A STUDY OF MOLD PIGMENTS FOR VITAMIN K ACTIVITY

by

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INTRODUCTION

One of the most interesting aspects of bio-chemistry is the study of the biological activities of the molds. These organisms, due to their adaptability, render themselves of vast economic importance. One may find them thriving practically anywhere and under the most adverse conditions, obtaining nourishment from almost any common organic compound such as sugars, acids, paraffins, formaldehyde, and starches. Their importance in food control cannot be overestimated, for no common food of man is outside their attack. Yet, on the other hand, they have proved themselves tireless workers for the production of many organic compounds such as enzymes, fats, acids, pigments, alcohols, and drugs. Because of their complicated structure and large number of metabolic products no systematic study was made until 1931 when Raistrick and his associates (15) devised new and careful methods for investigation.

Of the many pigments investigated since that time, a large number are yet unidentified and are without a known structural formula. Some of these are acids, but for the greater part they are hydroxyanthroquinones such as ravenelin described by Raistrick, Robinson and White (16), or auroglaucin, flavoglaucine and rubroglaucium described by Gould and Raistrick (9). Another quinone of simpler structure, fumigatain which contains a toluquinone nucleus, has been reported by Anslo and Raistrick (2).

Due to the particular quinoid organic grouping, it might be supposed that some of the compounds are capable of vitamin K activity due to their structural similarity with the known vitamin K active compounds.

It is known that bacteria produce vitamin K active compounds. Phthiocol, the natural occurring pigment of the human tubercular bacilli, possess anti-hemorrhagic properties. In fact, vitamin K compounds were found by McElroy and Goss (12) to be produced by bacteria and to appear in the rumen of cows on vitamin K deficient diets, and for a long time decayed fishmeal has been known to be a rich source of these compounds.

Some time ago a Penicillium was found by Dr. B. L. Smits, of Kansas State College, which when grown on a medium containing glucose as the carbon source produced a striking amount of pigment. Since no work has been reported on the study of whether molds produce vitamin K active compounds during the course of their metabolism, and because of the relationship of these pigments to vitamin K, a study was undertaken to determine if this particular Penicillium would produce such products.

DESCRIPTION OF VITAMIN K

History

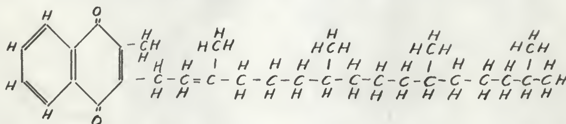
In 1935 a number of investigators were led to believe from the results of their work that there existed a fat soluble factor which was specifically involved in the blood coagulation of chicks, ducklings, goslings, and other birds. This was particularly noted when young fowl were placed on various nutritional diets which were deficient in this factor. There was a marked tendency for hemorrhage and anemia. The corrective factor was found to be present in vegetables, oils of grains and cereals, and in hog-liver fat. It was further found to be in the non-sterol fraction of the unsaponifiable matter of these materials.

In 1939 Dam and others (6) isolated vitamin K from alfalfa for the first

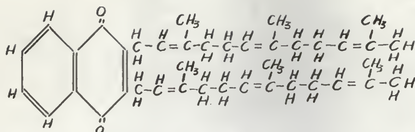
time. A little later McKee, Binkley and others (13) isolated a similar compound from putrified fishmeal. The former was named vitamin K_1 and the latter vitamin K_2.

Chemistry

Vitamin K_1 is a yellow oil of the following percentage composition: C, 82.2; H, 10.7; O, 7.1. It has been shown to have a characteristic absorption spectrum with maxima 243, 248, 261, 270, and 328 mu. It is unstable to light and yields a violet-blue color-reaction with sodium ethylate which turns red and finally brown upon standing. Binkley, Thayer, MacCorquodale and Doisy (3) reported that the condensation of phytyl bromide with the monosodium salt of 2-methyl-1, 4-napthoquinone to yield 2-methyl-3-phytyl-1, 4-napthoquinone which was identical with vitamin K_1. Its structure is as follows:



Vitamin K_2 is a crystalline product with a melting point of 50.5-52.0°C. The elementary composition was found to be C, 84.43 to 84.57; H, 9.73 to 9.87. The absorption spectrum of vitamin K_2 resembles that of vitamin K_1 for it has maxima at 249, 269 and 320 mu. Like vitamin K_1 it is unstable to light and alkaline substances. From the above work Fieser and his co-workers (8) suggested the structural formula of vitamin K_2 to be 2,3-difarnesyl-1, 4-napthoquinone whose structure is:



Vitamin K_2 is found to have approximately 66 per cent the physiological activity of an equal amount of vitamin K_1.

Many closely related compounds have been shown to have vitamin K activity. It is generally agreed that the highest potency is found in 2-methyl-1, 4-napthoquinone and substances which lack the 2-methyl group have relatively low biological activity. It is interesting to note that Kuhn and others (11) found that α-tocopherol-quinone has some vitamin K activity so that K activity is not strictly confined to the napthaquinone derivatives.

Assay

To date chemical determinations have not been found satisfactory, and as a result biological assay has been the basis for determining vitamin K concentrations. The Dam unit and the Ansbacher unit have been frequently used. Dam and Glavind (5) described a unit of vitamin K as that amount which, administered to the test animal per gram of body weight, reduces its R = 200 to one (R equals $\frac{K}{Kn}$, K equals concentration of the tissue extract necessary to complete clotting of the blood plasma in three minutes; Kn equals the corresponding concentration for normal plasma). The biological unit as described by Ansbacher (1) is described as that amount which, when administered orally to a two to four weeks old vitamin K deficient chick, will reduce the coagulation time to less than six minutes in six hours. A comparison of these tests shows the Ansbacher unit to be equal to 20 Dam units of vitamin K.

Role of Vitamin K in Human Pathology

Vitamin K avitaminosis in human pathology has been found in connection with obstructive jaundice, pregnancy, and hypoprothrombinemia. Butt, Snell and Osterberg (4) have reported the restoration of the prothrombin level in cases of obstructive jaundice to approximately normal two days after 240,000 Dam units per day were used together with one to two grams of bile salts. Shettles, Delfs and Hellman (17) reported that the prothrombin in the new-born child is lower than in the mother and that by giving vitamin K either to the mother before delivery or to the infant after birth this level may be raised. Gupwall and others (10) urged the use of vitamin K before all operations on infants in the first week of life and suggested the possibility of preventing intercranial hemorrhage at the partus. These conditions are particularly marked in the premature infant.

Dam and Glavind (5) reported the failure of vitamin K to influence the abnormal coagulation in hemophelia and thrombopenia. These observations have been confirmed many times by subsequent workers.

Mode of Action

There has been no complete explanation as to the mode of action of vitamin K. According to several investigators, Dam and others (7), the function in preventing hemorrhage consists in maintaining a prothrombin level in the blood. However, prothrombin itself has been found to have no vitamin K activity. It is presumed that vitamin K activity is confined to the liver in the production of prothrombin, but does not enter into the function of blood coagulation directly.

DESCRIPTION OF THE MOLD

Colony Form

When the spores were seeded on the surface of sterile potato agar the following colony characteristics were observed:

Surface. Young colonies appeared as small, white, flatly conical colonies, rather deeply embedded in the agar. White mycelium began to grow and on aging turned a light green; upon further aging and fruiting it became dark green to olive. Only the extreme margin where the fruiting had not begun remained white. The surface was flat with the exception of a slight conical center. The fruiting was heavy and resembled Penicillium oxalaticum except for a slightly different green.

Reverse Side. The following color change upon aging was observed: white first, then to a pale yellow which gradually changed to a yellow-orange and finally a permanent red color. This pigment was at least water soluble in part, as it readily diffused into the agar.

Microscopic. The conidiophore produced a terminal symmetrical whorl or verticil of sterigmata closely packed in the verticil. The organism was biverticulate. Its conidia were elliptical in form and measured three to four by two to three microns.

Mold Mat

Because the following medium gave such excellent growth with this Penicillium sp. it was used throughout this work:

Glucose	200.00 g
NH_4NO_3	1.40 g
$MgSO_4$-7HOH	.20 g
KOH	10.00 ml containing 1 g/l
H_3PO_4	25.00 ml containing 1 g/l
HOH	1000.00 ml

The solution was adjusted to pH 4.5.

The following trace elements were found to be beneficial to growth and were added in the following proportions:

Fe	5 parts per million
Cu	1 part per million
Mo	1 part per million

The medium was added in 500 ml quantities to 5.0 l fermentation flasks and autoclaved for 20 minutes at 15 pounds pressure. These flasks were then innoculated from 10-day-old stock cultures of Penicillium sp. and incubated at 37° C. for three weeks.

At the end of a three-week incubation period in the above medium the mold mat had completely covered the surface. Colorless crystals had collected on the lower surface of the mat and some had settled to the bottom of the flask. A yellow pigment was observed at several different places on the mat. The color of the medium had changed from a light yellow to a deep orange-yellow.

The mats were then removed from the flasks and allowed to drain through a cloth filter. The weights of these mats were from 40 to 50 g each. A moisture determination by heating in a vacuum oven or by use of a toluene moisture determination apparatus showed the moisture content to be from 55 to 60 per cent of the total weight. The ash content of the drained mat was found to be from .18 to .20 per cent of the total weight while the protein content was 25.69 to 28.75 mg per 100 g when the N×6.25 factor was used.

EXPERIMENTAL PROCEDURE

Extraction of Mat

Preliminary study of the acetone extractable material of the wet mat taken to dryness and then redissolved in part in Skellysolve showed at least two pigments when filtered through a 50 per cent MgO_2 silliceous-earth mixture absorption column. Further study showed a bright yellow pigment which was fat soluble and an orange pigment which was sparingly water soluble. Both of these pigments were readily soluble in acetone. Both pigments became a brilliant red when treated with sodium ethylate and, when reacidified, returned to their former colors.

To obtain these pigments in a more or less pure state for study of vitamin K activity, the following method of extraction was found to be the simplest and fastest. The wet mat and insoluble crystals were separated from the glucose medium by filtering through cheesecloth. The mat was then ground through a food chopper of medium grind. The entire mat and crystals were then placed in a Soxhlet extractor and extracted until no more color change was noted when the extract was tested with sodium ethylate. Complete extraction could be made in 12 to 16 hours. By the end of this extraction

period, the extractor flask was lined with a crystalline white material. These crystals were soluble in acetone and were extracted after the water had been removed from the mat.

When this white material was dissolved in acetic acid and reprecipitated by the addition of water, they crystallized in the characteristic unsymmetrical hexagonal crystals. Their solubility and reactions made them comparable to a lactone previously reported by Smits[1]. The lactone was identical with the original white insoluble crystals found in the medium.

The acetone fraction was then acidified with a few drops of acetic acid and chilled at a -5° C. for two days. At the end of this time a brown, gummy residue had separated out, which was filtered from the acetone fraction. The brown material gave a positive sterol test when treated with acetic anhydride and concentrated sulphuric acid. The filtrate was then evaporated by use of a fan and in the absence of light to approximately 25 ml. More of the white lactone separated out which was removed by filtration. The filtrate was then made up to a volume of approximately 100 ml with acetone.

To this fraction was added 50 ml of water and 50 ml of Skellysolve. The mixture was thoroughly shaken and the acetone water phase drawn off and rewashed with fresh Skellysolve. The Skellysolve fractions were then rewashed three times with a 50 per cent acetone-water solution. These Skellysolve fractions were then filtered and taken to dryness under reduced pressure. The yellow flocculent residue was taken up in absolute ethyl alcohol and stored in the absence of light until ready for use.

The acetone water fractions were combined and evaporated to approximately 50 ml. This fraction was then rewashed with Skellysolve and evaporated

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[1] Smits, B. L., and Andrews, A. C. A new dextrorotary, water insoluble lactone produced by a Penicillium sp. from glucose. Kansas State College of Agriculture and Applied Science. 1938. (Unpublished data).

to dryness under reduced pressure and taken up in absolute ethyl alcohol.

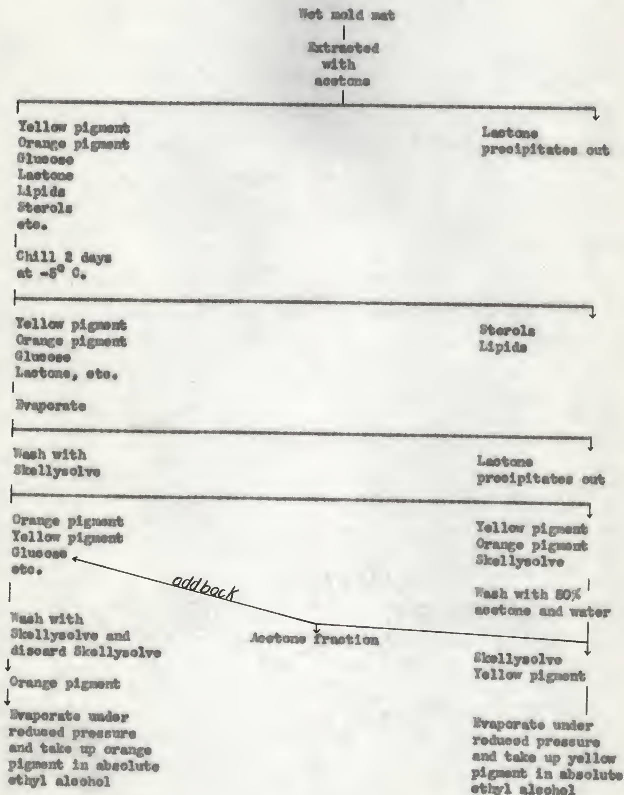
These solvents were found to separate the mold pigments better than any of the other common organic solvents such as ethyl alcohol, methyl ethyl ketone, isoamyl alcohol, acetic acid, etc.

Various attempts were made to crystallize these pigments but were without success. Raistrick (15) explained this difficulty with some mold pigments as being due to the pigments uniting with molecules of the solute. Some of the pigments have been crystallized by converting them to acetates and recrystallizing from acetic acid.

By the use of the above procedure, from 0.3 to 0.4 g of yellow pigment could be extracted per mat. The orange pigment was in all cases less, averaging from 0.15 to 0.2 grams. A solution containing the concentration of 0.1 g of pigment in 1 ml of absolute alcohol was used for vitamin K assay with the vitamin K deficient chicks. As a check on the pigments, the wet mat was finely ground and dispersed in alcohol so that a concentration of approximately .1 g per ml of alcohol was obtained.

Examination of the Medium. The aqueous phase at the time of innoculation was a light yellow, clear liquid. After four days to a week the medium becomes turbid due to formation of the lactone and the color changes to a deeper yellow. Further growth precipitates lactone crystals. The average weight of these crystals not adhering to the mat was 1½ g per 800 ml of medium. This estimation was made by first removing the mat and filtering the remaining solution through a clean, dried filter paper, washing the precipitate with cold, distilled water, and redissolving with methyl ethyl ketone. Examination of this material disclosed that the lactone alone was soluble in this solvent, and recrystallizing from acetic acid gave the

Scheme of Separation of Mold Pigments



unsymmetrical hexagonal crystals. The lactone is practically insoluble in water, but is soluble in methyl alcohol, ethyl alcohol, acetone, hot benzene, and is very soluble in methyl ethyl ketone and glacial acetic acid. Uniform crystals can only be obtained with acetic acid.

The orange pigment could be removed from the solution by repeated shakings with chloroform. However, the most satisfactory method of separation was made by making the solution slightly alkaline and extracting with amyl alcohol. This pigment was orange to yellow when in an acid solution and a dark red in a basic solution. The range of its color change in respect to hydrogen ion concentration is similar to that of phenolphthalein. No means was found for crystallizing this pigment. Among the solvents tried was Skellysolve, water, acetone, chloroform, methyl and ethyl alcohol, methyl ethyl ketone, benzene, carbon disulfide, and acetic acid. This pigment was the same orange pigment as that extracted from the mold mat.

A portion of this medium was clarified with lead acetate. The reducing power of the solution found by use of Fehling's solution to be equivalent to 28.6 g of glucose per 500 ml. When this clarified solution was reacted with phenylhydrazine, the osazone crystals were formed in four to five minutes, and examination gave only the characteristic glucoosazone crystals.

The optical rotation of the clarified solution was found to be 2.8 positive at 20° C.

When the medium was filtered and the water slowly driven off by the use of a steam cone until the residual material remained a thick, syrupy liquid, it was found that this material weighed but a fraction over 25 g.

The medium may be divided into two phases by driving off most of the moisture by use of an electric fan and dehydrating with absolute ethyl alcohol. The residual material is a white, sticky mass, and the yellow alcohol

upon standing precipitates a white material which is acidic and gives a positive Molisch test for carbohydrates. The residual alcohol contains the reversible orange pigment.

BIOLOGICAL ASSAY

In the biological assay of the pigments vitamin K deficient chicks were used. They were placed on a diet described by Ansbacher (1) which is here given.

Day-old chicks were placed on the following vitamin K free diet:

Polished rice (ground)	71.0 %
Fishmeal (ether extracted)	17.5 %
Brewers' yeast (ether extracted)	7.5 %
Salt mixture (Osborne and Mendel)	3.0 %
Codliver oil (Squibb)	1.0 %
Total	100.0 %

The yeast used in this ration was extracted ten times with ten volumes of ether and then dried at 50° C. and ground. The fishmeal was submitted to continuous ether extraction for seven days.

The Osborne and Mendel salt mixture was made up as described by Osborne and Mendel (14).

Osborne and Mendel Salt Mixture

$CaCO_3$	13.48 g
$MgCO_3$	2.42 g
Na_2CO_3	3.42 g
K_2CO_3	14.13 g
HCL	10.32 g

H_2SO_4	5.34 g
Citric acid H_2O	0.92 g
FeCi $1\frac{1}{2}$ H_2O	11.11 g
KI	0.6340 g
$MnSO_4$	0.0020 g
NaF	0.0079 g
$K_2Al_2(SO_4)_2$	0.0062 g
Lactose	246.00 g
Total	307.7901 g

The constituents were well mixed and fed to the chicks as their sole ration.

Tap water was supplied. The chicks were kept in a ventilated warm brooder having a mesh-screen floor. It was found in the first attempt to raise vitamin K deficient chicks that this type of floor was essential, as chicks are autocoprophagic and are able to obtain enough vitamin K from their feces to keep them from becoming deficient.

Most chicks were found to become deficient on this diet in two weeks, and unless given a vitamin K supplement, would die within four weeks. The typical hemorrhagic syndrome for vitamin K-avitaminosis of the chick was shown in the form of subcutaneous and intermuscular hemorrhages of the head, neck, breast, abdomen, back, wings, and legs.

Twenty-four one-day-old white Leghorn chicks were obtained from the Kansas State College Experimental Farm for use in this assay. Four chicks were placed on a recommended normal chick ration obtained from the Kansas State College Poultry Department and maintained as controls throughout the experiment. The remaining 20 chicks were placed on the vitamin K-free diet.

Within two weeks all had developed clinical signs of vitamin K-avitaminosis other than a prolonged blood-clotting time. These chicks were then divided into groups of five. The first group was used for the assay of the yellow pigment, the second group for the orange pigment, the third group for the mold mat, and the fourth group was used for controls with a known vitamin K active compound. The compound used in this instance was 2-methyl-4-hydroxy-napthoquinone. The concentration was .1 g per ml of absolute ethyl alcohol.

A variation of the Ansbacher method for determination of the clotting time was used. Ansbacher (1) described his method as follows:

> ....a small wing vein was punctured with a fine sewing needle; the blood was allowed to flow into a micro-test tube (3.5 x 0.2 mm; 0.1 mm thickness). The tube was placed at once in a water thermostat with a temperature of 38 to 40° C. and shaken by a constant speed mechanism. As the clotting time we considered the length of time necessary for a solid clot to form so that the tube can be inverted without dislodgement of the clot.

The variation used in this work was as follows: A small, wing vein was punctured with a sterile sewing needle and the blood allowed to flow into capillary tubes. (It was suggested by Ansbacher (1) that the left wing be tapped first if two observation times were to be made, as a greater mortality was observed from puncturing the right wing vein.) These tubes were left at room temperature and broken at various time intervals to determine whether the blood had coagulated. Room temperature was found to be constant enough so that no temperature control was used. The room temperature was 35° to 37° C.

The chicks were tested for coagulation time at the time of administration of the material for assay.

The concentrates were introduced into the chicks' crops in 1 ml quantities containing .1 g of pigment or total extract. The fowl was then allowed

Table 1. Preliminary study of coagulation time.

Diet	Bird number	Time in minutes to coagulate									
		5	7	10	15	20	25	30	35	40	Over 40 minutes
Normal	1	coag.									
	2	—	coag.								
	3	coag.									
	4	coag.									
K-deficient	5	—	—	—	—	—	—	—	—	—	coag.
	6	—	—	—	—	—	—	—	—	—	coag.
	7	—	—	—	—	—	—	—	—	—	coag.
	8	—	—	—	—	—	—	—	—	—	coag.
	9	—	—	—	—	—	—	—	—	—	coag.
	10	—	—	—	—	—	—	—	—	—	coag.
	11	—	—	—	—	—	—	—	—	—	coag.
	12	—	—	—	—	—	—	—	—	—	coag.
	13	—	—	—	—	—	—	—	—	—	coag.
	14	—	—	—	—	—	—	—	—	—	coag.
	15	—	—	—	—	—	—	—	—	—	coag.
	16	—	—	—	—	—	—	—	—	—	coag.
	17	—	—	—	—	—	—	—	—	—	coag.
	18	—	—	—	—	—	—	—	—	—	coag.
	19	—	—	—	—	—	—	—	—	—	coag.
	20	—	—	—	—	—	—	—	—	—	coag.
	21	—	—	—	—	—	—	—	—	—	coag.
	22	—	—	—	—	—	—	—	—	—	coag.
	23	—	—	—	—	—	—	—	—	—	coag.
	24	—	—	—	—	—	—	—	—	—	coag.

Table 2. Coagulation times six hours after administration of tested materials.

Diet	Material tested	Bird number	Time in minutes to coagulate									
			5	7	10	15	20	25	30	35	40	Over 40 minutes
Normal	—	1	coag.									
	—	2	—	coag.								
	—	3	coag.									
	—	4	coag.									
K-deficient	Yellow pigment	5	—	—	—	—	—	—	—	—	—	coag.
		6	—	—	—	—	—	—	—	—	—	coag.
		7	—	—	—	—	—	—	—	—	—	coag.
		8	—	—	—	—	—	—	—	—	—	coag.
		9	—	—	—	—	—	—	*			
	Orange pigment	10	—	—	—	—	—	—	—	—	—	coag.
		11	—	—	—	—	—	—	—	—	—	coag.
		12	—	—	—	—	—	—	—	—	—	coag.
		13	—	—	—	—	—	—	—	—	—	coag.
		14	—	—	—	—	—	—	—	—	—	coag.
	Total extract	15	—	—	—	—	—	—	—	—	—	coag.
		16	—	—	—	—	—	—	—	—	*	
		17	—	—	—	—	—	—	—	—	—	coag.
		18	—	—	—	—	—	—	—	—	—	coag.
		19	—	—	—	—	—	—	—	—	—	coag.
	K-active compound	20	coag.									
		21	coag.									
		22	coag.									
		23	coag.									
		24	coag.									

*Bled to death

to remain in the cage for six hours during which time it received neither food nor water. Finally a blood sample was taken and the clotting time determined.

No vitamin K activity was found in any of the above assayed concentrates. It is interesting to note that upon a previous attempt to assay this material Wesson Oil, the salad oil commonly sold on the market, was used as a vehicle. This gave a positive test for vitamin K, as all chicks had a normal coagulation time after the six-hour observation time.

CONCLUSIONS

This work was intended as an investigation of the vitamin K potentialities of the pigments of a mold. Consequently, the study was concerned primarily with the means of extracting the mold pigment. As a result, various experimental techniques were tested on this particular fraction.

The biological assay was conducted according to the outlines of previous workers with the exception of experimenting for a simpler means of determining coagulation time with chicks.

Certain technical conclusions can be drawn from this preliminary experimentation:

The particular mold studied produced at least two different pigments in its metabolism on a glucose medium. One of these pigments was yellow when in an acid solution and was fat soluble. The other pigment in an acid solution was orange and was sparingly water soluble. Both pigments became red in an alkaline solution and the color-reaction was reversible.

No means was found for crystallizing these pigments and as a result they are comparable to pigments mentioned in literature of the anthroquinoid type.

Due to their lack of vitamin K activity, the nucleus could not be of the napthoquinoid type.

Besides the particular pigments mentioned above, the mold produced considerable quantities of a complex water-insoluble lactone which could be crystallized from dilute acetic acid.

A certain amount of sterol material was isolated.

The mold was found to be a biverticulate Penicillium.

A simpler means of testing the blood coagulation time in chicks was

tried; it compared favorably with other methods described in literature. Vitamin K activity was not found in any of the pigments of the mold nor in the mold mat itself.

These conclusions when summarized are:

1. The mold (Penicillium sp.) was found to have at least two different pigments. One was yellow and fat soluble; the other, orange and sparingly water soluble.

2. The pigments of this mold and the total extract showed no vitamin K activity when assayed with vitamin K deficient chicks.

3. The use of a capillary tube for determining clotting time appears to be satisfactory.

4. Wesson oil was found to show vitamin K activity.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Ansbacher, S.
A quantitative biological assay of vitamin K. Jour. Nutr. 17:304-306. 1939.

2. Anslo, W. K. and Raistrick, H.
Fumigatin (3-hydroxy-4-methoxy-2:5-toluquinone) and Spinulosin (3:6-dihydroxy-4-methoxy-2:5-toluquinone), metabolic products respectively of Aspergillus fumigatus Fresenius and Penicillium spinulosum Thom. Biochem. Jour. 32:687-695. 1938.

3. Binkley, S. B., Thayer, S. A., MacCorquodale, D. W., and Doisy, E. A.
The isolation of vitamin K_2. Jour. Biol. Chem. 130:220-231. 1939.

4. Butt, H. R., Snell, A. M., and Osterberg, A. E.
Further observation on the use of vitamin K in the prevention and control of the hemorrhagic diathesis in cases of jaundice. Proc. of Staff Meetings of May Clinics. 13(48):753-764. 1938.

5. Dam, H., and Glavind, J.
Vitamin K_1 den Menneskelige Patologi. Ugeskrift for Laeger. 100:248-250. 1938.

6. Dam, H., Glavind, J., Karrer, P., Karrer, W., Rothschild, E., and Salomon, H.
Isolierung des vitamins K in Hochgereinigter form. Helvetica Chim. Acta. 22:310-313. 1939.

7. Dam, H., Schonheyder, F., and Tage-Hansen, E.
Studies on mode of action of vitamin K. Biochem. Jour. 30:1075-1078. 1936.

8. Fieser, L. F., Campbell, W. P., and Fry, E. M.
Synthesis of quinones related to vitamin K_1 and K_2. Amer. Chem. Soc. Jour. 61:2206-2218. 1939.

9. Gould, B. S., and Raistrick, H.
Crystalline pigments of species in Aspergillus glaucus series. Biochem. Jour. 28:1640-1656. 1934.

10. Gupwall, E.
Zur Klinik und Pathologie des Hereditarin Hamolytischen Ikterus. Acta Medica Scandinavica. 96:106. 1938.

11. Kuhn, R., Wallenfels, K., and Weygand, F.
Kurze Originalmitteilungen zur Spezifitat des vitamins K. Die Naturwissenschaften. 27:518-519. 1939.

12. McElroy, L. W., and Goss, H.
Quantitative study of vitamins in rumen contents of sheep and cows fed vitamin-low diets. Jour. Nutr. 20:527-539. 1940.

13. McKee, R. W., Binkley, S. B., Thayer, S. A., MacCorquodale, D. W., and Doisy, E. A.
The isolation of vitamin K_2. Jour. Biol. Chem. 131:327-343. 1939.

14. Osborne, T. B., and Mendell, L. B.
The inorganic elements in nutrition. Jour. Biol. Chem. 34:131-139. 1918.

15. Raistrick, H.
Biochemistry of microorganisms. Roy. Soc. London, Phil. Trans., Ser. B 220:209-244. 1931.

16. Raistrick, H., Robinson, R., and White, D. E.
L. Ravenelin (3-methyl-1,4,8-trihydroxyxanthone) a new metabolic product of Helminthosporium Ravenelii Curtis and of H. Turcicum Passerini. Biochem. Jour. 30:1303-1313. 1936.

17. Shettles, L. B., Delfs, E., and Hellman, L. M.
Factors influencing plasma prothrombin in newborn infant. Subtitle: II. Antepartum and neonatal ingestion of vitamin K concentrate. Johns Hopkins Hosp. Bul. 65:419-426. 1939.