recent investigations of Greaves indicate that Vitamin K is not stored in appreciable quantities in the liver of rats. (40) This necessitates the finding of a possible endogenous source of Vitamin K, in order to explain how, in the absence of Vitamin K from the diet, experimental animals maintain prothrombin levels within normal limits for long periods of time.

Greaves (39) investigated the possible manufacture or synthesis of Vitamin K in the intestinal tract. He found the anti-hemorrhagic factor in the droppings of chicks, which he collected and extracted and gave to chicks suffering from low prothrombin levels and hemorrhagic tendencies. The extract of droppings was added to a diet of K avitaminosis. These findings apparently substantiated the assumption that Vitamin K was synthesized in the intestinal tract by bacterial action.

Almquist and Stokstad (3) confirmed the above findings and concluded that the vitamin was evidently synthesized by bacterial action, either after the droppings were voided, or within the lower portion of the digestive tract where absorption could not readily take place, or both. In order to ascertain where the synthesis took place, they performed the following experiment.

- 21 -
The modified basal diet of Almquist (3) was given to chicks up until they were four weeks of age. The basal diet is as follows:

1. Ether extracted fish meal 17.5
2. Ether extracted dried Brewer’s yeast 7.5
3. NaCl plus small amounts of Cupric and Ferrous Sulfate 1.0
4. Cod Liver Oil 1.0
5. Ground polished rice 73.0

This diet contains ingredients approximately, as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>18.2%</td>
</tr>
<tr>
<td>Ether Extract (3)</td>
<td>2.6%</td>
</tr>
<tr>
<td>Ash</td>
<td>4.9%</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>0.5%</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.9%</td>
</tr>
</tbody>
</table>

The above diet, when supplemented with the anti-hemorrhagic factor, allows chicks to maintain good health and normal growth. The diet not so supplemented causes severe hemorrhages and eventual death.

Droppings from these four week old chicks were collected after the feces had dropped into trays containing 1% Phenol in aqueous solution. The rationale of the Phenol was to inhibit further bacterial action. The droppings were collected at 24 hour intervals. The droppings were then dried by several changes of ether.
The alcohol and ether extracts were mixed and diluted with a large amount of water. The ether solution was washed several times with sodium hydroxide solution and water. As a source of anti-hemorrhagic vitamin, the ether solution was found to be adequate at an equivalent level of 8%, marginal at 4% and inadequate at 2%. The potency of the solution was somewhat less than that of the extract obtained from droppings falling on the ground.

Later, Almquist, et al. (9) showed that bacteria, in the following manner, are essential to the synthesis of Vitamin K:

1. Fish meal sterilized gave no protection to chicks;
2. Fish meal, plus bacteria, protected chicks against hemorrhage.

Recent observations of Almquist, Pentler, and Meechi, (9) and Snell (67) indicate that an anti-hemorrhagic factor possessing similar qualities as found in Vitamin K, is present in certain species of bacteria, namely, Escherichia coli, Staphylococcus aureus, Bacillus subtilis and many microorganisms.

Recently, Ansbacher and Fernholz demonstrated the Vitamin K activity of phthiocol, which contains the naphthoquinone nucleus. This nucleus is found in Vitamin K1 and Vitamin K2 and the pigment of Mycobacterium tuberculosis. (13)
These observations indicate Vitamin K, or Vitamin K like substances, are present in certain species of bacteria and are the product of bacterial metabolism.

While much has been accomplished, there still remains a considerable amount of investigation of the role played by bacteria in the production of Vitamin K, or Vitamin K like substances, in the intestinal tract. Whether such a role will prove important or not, remains to be disclosed in the future.
CLINICAL ESTIMATION

The presence of Vitamin K deficiency is detected clinically by the demonstration of a decrease in plasma prothrombin.

Howell (14) used the clotting time of recalcified plasma as a measure of the prothrombin present. However, Quick (53) pointed out that in reality, the so-called "Howell's Prothrombin Time" method does not apparently determine prothrombin time, as the thromboplastin from the platelets is even more important in influencing the clotting time than is prothrombin.

Warner, et al, (70) originated a method for the quantitative estimation of plasma prothrombin which, although very exact and precise, is regarded as too complex for routine use in the clinical laboratory. Their method for the quantitative estimation of plasma prothrombin (70) differs from Quick's method in the following manner. Quick, et al, use the total clotting time, which is made up of the prothrombin conversion time and the time required for the thrombin to react with the fibrinogen. Warner, et al, separated the two phases by converting the prothrombin to thrombin, then, by means of a serial dilution technique, the thrombin that is formed may be titrated.
Previously, the prothrombin was partially altered in preliminary purification, or was converted incompletely into thrombin. Often the thrombin formed was allowed to disintegrate partially before titration, with resultant weak thrombin solutions which represented a small and variable fraction of the total plasma prothrombin.

In Warner's method, care has been taken to effect complete and rapid conversion of prothrombin to thrombin. Also, by making the serial dilutions before activating the prothrombin instead of after the thrombin has been formed, the possibility of thrombin disintegration has been mitigated. This so dilutes the anti-thrombin that it does not interfere with the titration. "The problem of fixed standards has been solved by using a standard clotting interval as a fixed point. The degree to which the unknown solution is diluted in reaching this point, gives a measure of the amount of thrombin present. We are then able to express this amount in units of dilution." (70)

"The prothrombin of plasma can be converted completely into thrombin by adding an optimal amount of calcium and an excess of thromboplastin. For convenience in this work, we have defined thrombin of unitary concentration as being of just sufficient strength so that in
the presence of fibrinogen (0.08 - 0.10 percent), it will form a clot in fifteen seconds. The pH of the reagents is adjusted to 7.4 and the titration is carried out at a room temperature of 28 degrees C. Prothrombin of unitary concentration is defined as being of sufficient strength, so that when completely converted, it will form an equal volume of unitary thrombin." (70)

The reagents used, are as follows:

1. Oxalated saline;
2. Tissue extract (thromboplastin);
3. Oxalated plasma;
4. Fibrinogen;
5. Calcium;
6. Heparin.

For the preparation of the above mentioned reagents, the reader is referred to the original article of Warner (70).

The plasma is defibrinated before titration is carried out. This is accomplished by using thrombin for the defibrination. The thrombin is prepared by mixing three drops of tissue extract, calcium and plasma, with six drops of saline. The thrombin is expressed from this mixture and used at once. "Thirty drops of the plasma were now defibrinated by adding three drops of thrombin. A clot is formed in eighteen seconds. After allowing fifteen minutes to insure complete clotting and destruction of excess thrombin, the fibrin was
rolled out. The expressed fluid, containing the pro-
thrombin was diluted 1-20, 1-30, and 1-40, with
oxalated saline. When tested for thrombin, these
solutions did not clot fibrinogen. To test them for
prothrombin, we mixed three parts of each with three
of saline, three of calcium and three of tissue ex-
tract. After suitable periods of incubation, we
added three drops of fibrinogen to twelve drops of
each incubated mixture. The clotting was then meas-
ured with a stop watch. From data already given,
the total plasma dilution can be calculated. " ( 70 )
"The thrombin concentration typically rises to a
plateau, which is maintained for a safe interval of
time." ( 70 )

In their example, the plateau of thrombin
showed a fifteen second clotting time with the plasma
diluted 1-223. Therefore, the prothrombin unit concen-
tration would be 223, since one unit of thrombin,
according to Warner's definition, is equal to one
unit of prothrombin.

The above findings were of dogs. In estimating
the prothrombin content of human plasma, lower concen-
trations will be found. Two modifications were made,
later. The plasma is defibrinated by their thrombin
preparation, which is added to the plasma. The thrombin
is so diluted that a clot forms in 15-18 seconds. The second change is the usage of acacia. The result is that higher "prothrombin unitage" is possible. (77)

Quick, et al. (53) by means of a new test, were able to determine prothrombin levels in the plasma. A complete account of the procedure can be found in the original article (53). 9 c.c. of blood were withdrawn rapidly and mixed with 1 c.c. of M/10 sodium oxalate, and then centrifuged at low speeds for 5 minutes. Small amounts of the plasma, 0.1 c.c., were transferred to a dry clean test tube, 13x100 m.m., and mixed with equal amounts, 0.1 c.c., of thromboplastin solution. Next, 0.1 c.c. of M/40 calcium chloride was added and the mixture quickly shaken and placed in water, both at 37 degrees C. "The exact time required for the formation of a solid clot is recorded." (53)

If active thromboplastin is used, normal human plasma should clot in 22 to 25 seconds. Assuming that the clotting time is dependant on the prothrombin concentration, any dilution of plasma should produce a delay in the coagulation. This actually occurs, as Quick has proven.

Increased amounts of thromboplastin are added to the plasma, which has been diluted so that the prothrombin has been reduced to 1/5 of normal. The
clotting time is increased to 65 seconds. In order to mitigate the possibility that the prolonged clotting time is due to dilution of some other constituent in the plasma, dilutions were made with normal saline and plasma that had been treated with aluminum hydroxide. The aluminum hydroxide removes or inactivates prothrombin with no apparent change resulting to the other constituents of the plasma. The results were the same, indicating that the decrease in plasma prothrombin is responsible for the plasma clotting time. In order to have standard results and carry out the prothrombin test successfully, it is necessary to have an active and stable thromboplastin.

Rabbit's brain proved most efficacious as the source, since it is both active and stable. The preparation is as follows: Remove superficial blood vessels from the brain of rabbit, wash and grind to a paste and spread on a glass in thin layers; dry at 37 degrees C.; remove material and place in stoppered container; then add 0.2 gm. of this material to 0.3 c.c. of 0.85% sodium chloride and mix, then incubate for 15 minutes. This emulsion, when added to plasma (human), causes clotting in 22 to 25 seconds. This emulsion is stable.
Quick brings out the fact that Fibrinogen, which is in the plasma normally at concentrations of 0.3 to 0.75%, plays a passive part in the role of clotting. (53) However, total absence of Fibrinogen has been reported by Rabe and Salomon (58'), and Opitz and Frei (50'), with ensuing fatal hemorrhage.

Regarding calcium, Quick says that, contrary to common belief, it seems more likely that only organically combined calcium is directly essential for the formation of thrombin, and not the ionized calcium of the blood. Quick uses Scott's and Chamberlain's (49) findings in this article. They demonstrated that the quantitative removal of ionized calcium from the blood with oxalate, will not prevent clotting.

Quick (54) later revised the quantitative determination of prothrombin, as follows:

1. 4.5 c.c. Blood plus 0.5 c.c. Sodium Oxalate solution, centrifuged;
2. 0.1 c.c. plasma plus 0.1 c.c. thromboplastin;
3. 0.1 c.c. Calcium Chloride then added quickly, after which, observe the length of time it takes to clot.

Normally, this will be 12 to 13 seconds. With a decrease in prothrombin, the clotting time is delayed.
By means of the following chart, the prothrombin content can be determined:
A simple bedside test for the clotting time has been perfected by Ziffren, Owen, Hoffman and Smith (75). It is a simplification of Nick's test, and like his so-called "prothrombin test", measures not only prothrombin, but the summation of several variables, thus giving a practical test for the tendency to bleed.

The test is run as follows:

1. 0.1 c.c. of thromboplastin is placed in a 3 c.c. tube;
2. 1.0 c.c. of freshly drawn blood is run into the tube;
3. The tube is inverted once, and then gently tilted every few seconds;
4. The clotting time is observed;
5. Repeat test on a normal individual, then the unknown is expressed in percentage of the normal, as expressed by the following formula:

\[
\text{Clotting Activity (In \% of Normal)} = \frac{\text{Clotting time of normal}}{\text{Clotting time of unknown}} \times 100
\]

The thromboplastin is prepared as follows:

"Extract 10 gm. ground brain or lung (ox or rabbit), 2 hours with 10 c.c. saline; strain and preserve in refrigerator." "Variable potency does not affect the clotting time ratio of unknown to control. If normal values exceed 60 seconds, the thromboplastin is rejected; if less than 25 seconds, one should dilute the thromboplastin with saline." (75)
The originators of this new test compared this test with the quantitative prothrombin test of Warner. Ten cases were used for the comparison.  

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Sex</th>
<th>New Test</th>
<th>Quant. Prothrombin Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(%) of Normal</td>
<td>(%) of Normal</td>
</tr>
<tr>
<td>Pernicious Anemia</td>
<td>F</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>Aplastic Anemia</td>
<td>M</td>
<td>93</td>
<td>83</td>
</tr>
<tr>
<td>Cholecystitis</td>
<td>F</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>Obstr. Jaundice</td>
<td>M</td>
<td>76</td>
<td>80</td>
</tr>
<tr>
<td>&quot;</td>
<td>M</td>
<td>62</td>
<td>73</td>
</tr>
<tr>
<td>&quot;</td>
<td>M</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Biliary Fistula</td>
<td>F</td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>Obstr. Jaundice</td>
<td>M</td>
<td>41</td>
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<tr>
<td>&quot;Toxic Hepatitis&quot;</td>
<td>M</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>Obstr. Jaundice</td>
<td>M</td>
<td>22</td>
<td>13</td>
</tr>
</tbody>
</table>

For all intents and purposes, the above test or Quick's test, prove satisfactory for the clinical use. Many clinicians and technicians modify the Quick test by using less blood. The ratio of the remaining constituents is not changed.
BILIARY FISTULA AND 
OBSTRUCTIVE JAUNDICE.

"The earliest reported case of fatal bleeding in a patient having jaundice was made by Wedelius, in 1683." (77) (25) Since the advent of aseptic surgery, the tendency to bleed in patients having jaundice has been helplessly noted. This condition has caused grave concern to surgeons, innumerable times. The syndrome, jaundice, then bleeding, and then death, occurred so often it became almost an edict of surgical hierarchy, that to operate on such cases meant death for the patient.

From the time Wedelius first reported the syndrome in the literature, up until Quick made his observations, little effective treatment was forthcoming to the afflicted. This was not because effort had not been expended, but quite the contrary, for investigators have literally turned the gall bladder inside out, seeking the answer.

Greaves and Schmidt (77), in 1933, demonstrated that little or no irradiated ergosterol is absorbed from the bile fistula rat (—). However, when desoxycholic acid was administered by mouth, it served as a carrier of irradiated ergosterol across the intestinal wall of the bile fistula rat. This work proved that
bile salts are needed for the absorption of fat soluble vitamins, such as Vitamin D.

Their work was the outgrowth of Pavlov, Looser, Wisner, Whipole, Seidal and other early investigators, who pointed out the fact that exclusion of bile from man and certain other animals leads to Osteoporosis. Seifert (47) suggested that in the absence of bile, Vitamin D is not absorbed, which consequently leads to a negative calcium and phosphorous balance.

This work played an important part in discovering the mechanism of Vitamin A and Vitamin D. Years later, it again was to be used in explaining the mechanism of another vitamin. This Vitamin is K.

Dam and his associates, while working on experiments with chicks, found that if certain factors are missing from the diet, a hemorrhagic condition ensues. They later found certain foods would alleviate this condition. Thus, Vitamin K was born into the world, and from that time on, has been subjected to the scientific scrutiny of investigators anxious to clear up the enigma of hemorrhage in jaundiced patients.

Vitamin K was found in alfalfa and other leafy substances, the photosynthetic portion of plants. Next, it was found in putrified fish, in eggs, in hog liver oil, in hemp seed and many other sources.
studying these factors. They, therefore, developed a method for prothrombin detection and for the preparation of a stable and active thromboelastin. "In studying the plasma from various types of jaundiced patients by this new method for determining prothrombin, it was found that this component was greatly diminished. Instead of obtaining a normal clotting time of 22 to 25 seconds, a delay, which in one case was as long as 90 seconds, was obtained." (53)

"These findings, therefore, suggest that the hemorrhagic tendency in Jaundice is brought by a diminuation of prothrombin." (53)

This suggestion by Quick proved to be a tremendous boost for the search of the cause of hemorrhage in Jaundice.

The first direct evidence reported in support of the new theory was supplied by Greaves and Schmidt (42). They showed that when rats with biliary fistulas were maintained on a low fat diet for periods of 30 to 50 days, a small scratch would result in prolonged bleeding, which in some cases led to the death of the animal.

Quick's method for determining the prothrombin found that the loss of prothrombin closely paralleled the hemorrhagic tendency. It was further noted that the
prothrombin content of these animals would have to be reduced to 20% to 30% of normal, before the coagulation time of the blood was markedly reduced. A marked rise in the prothrombin content of the blood was noted to take place within 2 to 4 days after the administration of 2 to 3 c.c. of beef bile. With the increase in prothrombin content, a decrease in clotting time was noted.

Vitamin K extracts, when fed to the bile fistula rats, were effective in decreasing the clotting time and increasing the prothrombin content.

Next, the rats were made icteric by ligation of the bile duct, and fed a stock diet. The stock diet contained Vitamin K. A decrease in prothrombin content was noted, and soon, the re-establishment of the flow of bile into the intestinal tract and an increase of prothrombin was noted in the plasma. Only when massive doses of Vitamin K, without the bile salts or bile itself, were fed, did the prothrombin rise to normal levels. They concluded, therefore, "that (1) bile fistula rats show loss in blood coagulability and a decrease in the prothrombin level; (2) this condition can be relieved by administration of Vitamin K concentrate". (‡2)
Two months later, Warner and associates (73) reported the first successful treatment of human patients. They say in their article, "We have followed the prothrombin level in human cases, in which bile feeding was supplemented with a fat soluble extract of alfalfa meal. The residue of a petroleum ether extract of 200 to 400 gm. alfalfa meal was emulsified in human bile, or, in some cases, in 2% sodium taurocholate solution, and fed daily. In a number of patients given this alfalfa-bile mixture, even with persistence of Jaundice, the rise in prothrombin was very rapid. If bleeding existed, it soon ceased. In three cases of Obstructive Jaundice, the prothrombin level before therapy was 43, 20, and 37%. Alfalfa with bile or bile salts was given for 6, 8, and 6 days, respectively. At the end of the period, the prothrombin values had risen to 66, 107, and 102%. The rise in these cases is considerably faster than with simple bile feeding." (73)

Shortly after Warner and associates published their article, Butt, Snell and Osterberg of the Mayo Clinic, reported their findings regarding the usage of Vitamin K and bile salts in the treatment of low prothrombin content of patients suffering from Obstructive Jaundice. (73) Their report confirmed the findings
of Warner, et al. Eighteen patients with Obstructive Jaundice were used in their clinical trial of Vitamin K. They found that Vitamin K is not effective unless given with bile salts. Bile given alone, will effect a decrease in the clotting time, but it is a gradual and relatively slow decline, whereas, bile salts plus Vitamin K, effect a rapid decrease in the clotting time.
The clinical findings and observations of Stewart (47), Olson (46), Rhoades (59), Johnston (46), Scallon, et al. (62), Dam and Glavind (37), and others, have confirmed the reports of Warner, et al., and Butt, et al., which showed that the bleeding tendency in biliary fistula and obstructive jaundice was due to a lack of Vitamin K absorption in the absence of bile from the intestinal tract. This led to a low prothrombin content and a subsequent increase in the clotting time of the blood.

Greaves (39) added conclusive proof to these clinical findings with his experimental findings upon rats. He showed that in the rats, Vitamin K is carried across the intestinal tract by means of bile salts. In the absence of Vitamin K or bile salts in the rat, a lowered prothrombin time occurs, with an increase in the clotting time. Hugh R. Butt, et al. (25) state the following: "Even current figures indicate that cholemic bleeding has accounted for about fifty percent of the mortality accompanying surgical intervention on patients having jaundice, and that cholemic bleeding, of itself, imposes a surgical risk of approximately five percent."

The understanding of cholemic bleeding that these investigators and others have given the medical profession should prove to be "a big stick" in striking
down the mortality and surgical risk on patients so afflicted. In disclosing the causitive factor, they disclose the treatment, for from their work, we see that the cause is mainly due to a deficiency of Vitamin K, a deficiency caused by lack of absorption through the intestinal tract in the absence of bile salts. Thus, a vicious cycle can be broken by addition of bile and an adequate amount of Vitamin K.

The writer, in this paper, has endeavored to set out in chronological order, the research and discoveries that have lead to the present knowledge of Vitamin K's role in jaundice, due to biliary fistula and obstruction.

In summarizing, we find the following facts:
1. Cholemic bleeding has been known for centuries.
2. In obstructive jaundice and biliary fistula, there is an absence of bile from the intestinal tract.
3. Bile salts are necessary for the absorption of Vitamin K across the intestinal tract.
4. Vitamin K in large dosages will restore the prothrombin content to within normal levels.
5. Bile salts taken with Vitamin K prove to be more efficacious as a method for restoring the prothrombin content to normal.
Cantarow has covered the treatment of biliary fistula and obstructive jaundice more thoroughly than other investigators, and we quote from him:

"Vitamin K therapy at the moment is in a transitional stage, owing to the recent demonstration of the chemical nature of the vitamin and the availability of synthetic preparations which will undoubtedly replace extracts which have been employed heretofore. Preparations made from alfalfa and other grains (wheat and oats) and fish meal have provided the most potent sources until recently. The commercial preparations in most common use are Klotogen (Abbott) and Cerophyl (Cerophyl Laboratories). The latter is available in tablet and powder form and the former in soft gelatin capsules (1000 Almquist and Stokstad units; 37,500 Dam units) and in 50 cc. vials, each cubic centimeter of which contains 1250 Almquist and Stokstad units. The Dam unit is defined as the amount of antihemorrhagic material contained in 2 mg. of dried spinach. These measures of unit value are obviously of limited usefulness and will undoubtedly be discarded now that pure preparations of the vitamin are available. It has been suggested recently that a unit of Vitamin K activity should be regarded as equivalent to the
antihemorrhagic effect of 1 microgram of pure 2 methyl -1, 4-naphthoquinone.

"In the treatment and prevention of the hemorrhagic tendency of patients with obstructive jaundice and bile fistula, before and after operation, Cerophyl has been given effectively in doses of 5-25 Gm. daily. Klotogen is usually given in the dosage of one capsule three times daily or, in severe cases, 8 cc. of the solution in oil may be administered daily by duodenal tube. In such cases bile salts should be administered in conjunction with the vitamin. Whole bile or bile concentrates may be given. Bile salts are perhaps to be preferred, in doses of 1-2 Gm. of sodium taurocholate and glycocholate or, preferably, deoxycholic acid, in the dosage of 3 grains (0.2 Gm.) three times daily. Synthetic, crystalline Vitamin K or one of the several similar naphthoquinones which have been shown to possess Vitamin K activity are now being used. These preparations seem to be effective in doses of 1-2 mg. daily, by mouth. Bile salts should be given simultaneously, as stated above. They have also been found to be effective when given parenterally.

"In the great majority of cases of obstructive jaundice and bile fistula, the plasma prothrombin increases promptly after the institution of this form of
therapy and usually returns to normal in a few days. A similar effect may frequently be produced by the oral administration of whole bile, bile concentrates or bile salts, without vitamin K, the latter being present in the food and in the intestine but inadequately absorbed because of the absence of bile salts. However, the increase in prothrombin under such circumstances is usually much more gradual than if vitamin K is given in excess simultaneously. As stated previously, occasional failure of vitamin K therapy may be due to the presence of extensive hepatocellular damage.

"According to Quick, if the prothrombin content of the blood is below 15 per cent of normal, transfusion should be resorted to. The effects are transitory and this procedure should be used only as an emergency measure to supplement vitamin K and bile-salt therapy. The observations of Lord, Andrus and Moore indicate that the changes in plasma prothrombin of the recipient of a transfusion are dependent on the prothrombin content of the blood of the donor, and may be calculated on the basis of addition. Stewart found an increase of only 6 per cent in one case after transfusion of 600 cc. of blood. Blood from "blood banks" should not be used because its prothrombin content is
apt to be low. According to Rhoads and Panzer, blood that has been preserved for a week or more is practically useless for this purpose and that kept for 3 days is of only slight value.

"Since the plasma prothrombin may fall quickly, especially after operation, determinations of this factor must be made frequently during the pre- and post-operative periods, even though no deficiency is found, and adequate Vitamin K and bile-salt therapy must be provided before and after operation upon patients with obstructive jaundice and bile fistula." (27)

The above finding agrees with the majority of investigators in this field. (23, 24, 25, 26, 34, 45, 47)

70. 54, 39, 60, 62, 60, 68, 75, 75.
SUMMARY

1. A new vitamin has been discovered and named "Vitamin K" by Hendrik Dam.

2. Recently, another form of the vitamin has been isolated, namely, Vitamin K2. The structural and empirical formula has been found for Vitamin K1 and Vitamin K2. The synthesis of Vitamin K like substances has been performed in the laboratory.

3. The vitamin is found in varying amounts in plants, fish and bacteria.

4. The vitamin contributes to the formation of prothrombin in the body, without which aid, the tendency to hemorrhage appears.

5. The method for estimating the Vitamin K utilization in the body is based upon the prothrombin content of the plasma. A low prothrombin content signifies a low intake of Vitamin K, or poor utilization by the body.

6. Bile salts convey the vitamin across the intestinal tract. A deficiency of bile salts results in decreased absorption of Vitamin K by the intestinal mucosa and a subsequent increase in the clotting time. In obstructive jaundice and biliary fistula, bleeding makes operating a poor risk.
7. Therapy is designed to raise the prothrombin time and decrease the clotting time. Bile salts with Vitamin K are given preoperatively and postoperatively. The result of therapy is gauged by prothrombin estimation.
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