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Isolation and partial characterization of flavins from plants

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ISOLATION AND PARTIAL CHARACTERIZATION OF FLAVINS FROM PLANTS

by

George Edward Treadwell, Jr.

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Dean of Graduate College

Iowa State University
Ames, Iowa

1970
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DEDICATION

To my parents
And for all this, nature is never spent

Gerard Manley Hopkins
ABSTRACT

A mild method for the isolation of flavins from plant tissues was developed and applied. The method consists of an aqueous extraction in the presence of enough ammonium sulfate to form a saturated solution, and isolation using 1) columns of a resorcinol-formaldehyde resin, 2) columns of unwashed talc, and 3) thin-layer chromatograms of Silica Gel H. Amounts of riboflavin as small as 1.5 ng. obtained from 1/40 of one corn coleoptile were detectable.

Using this method, a compound which was characterized as lumichrome was detected when etiolated seedlings of corn and of oats, and cells of yeasts were exposed to six hours of sunlight or to 1100 foot-candles of artificial light for the same period of time.

Compounds which are probably natural derivatives of riboflavin were found in etiolated seedlings of oats and Job's tears. The work with oats confirmed an earlier report of a new flavin and showed this flavin to be negatively charged, and apparently substituted on neither the ring nor the second or third carbon of the side chain. The two compounds from Job's tears were not charged, and apparently not substituted on the ring, the second, or third carbon of the side chain. These compounds did not correspond chromatographically to any derivatives of riboflavin whose structure is now known.
INTRODUCTION

For many people the tale of flavins ended with the discovery of co-enzymic functions for the two flavin nucleotides. But the tale seems far from ended. Recent studies have shown the existence in tissues of a number of flavins other than riboflavin, riboflavin mononucleotide, and riboflavin adenine dinucleotide. These studies are all the more interesting because of the large amount of evidence that a flavin may be the receptive pigment in photo-responses of plants. The situation is most strikingly shown by the study of flavins in oats (Avena). Long years of debate have assumed that the significant pigments in phototropic responses in Avena had to be riboflavin and/or a carotenoid. Recent examination of the pigments in etiolated coleoptiles of Avena suggests differently, and points out the need for more study.

This work was undertaken to develop methods for the isolation of free flavins from plants. Very mild conditions were sought to insure that naturally occurring flavins could be isolated without degradation. The developed methods were applied to seek flavins other than riboflavin, its mononucleotide and its adenine dinucleotide in plants. Both natural derivatives and photo-products of riboflavin were sought.
LITERATURE REVIEW

Roles of Flavins

A primary reason for studying flavins in plants is that possibly they function as receptors for photo-responses. A. Galston (30) first considered riboflavin as a possible receptor. He suggested it as the receptive pigment for the first positive phototropic response of the *Avena* coleoptile. Other responses to blue light for which riboflavin has been postulated as the receptor are: high intensity phototropic responses of *Avena* (138, 144), the phototropic responses of *Phycomyces* (15, 16, 20, 22), the phototropic response (79) and the development of trophocysts in *Pilobolus* (78), inhibition of sporulation in *Alternaria* (63, 64), photo-induction of sporulation in *Trichoderma* (57), photo-induction of synthesis of carotenoids in *Fusarium* (90) and *Neurospora* (143), photo-stimulation of oxygen uptake in *Chlorella* (54, 83, 84), the potentiation of photosynthetic evolution of oxygen in *Acetabularia* (118), the photo-induction of polarity in spores of *Equisetum* and zygotes of *Fucus* (70), photo-control of primary pulvini of *Mimosa* (27), photo-morphogenesis in the gametophytes of ferns (72, 142), and photodinesis (96, 122), changes in cytoplasmic viscosity (96, 127, 128, 129), and the movement of chloroplasts (96, 128, 146, 147), in a number of species.
Direct proof of the nature of the photo-receptive pigment is hard to obtain. Nevertheless, the experimental approaches used have given significant information, and strong support for a role for riboflavin in some of the processes. Thus excellent action spectra have been obtained for: the first (100, 121) and second (25) positive phototropic responses of coleoptiles, the phototropic responses of sporangiophores (20, 22), the stimulation of oxygen uptake (54, 83, 84), the photo-induction of carotenoid synthesis (90), and the movement of chloroplasts (96, 146) and related changes in cytoplasmic viscosity (96). Less detailed action spectra have been determined for the photo-induction of sporulation (57), light induced changes in the viscosity of the cytoplasm (127), photodinesis (122), movement of pulvini (27), induction of polarity in zygotes (70) and spores (70), and the development of trophocysts (78).

In general, these action spectra are exact fits of the absorption spectra of neither flavins nor their chief contenders, the carotenoids. (Comparisons of the action spectrum for tropism to the absorption spectra of various compounds is given by Thomas (122) and by Thimann and Curry (121).) When in vivo modifications of the spectra are considered either a flavin or a carotenoid could be the receptor. Asomaning and Galston (4) suggest that both pigments act in phototropism and both shape the action spectrum.
One of the pigments could be active and the other could modify the action spectrum by internal screening effects. However, mathematical studies of spectra resulting from screening considerations give no support to this theory (121).

Modifications of the chemical environment of riboflavin can cause its absorption spectrum to become impressively similar to the action spectrum of the first positive phototropic response of *Avena*. Thus the absorption spectrum in castor oil by Thomas (122) and improved by Kurtin and Song (59) is a very close fit.

Moreover, evidences for an effect in the near ultraviolet for phototropism in *Phycomyces* (16, 20, 22), photoinduction of synthesis of carotenoids (90), stimulation of oxygen uptake (54, 83, 84), potentiation of oxygen release during photosynthesis (118), induction of polarity in spores of *Equisetum* (70), movement of chloroplasts (96, 146), and the first (100, 121) and second (25) phototropic responses of *Avena* add increased support for a flavin (or a cis-carotenoid) being the receptor (121, 122). (The cis-carotenoids have absorption in the ultraviolet, but have not been found in plants (121)). Many of the action spectra of other responses were determined of the visible only. New determinations for these responses may show that many of them are sensitive to the ultraviolet.

In other approaches to the problem, model, *in vitro*
systems have shown that riboflavin can photo-destruct indoleacetic acid (30, 33, 76). Carotenoids and pure chlorophylls are unable to do this (76). Galston and Baker (31) reported that light inhibits growth in sub-apical sections of etiolated pea stems in the presence of indoleacetic acid and riboflavin. Galston (29) later reported that this was because of destruction of auxin in the solution. This interpretation seems reasonable since Mer (67) reported that riboflavin apparently does not enter the protoplasts of cells in coleoptiles.

Considerations of quantum yields show that direct destruction of auxin could not be the means of action in the first positive phototropic response (121). Furthermore, excellent evidence exist for the lateral transport of auxin with little or no destruction of the hormone (119, 144, 145).

Destruction of auxin may have a role in some photo-processes, however. An example is the high energy curvature which comes to center in the base of coleoptiles. The action spectrum is similar to the absorption spectrum which one might expect for a bound auxin (21), but direct photolysis of auxin apparently does not occur (121). The process may or may not involve riboflavin (21, 138).

Direct photo-destruction of auxin may occur in tissue. Zenk (144, 145) has shown the process to occur in coleoptiles. He detected photolysis of only 1% of the free auxin within the cells. Nevertheless his work shows that flavin can be
photolytically active in vivo under intensities of light which induce the second and third positive phototropic responses (144).

One theory would allow amplification of the initial photolytic process. Pohl (69) suggests that a photo-degradation product of indole-acetic acid inhibits polar transport of auxin. The product, indole-3-aldehyde, is converted, enzymically, to indolecarboxylic acid. The latter compound is subject to photo-degradation. Combinations of the photo- and enzymic processes could explain the positive and the negative responses of Avena. Such inhibitions have been observed (68, 69), but at intensities far greater than those for the first positive response. Thimann and Curry (121) point out that if such a system were operative, an increased in vivo efficiency would be needed.

A photo-enzymic system has been suggested for the in vivo destruction of auxin. The enzyme indole-acetic acid oxidase was postulated to be stimulated by light (29, 32, 34, 121, 136). Photo-activation of part of the system gives an action spectrum which is similar to the absorption spectrum of riboflavin (32).

In addition, photo-inactivation of enzymes in the presence of riboflavin has been demonstrated (29, 31). An additional mode of action might be interaction of light to overcome the protective effect of riboflavin
phosphate in an enzymic system (136).

Other attempts to identify the receptor used plants with modified concentrations of pigments. Classic among these is use of plants with very low concentration of carotenoids (4, 29, 133). Most of these plants give normal responses. But the problems are many. Most albino plants contain enough carotenoid to effect a response (120, 132, 138); standard exposures and species were not always used (120); and some workers obtained responses which were indeed lower (120, 121).

Other workers have attempted modification of the content of pigment in normal plants. Page found that diphenylamine inhibited synthesis of carotenoid in Pilobolus but had no clear effect on the formation of trophocysts (78) or on the phototropic response of sporangiophores (79). Lyxoflavin, a competitive inhibitor for riboflavin, suppressed formation of trophocysts (78). The suppression was overcome to a small degree by an increase in exposure. This fact was taken as evidence that the interference was at the point of absorption of light. Addition of riboflavin overcame the inhibition. Carlile (15) found that mepacrine and lumichrome, which act as inhibitors of systems requiring riboflavin, had a greater effect on growth of illuminated Phycomyces than on the un-illuminated organisms. Bara and Galston (8) blocked synthesis of carotenoids in excised coleoptiles of Avena by use of
inhibitors. The responses were normal. Kumagai and Oda (57) treated the fungus *Trichoderma viride* with diphenylamine. A reduction in photo-response occurred. However, no carotenoids were found in normal hyphae and diphenylamine is known to combine with DNA (57). The same authors used atebrin, an antagonist of flavin, and got no change in response. When they used phenylacetic acid for the *in vivo* photo-inactivation of flavin no change in response occurred. When Hart and Filner (44) used phenylacetic acid in an earlier test with coleoptiles of *Avena*, definite inhibition of phototropism occurred. A further discussion of earlier works of this type may be found in an article by Thimann and Curry (121). 

Studies with the electron microscope showed crystal-containing bodies in coleoptiles of oats and sporangiophores of *Phycomyces* (123). *Zea mays* contained somewhat similar structures. These bodies could be the photo-receptive organelles; however, their general appearance is that of peroxisomes.

Yet another approach has been the study of the contents of pigments in the photo-responsive tissues. These studies have been largely of the coleoptiles of *Avena*. Büning (12) found a high concentration of carotenoid just below the tip. Thimann and Curry (120) reported the carotenoid to be localized just back of the area of highest sensitivity. Asomaning and Galston (4) found a higher concentration of
carotenoid in the tip than in the base. They suggested that flavins and carotenoids contributed equally to lessening of light across the receptive region. Thimann and Curry (120) reported that the content of riboflavin was moderate throughout the coleoptile and not concentrated in the tip. Asomaning and Galston (4) reported riboflavin mononucleotide to be most concentrated in the tip. More recently, Zenk (144, 145) has shown a compound of unknown structure to be the major flavin in etiolated coleoptiles of *Avena*.

There are other, even less understood, roles of flavins in plants, which, in time, may prove to be of fundamental significance. A very interesting process is the increased biosynthesis and secretion of riboflavin during deficiencies of iron, molybdenum, zinc, or manganese (73, 97, 98, 107). Riboflavin is also secreted during guttation (82).

Definite interactions between riboflavin, growth hormones, and chlorophyll apparently exist in plants (1, 2, 3, 80, 141). Gibberellin lowers the content of riboflavin in leaves (2, 141). Moreover, gibberellin decreases synthesis and accelerates destruction of chlorophyll; riboflavin reverses these effects (3). The amount of chlorophyll which is less strongly bound to protein is affected especially (1). Gibberellin and riboflavin also seem to increase the rate of photo-phosphorylation (2).

Finally, evidence exists for riboflavin having important
roles in the machinery of photosynthesis. A complex containing manganese, galactosyl diglyceride, linolenic acid, and flavin has been suggested to take part in the release of oxygen (126). (Interestingly, this process is influenced by blue light (118)). In addition, an in vitro complex of flavin and chlorophyll a resembles the P700 pigment (125).

Types of Flavins

Flavins other than riboflavin and the flavin nucleotides may have roles in the above processes or in processes yet to be discovered. The most noteworthy is the compound isolated by Zenk (144, 145). This pigment, referred to as FX, was found to be the dominant flavin in etiolated leaves and coleoptiles of Avena. FX and riboflavin accounted for 94% of the flavin in the etiolated coleoptile. FX seemed free in the water-soluble contents of the cell, but about 10% seemed to be bound in fraction containing the cell-wall. The absorption spectrum was characteristic of a flavin. Under mildly acidic or alkaline conditions FX was hydrolyzed to riboflavin. No phosphate was found after hydrolysis.

An enzyme which forms a glucoside and a galactoside of riboflavin has been found in higher plants (110, 111). Thus, the glycosides might occur in higher plants. In fungi and bacteria the glycosides have been postulated as carriers in the formation of oligosaccharides (48, 109).
In animals they have been postulated as intermediates in the biosynthesis of flavin adenine dinucleotide (134, 135). Phosphates of riboflavanyl glucoside have been reported (113, 114, 115, 116).

A flavin of undetermined structure, which gives riboflavin on hydrolysis, has been isolated from the yeast, Candida curvata (98).

Products from the breakdown of riboflavin have been found in plants. Lumichrome has been detected in seedlings of Bengal gram which was grown in light (74). Schopfer (95) reported the presence of lumiflavin and lumichrome in the vacuoles of cells, but details of the determination were not given. Stocks (107) obtained suggestive evidence that these compounds, plus several others, might be formed. Flavoviolet has been reported in pea leaves cultured in solutions of riboflavin (46). Enzymic action by riboflavin hydrolase produces lumichrome in spider lily (58) and in water cress (117).

Concentrations of Flavins

Marked changes in concentration of riboflavin during growth of plants have been reported. Unfortunately, the exact nature of the flavins in many tissues is not known, and derivatives of riboflavin may have been counted as riboflavin in some cases. However, the data do indicate trends in flavin
Large changes in riboflavin content occur during germination (7, 13, 41, 74, 75, 101). In germinating green beans, flavin is synthesized as free riboflavin (62).

In growing plants, the concentration is often low in stems and roots (45). Generally it is highest in leaves (45, 74), but it may be high in flowers, also (45). Indeed, stigmas of Crocus sativus L. are reported to be the richest known source of riboflavin (88). In wheat, riboflavin increases chiefly during vegetative growth (14). In peas, the increase becomes only slightly less after fructification (14). For Bengal gram, the rate of synthesis is very high during flowering and formation of seeds (74).

The content of flavin within an organ may vary greatly during development. In tomato, the highest concentration of riboflavin is found in leaves and stems while these organs are still immature (37). In Bengal gram the amount of total riboflavin in the leaves increases from 50% to 70% as the plant matures, but the amount in pods and stems decreases (74). In many plants the amount of flavin in the fruit or seed may increase 2-5 fold during the formation of seeds (45). In some, such as Bengal gram, the accumulation of riboflavin in the developing seed may be slower than the accumulation of dry weight, and thus the concentration may decrease with maturity (74). Apparently, riboflavin synthesized in the leaves is
transported to other parts of the plant (37, 52, 71, 74) and may be transported into the maturing seeds (52, 74).

The changes continue after maturation. During desiccation at the harvesting stage, the content of riboflavin suddenly drops for Bengal gram (74). During withering, the contents in peppers and egg-plants may drop to one third that of the fresh vegetables (26). On the other hand, the content of riboflavin in ungerminated seeds from cereals and legumes may double during nine months of storage (91). The concentration in seeds of certain varieties of cotton may increase 300% if the seeds are heated to 45°C. during dormancy (85).

The effect of light on the amount of flavin (4, 13, 32, 38, 51, 74), is not as clear as the effect on the form of flavin. In oats, the acid-liberated flavin (presumed to be bound to protein) decreases more than 50% over a period of 15 hours when germination occurs in light. Interestingly, this change is not found in light-insensitive Avena (43). For the epicotyls of seedlings of horse bean, the ratios of esterified to total flavin changes from 58% in the dark to 71% in the light (51). Etiolated leaves of oats contain 83% FX and 17% flavin phosphate; green plants of the same age contain 60% flavin phosphate (145). Riboflavin phosphate increases in blue or red light (4).

Only slight changes in concentration of riboflavin occur
in green plants due to exposure to light of varying intensities (38). The concentration is not affected by exposure of the plants to cycles of light which induce flowering (60). However, plants exposed for increasing lengths of time have increasing concentrations of riboflavin (39).

Temperature definitely affects the concentration of riboflavin in plants, but the effect varies with species (40).

Infection and wounding effect the concentration of flavin. Following wounding the tissue increases biosynthesis of riboflavin parallel to increases in respiration. Free riboflavin increases but bound decreases. (Light does not affect the process) (87). During infection of tomato with tobacco mosaic virus the content of riboflavin in the leaves decreases to half of that in leaves of healthy plants. The content of the roots increases slightly (53).

The concentration of minerals greatly affects the concentration of flavin in plants. Thus soil and fertilizer (103, 104, 105) are important. Phosphorus (103) and nitrogen (36) are significant, of course. But iron (86, 137), cobalt (24), magnesium (61), and the micro-nutrient elements in general are important (65). Large amounts of riboflavin are excreted from roots of plants deficient in iron, or high in the other elements. Iron may be necessary for the binding of riboflavin; the other elements, in excess, may compete with iron and cause the same effect as a deficiency in iron.
The excretion of riboflavin by higher plants (and by micro-organisms) means that riboflavin occurs in soil (17). This flavin possibly may be taken up by other plants (107).

Isolation of Flavins

The most widely used methods for extraction and assay of flavins have been well reviewed by Yagi (140), Pearson (81), Beinert (9), and Strohecker and Henning (108). These methods contain a number of harsh conditions which make them less than ideal for work with certain flavins. Acid in the form of HCl and/or trichloroacetic acid is often used to remove protein during or following extraction. These acidic conditions lead to accelerated decomposition of flavin adenine dinucleotide (81), FX (144), and possibly riboflavinyl glucoside (99, 139). Oxidation with potassium permanganate has been used extensively to decompose interfering fluorescent compounds. Such treatment may lead to a nonspecific blue fluorescence (50) and to oxidation of flavins (28, 124). Methods using alkaline photolysis have the disadvantage of converting most, if not all, flavins to a mixture of photoproducts (124). Without methods of separation, direct or indirect fluorimetry can tell nothing of the nature of the flavins.

Thus with increasing types of flavins being discovered,
the need for methods which will allow the isolation and separation of flavins increases. Since Crammer's work (19) in 1948, paper chromatography has been used extensively for separation of flavins. Metzler's group (102, 106, 124) has obtained improved separations of flavins by using silica gel in thin-layer chromatography. Harris (42) claimed best separation of flavin nucleotides with use of cellulose.

For the best use of chromatography, flavins from tissue need to be separated from substances which interfere with their migration and detection. Methods for this purpose which were not reviewed in the above references are those using phenolic resins (56) and talc (130, 131).
MATERIALS AND METHODS

Materials

All chemicals were reagent grade. Redistilled water was used.

Silica Gel G or H from Brinkmann and Florisil (60-100 mesh) from Matheson Coleman and Bell were used. Silica Gel 7G and Florisil TLC were gifts from J. T. Baker Chemical Company and from Floridin Company, Hancock, West Virginia, respectively. Coarse talc was obtained by repeated washings of talc obtained from Mallinckrodt Chemical Works. For columns with unwashed talc and for thin-layer chromatograms, talc (U.S.P.) from J. T. Baker Chemical Company was used. Resin R-15 (a resorcinol-formaldehyde resin) was prepared according to the procedure of Koziolowa and Koziol (56). Flavin glycosides were prepared according to the procedure of Suzuki and Uchida (111). Medium for yeast was obtained from Difco Laboratories, Detroit, Michigan.

Bakers' yeast (Saccharomyces cerevisiae Meyen) was obtained from the Department of Bacteriology at Iowa State University. Seeds of wheat (Triticum aestivum L., variety Gage) and of oats (Avena sativa L., variety Multiline-E-69) were obtained from the Seed Laboratory of Iowa State University. Seeds of oats (Avena sativa L., variety Clinford) were obtained from Dr. J. A. Browning of the Department of Botany and Plant Pathology at I.S.U. Seeds of cotton
(Gossypium herbaceum L., variety Coker-413-68) were obtained from the Central Alabama Cooperative, Selma, Alabama. Irish potato (Solanum tuberosum L.) tubers and sweet potato (Ipomoea batatas Poir.) tubers were obtained from Hy-Vee Grocery Store in Ames. Leaves of Japanese Magnolia (Magnolia soulangeana Soul.) were obtained in June from a bush which was growing on campus. Roots of beets (Beta vulgaris L.) were of an unknown, locally grown variety. Seeds of corn (Zea mays L., variety Snowcross) and Job's tears (Coix lacryma-jobi L.) were obtained from W. Atlee Burpee Seed Company, Clinton, Iowa. Snowcross corn was selected because it was used by Briggs (11) in studies of phototropism.

Methods

Growth

Seeds were soaked for half an hour in 1% (v/v) Clorox and then placed between moist paper towels, or in moist Vermiculite. Seedlings were grown in darkness except for exposure to dim red light during watering and harvesting.

Bakers' yeast was cultured in a medium consisting of 30 g. of glucose, 3.0 g. of yeast extract, 5.0 g. of peptone and 3.0 g. of malt extract per liter of distilled water. After the medium was autoclaved for 40 minutes at 121.6°C and 15 p.s.i. of pressure, one loopful of suspension of yeast
was transferred to 25 ml. of medium contained in 125 ml. Erlenmeyer flask. These flasks were rotated at a speed of four on a Gyrotory Shaker (Model G-78) by Brunswick Scientific Company. On the beginning of the third day of growth the yeast was transferred to Fernbach culture flasks containing 600 ml. of medium. Cells were grown in the dark at room temperature and harvested on the second or third day after transfer.

**Preparation**

Harvested tissue was added to a Waring blender with two or more times its weight of water at 22°C and enough \((\text{NH}_4)_2\text{SO}_4\) to give a saturated solution. Blending normally consisted of two runs of 30 seconds each. The homogenate was centrifuged for 30 minutes at 16,000 r.p.m. and 0°C. Centrifuged extracts containing suspended debris were filtered. Solid residue was removed from large samples by filtering through cheese cloth before centrifugation. In investigations for unknown flavins 100 g. of tissue were used. For most other studies one to ten grams were used.

For determinations of dry weights, randomly selected samples were dried at 80°C until a constant weight was obtained.

For exposure to light, samples were placed 15.0 cm. below two 40 watt white Westinghouse fluorescent tubes for six hours. This exposure gave an average intensity of
1100 foot-candles as determined with a Weston Illumination Meter (Model 735). For the quantitative study with corn, seedlings were exposed for six hours to sunlight. (The exposure was from 10:44 A.M. to 4:44 P.M.; the maximum intensity was 6000 foot-candles.) One sample of corn was exposed to sunlight for eight hours on August 14.

After illumination, the yeast cells were removed from the medium by centrifugation at 5000 r.p.m. for 20 minutes. The cells were twice washed with 3.5% glucose, suspended and recentrifuged. The resulting preparation was treated in a manner similar to that given above for the seedlings.

During purification, solutions were evaporated at 40°C in a rotary evaporator.

**Phenolic extraction**

Phenolic extraction was according to a procedure similar to that used by Yagi (140) and by Zenk (145). When extracts from corn and oats were allowed to migrate on thin-layer chromatograms, large amounts of blue fluorescent materials obscured parts of the chromatograms. However, the second yellow fluorescent compound from oats was clearly visible and no compound of corresponding migration was detected from corn.
Chromatography

Florisil Columns were packed using aqueous slurries of Florisil (60-100 mesh) and gravitational flow of solvents. Flavins were applied in aqueous solution and then washed onto the column with 25 ml. of water. The flavins did not bind in a distinct, narrow band.

No narrow bands were obtained during elution. Of numerous mixtures of acetone, acetic acid, and water, and of pyridine and water, pyridine-water (1:9) gave the sharpest band. With pure acetone, acetone-water (1:4), acetic acid-water (1:1), acetone-water (1:9), and pyridine-acetic acid-water (1:1:16) riboflavin spread over the entire length of the column. With acetone-water (1:1) a diffuse band approximately half the length of the column was obtained. With acetic acid-acetone-water (1:4:5) a diffuse band approximately one third the length was obtained. With pyridine-acetic acid (1:1) riboflavin moved as a band but with tailing. Pyridine-acetic acid-water (1:1:2) gave rapid elution but with a band covering one third the length of the column. Pure pyridine gave rapid elution but no distinct band; pyridine-water (1:19) caused streaking over the entire column.

The Florisil contained soluble materials having blue fluorescences. Some of these materials were removed by repeatedly washing with distilled water (93). However, enough remained to severely handicap the identification of the
flavins.

When 60-100 mesh Florisil was used for thin-layer chromatograms, no distinct separations were obtained with 2-butanone-acetic acid-methanol-benzene(1:1:4:14), n-butanol-acetic acid-water(4:1:5), acetone-water(1:1), acetone-water (1:4), or pure acetone. Little migration was obtained with acetone-water(1:9) or with 0.01 N HCl. In general, this mesh of Florisil was too coarse.

On Florisil TLC, riboflavin, lumichrome, lumiflavin, and formylmethylflavin did not migrate in 2-butanone-pyridine-methanol-benzene(1:1:4:14), in 2-butanone-pyridine-methanol-benzene(2:1:4:14), in acetone-0.01 N aqueous HCl(1:9), or in distilled water. Slight migration of formylmethylflavin was obtained with n-butanol-acetic acid-water(4:1:5). With 2-butanone-acetic acid-methanol-benzene(1:1:4:14) the flavins streaked, but did not migrate as spots. With pyridine-water (1:9) there was extreme streaking. As with the columns, the blue fluorescent impurities interfered with determinations of the flavins.

R-15 resin The resin was prepared according to the procedure of Koziolowa and Koziol (56). A 4 x 80 mm. column was used for samples from one to five grams of fresh tissue. For larger samples, a 100 mm. bed of resin in a 10 mm. column equipped with a coarse sintered glass disc and a 1.0 mm.
capillary stopcock was used. The column gave rapid flow-rates (up to 1 ml./min.) which could be controlled by adjusting the stopcock. When aqueous solutions of centrifuged, filtered extract were applied to the column, salts, remaining protein, and most other impurities passed directly through the column. Flavins (as well as some other pigments) remained firmly attached. Most, if not all, of the flavin nucleotides were removed (56). They were held from salt solutions, but lost during washing with water. For samples of from 10 to 100 g. (fresh wt.), 500 ml. of water were used for washing and 100 ml. of acetone-water(1:1) for elution. For most samples of less than ten grams, 250 ml. of water and 50 ml. of acetone-water(1:1) were used.

In general, the non-flavin material remaining after passage through resin (and to some degree, talc also) increased when etiolated tissue was exposed to light and when extracts in aqueous solutions were heated to 80°C (to precipitate proteins).

Use of resin without further purification gave samples in which riboflavin was readily detectable on chromatograms with extracts from Irish potato and cotton. Samples from leaves of magnolia were fairly pure after use of the resin, but enough impurities remained to cause migration different from that of authentic riboflavin. However, after the flavin was purified on thin-layer chromatograms in W, its migration
in KMB and BAW was the same as that of authentic riboflavin (for composition of solvent systems, see Table 2).

However, the resin alone was not a satisfactory system for purification of flavins from most plants. Extracts from corn coleoptiles gave two major impurities and traces of other impurities even after washing with 500 ml of water, 100 ml. of 0.1 N (NH₄)₂SO₄ and 200 ml. of water, or 100 ml. of 0.1 N HCl and 200 ml. of water. Washing with (NH₄)₂SO₄ (or HCl) and water was superior to washing with water alone. Washing with (NH₄)₂SO₄ rather than HCl avoided breakdown of any nucleotides to riboflavin (56). The major impurities could not be removed by fractionation.

In samples from beets, carrots, yeast, spinach, and oats enough blue fluorescent impurities remained to largely or totally obscure the detection of riboflavin on thin-layer chromatograms. In samples from beets, the impurities obscured the region between riboflavin and the origin. In BAW, one distinct dull blue fluorescent spot with a Rf value of approximately 0.10 was detected. Carrots contained blue, orange and dull green fluorescent impurities in such concentrations as to cause severe streaking. Yeast and sweet potato contained large amounts of bright blue fluorescent material. With AKMB extracts from sweet potato gave a bright blue fluorescent spot which was so located that it totally obscured riboflavin, a blue-violet fluorescent spot at about one third the distance
travelled by riboflavin and a similar colored spot at the origin. In BAW, Irish potato gave dark blue spots fluorescent spots with Rf values of 0.45 and 0.20. Material with a whitish-blue fluorescence streaked upward from the origin. In AKMB, most of these compounds were well below riboflavin. During electrophoresis for three fourths of an hour a blue fluorescent spot moved 1.5 cm. toward the negative electrode and a black spot moved 10.3 cm., but no yellow fluorescent spots were observed. Samples from cotton seeds were comparatively clean but some bluish-white fluorescent material streaked upward from the origin on chromatograms which had been placed in BAW and AKMB.

Spinach and sweet potato also contained a number of fluorescent impurities. For spinach, dark green compounds migrated in the area from 5.0 to 1.8 cm. toward the positive electrode and from 0.5 to 1.7 cm. toward the negative electrode. A blue compound migrated in the area from 1.9 to 2.6 cm. toward the negative electrode. In BAW, some faint blue material occurred in the area from slightly above riboflavin to near the solvent front. Some impurity remained at the origin.

During electrophoresis of samples from sweet potato, materials migrating toward the negative electrode were a blackish substance at 0.7 cm. and a blue one at 1.8 cm. Those toward the positive electrode were a blue-green band at 1.2
cm., a blue one at 3.2 cm., a whitish-yellow one at 9.8 cm., a blue one at 12.6 cm. and a very faint whitish-blue one at 16.2 cm. A dirty white material remained at the origin.

With later samples of sweet potato the best separation with electrophoresis was obtained at 4000 volts for 45 minutes. Under these conditions the yellow compound migrated 4.8 cm. toward the positive electrode and a blue compound, 2.1 cm. A yellow compound migrated 1.3 cm. toward the negative electrode and a blue one 2.4 cm. A whitish-aqua material remained at the origin.

Dowex\textsuperscript{1} Solutions from R-15 resin were applied to Dowex Gl-4X (in OH form). The column of Dowex was washed with acetone-water(1:1), and then with 0.005 M NH\textsubscript{4}Cl. Then 0.1 M NH\textsubscript{4}Cl was applied for elution of the flavins. After applying NH\textsubscript{4}Cl the eluate changed from pH 7 to pH 9-9.5. The fractions containing flavin were applied to R-15 resin for desalting. The resulting sample contained fewer impurities than samples passed through the resin alone, but large amounts of blue fluorescent impurity remained. Riboflavin was the only flavin obtained from extracts of oats.

Talc Wahba and Fahmy (130, 131) reported a system for the isolation of riboflavin from mixtures of vitamins

\textsuperscript{1}Matsui, K., Osaka City University, 459, Sugimoto-cho, Sumiyoshi-ku, Osaka, Japan. Use of Dowex for separating flavins. Private communication. 1969.
and from urine which used columns of large particles of talc. In the present work, Mallinckrodt Talc gave the best yield of the sized particles used by Wahba and Fahmy.

In the original method the talc columns were washed with dioxan-water (1:9) and eluted with dioxan-water (1:4). To avoid peroxides, which could decompose flavins, acetone was substituted for dioxan. Rates of flow were slow (4 ml./hr. for a 5 x 100 mm. column having a 60 cm. head of solvent). Mixing Celite with the talc did not improve the rates, and flow was completely stopped by small amounts of protein.

For the isolation of flavins from plants, unwashed talc by Baker Chemical Company had sufficient binding power. Columns were prepared by adding enough slurry of talc-water (1:2) to form a 30 to 50 mm. bed in a 10 x 100 mm. tube fitted with a sintered glass disc. Suitable flow was obtained by use of suction. The flavin, which were applied in aqueous solution, formed a narrow band near the top of the bed. During washing with acetone-water (1:9) the flavin very slowly moved down the column as a sharp band. Elution was accomplished by continuation of this washing, or by changing to acetone-water (1:1).

With samples in which the flavin content was small relative to that of other pigments, the flavin was sometimes lost from the columns before the impurities were removed. However, if the column was washed with water periodically
during the application of the sample, the flavin remained near the top of the column.

When high concentrations of materials from talc columns were applied to chromatograms, dull blue and bluish-white impurities were observed near the origin for some samples from yeast, corn and wheat. These results were obtained when large samples were applied to talc columns or when, in order to insure complete recovery of flavins, fractions larger than those containing the flavin were collected. Use of a second talc column removed most, if not all, of these impurities. Some impurities in the upper region of chromatograms came from silica gel and solvents. In samples from carrot, an extremely faint dark blue fluorescent spot was observed in the upper area of two-dimensional chromatograms.

Extracts which contained appreciable protein stopped flow through the column. Thus columns of fine talc could best be used after removal of protein (for example, as a second purification step after the resin), or with tissues which contained little protein. Flavins isolated from small samples which were relatively low in protein gave satisfactory separations. Thus when extract from 150 coleoptiles of corn was applied to a talc column, without prior use of resin, riboflavin, but no impurities, was detected on chromatograms.

For thin-layer chromatography, a mixture of 40 g. of talc (J. T. Baker Company, Phillipsburg, N.J.) and 65 ml. of
water was spread to give five and one half 20 x 20 cm. chroma-
tograms of 250 μ. thickness. Chromatograms were air-dried, but drying at 100°C for one or for two hours caused no dif-
ference in separation. Preparative chromatograms were made by using 45 g. of talc and 70 ml. of water to give one 20 x 
20 cm. chromatogram.

Attempts to use talc chromatograms for separation of flavins proved futile. AKMB, BAW, acetic acid-acetone-water 
(1:3:21), acetone-water(1:1) and pure acetone were unsatis-
factory. Little or no migration was obtained with methanol-
water(1:19), methanol-water(3:17), acetone-water(1:9) or 
with 0.01 N HCl.

The talc chromatographic system was of significant value in purification of flavins from plants, however. With ex-
tracts from etiolated shoots of corn seedlings most of the impurities migrated away from the origin. The major impuri-
ties had a Rf value of approximately 0.70 after one mig-
ration in acetone-water(1:9), 0.01 N HCl, or a combination of the two systems. After two migrations, the major impuri-
ties were concentrated at the top of the chromatogram. Riboflavin, lumiflavin, lumichrome, and solutions from the photolysis of riboflavin which were rich in compounds "S" 
(124) and "A" (124) showed no migration in 0.01 N HCl and 
only slight migration after two migrations in acetone-
water(1:9).
The capacity of talc thin-layer chromatograms was approximately 0.5 μg. per spot of 5.0 mm. diameter. When this capacity was exceeded, streaking upward from the spots occurred. By carefully applying extracts in a band, movement of the flavin could be obtained. The movement of the flavin away from the origin allowed its separation from a non-flavin, fluorescent residue which remained at the origin.

The flavins often did not move in an even, narrow band. There was diffuse and slightly uneven migration to a Rf value of about 0.05 with acetone-aqueous 0.01 N HCl(1:9). However, by examining the chromatogram under ultraviolet and visible light the distance between the flavins and the first impurity could be determined. This distance was large and allowed removal of a wide band to insure recovery of all the flavin without impurity. The band could be removed arbitrarily during later separations of the same type, and thus avoid exposure of the flavins to light. For isolation from corn seedlings, a band 0.05 to 7.0 cm. from the origin was removed. Flavins from this band contained only one minor impurity.

Elution of the flavin from the talc scraped off the chromatograms was accomplished with acetone-water(1:1). A series of elutions was tried using pure acetone and mixtures of acetone and water down to a ratio of 1:9. The range from 2:8 to 9:1 proved satisfactory.
The use of talc in preparative chromatograms offered no advantage over the normal chromatograms. A major disadvantage was that the thick layers of talc made detection of the flavins very difficult.

Because of their low capacity talc thin-layer chromatograms sometimes proved inferior to talc columns. This is especially true with materials such as roots from carrot where flavin is a minor water-soluble fluorescent pigment.

Silica gel Methods for using Silica Gel G and H are given by Treadwell, Cairns, and Metzler (124).

A smoother layer was obtained when the silica gel was spread as a suspension in acetone (89). The clarity of separation was not enhanced with activated or unactivated chromatograms, and therefore this method was not generally used.

Silica Gel 7G was used according to the packaging directions. The separations obtained were comparable to those obtained with Brinkmann's Silica Gel G or H. In AAW good separations were obtained even on unactivated chromatograms. Lumiflavin was only slightly above riboflavin. The ratio of migration for riboflavin to lumichrome was 0.50. Good separations were also obtained in AKMB on unactivated chromatograms. With the above system, for activated and unactivated chromatograms, the distance between riboflavin
and compound "A" (124) was slightly greater than with Silica Gel G or H. For unactivated chromatograms the ratio of distances of migration for riboflavin with reference to those of lumichrome was 0.31; for activated, 0.29.

All types of silica gel gave a large area of faintly blue fluorescent material diagonally across from the origin when two-dimensional chromatograms were run. This area made detection of small amounts of lumichrome difficult. The material could be caused to migrate slightly beyond the area of lumichrome by repeatedly allowing the solvent to migrate to the top of the chromatogram. Methanol, ethanol, butanol, and silica gel were found to contain blue fluorescent impurities.

The best of the solvent systems which were tested are given in Tables 1 and 2. The new systems were developed by direct modifications of known systems, except for WMBC. The latter system was developed especially for polar compounds which are subject to acidic hydrolysis. For it, varying amounts of the components were tested; the present composition gave best resolution.

Several points about other systems should be noted. Systems containing formic acid or acetone often gave wide variations in Rf values. Those systems containing ammonium ions gave two or more spots from lumichrome. This apparent formation of derivatives was used as a test for lumichrome.
Table 1. Migration of flavins on thin-layer chromatograms

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\(^a\) Time: hours needed for front to reach 15.5 cm.

\(^b\) Rating based on general clarity of separation.

\(^c\) Devised in present study.

\(^d\) LC = Lumichrome, FMF = Formylmethylflavin, LF = Lumi-flavin, HEF = Hydroxyethylflavin, RF = Riboflavin, FMN = Riboflavin mononucleotide, FAD = Riboflavin adenine dinucleotide, ARF = 3-Acetic acid riboflavin, B = Compound B (from photolysis), GUF = Riboflavinyl glucoside, GALF = Riboflavinyl galactoside.
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<tr>
<td>.000</td>
<td>.49</td>
<td>.47</td>
<td>.48</td>
<td>.63</td>
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</tr>
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<td>.000</td>
<td>.000</td>
<td>.075</td>
<td>.10</td>
<td>.000</td>
<td>.000</td>
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</tr>
<tr>
<td>.55</td>
<td>.57</td>
<td>.69</td>
<td>.57</td>
<td>.69</td>
<td>.68</td>
<td></td>
</tr>
<tr>
<td>.000</td>
<td>.000</td>
<td>.21</td>
<td>.18</td>
<td>.052</td>
<td>.045</td>
<td></td>
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<tr>
<td>.000</td>
<td>.000</td>
<td>.000</td>
<td>.017</td>
<td>.034</td>
<td>.029</td>
<td></td>
</tr>
<tr>
<td>.026</td>
<td>.043</td>
<td>.092</td>
<td>.16</td>
<td>.24</td>
<td>.18</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Composition of solvent systems for the separation of flavins on thin-layer chromatograms

<table>
<thead>
<tr>
<th>No.</th>
<th>Solvent System</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-butanol: acetic acid: water: ethyl ether (8:1:1:5) (BAWE)</td>
</tr>
<tr>
<td>3</td>
<td>iso-amyl alcohol: 2-butanone: acetic acid: water (8:8:1:2.5) (AKAW)</td>
</tr>
<tr>
<td>4</td>
<td>iso-amyl alcohol: 2-butanone: pyridine: water (8:8:1:2.5) (AKPW)</td>
</tr>
<tr>
<td>5</td>
<td>chloroform: ethyl acetate: acetic acid (1:2:1) (CEA)</td>
</tr>
<tr>
<td>6</td>
<td>n-butanol: formic acid: water (1:1:1) (upper layer) (BFW)</td>
</tr>
<tr>
<td>7</td>
<td>benzyl alcohol: acetic acid: water (3:1:3) (lower layer) (ZAW)</td>
</tr>
<tr>
<td>8</td>
<td>iso-propanol: pyridine: water (1:3:1) (PPW)</td>
</tr>
<tr>
<td>9</td>
<td>t-amyl alcohol: pyridine: water (5:1:5:3:5) (APW)</td>
</tr>
<tr>
<td>10</td>
<td>water (W)</td>
</tr>
<tr>
<td>11</td>
<td>n-butanol: acetic acid: water (5:1:2.2) (BAW)</td>
</tr>
<tr>
<td>12</td>
<td>n-butanol: acetic acid: water (4:1:5) (BAWa)</td>
</tr>
<tr>
<td>13</td>
<td>iso-amyl alcohol saturated with water (AW)</td>
</tr>
<tr>
<td>14</td>
<td>n-amyl alcohol: acetic acid: water (3:1:3) (upper layer) (AAW)</td>
</tr>
<tr>
<td>15</td>
<td>acetic acid: acetone: methanol: benzene (1:1:4:14) (AAMB)</td>
</tr>
<tr>
<td>16</td>
<td>acetic acid: 2-butanone: methanol: benzene (1:1:4:14) (AKMB)</td>
</tr>
<tr>
<td>17</td>
<td>acetic acid: 2-butanone: methanol: benzene (5:6:25:80) (AKMBa)</td>
</tr>
<tr>
<td>18</td>
<td>pyridine: 2-butanone: methanol: benzene (1:1:4:14) (PKMB)</td>
</tr>
<tr>
<td>19</td>
<td>methanol: benzene (1:4) (MB)</td>
</tr>
<tr>
<td>20</td>
<td>2-butanone: methanol: benzene (1:4:15) (KMB)</td>
</tr>
<tr>
<td>22</td>
<td>n-butanol: acetic acid (2.5:7.5) (BA)</td>
</tr>
<tr>
<td>23</td>
<td>dioxane: water: acetic acid (80:20:0.4) (DWA)</td>
</tr>
<tr>
<td>24</td>
<td>acetic acid: acetone: ethanol: benzene (5:5:20:70) (AAEB)</td>
</tr>
<tr>
<td>25</td>
<td>pyridine: acetic acid: water (10:1:40) (PAW)</td>
</tr>
<tr>
<td>26</td>
<td>2-butanone: acetic acid: methanol: chloroform (1:1:4:14) (KAMC)</td>
</tr>
<tr>
<td>27</td>
<td>chloroform: methanol (1:4) (CM)</td>
</tr>
<tr>
<td>28</td>
<td>water: methanol: n-butanol: chloroform (2:2:8:1.5) (WMBC)</td>
</tr>
</tbody>
</table>
Aqueous solutions of salts, such as 5% boric acid or NaHPO$_4$, caused severe streaking. With charged compounds PAW seemed to separate into two components.

W was of value because charged compounds seemed to migrate to a position near the solvent front; uncharged compounds remained in the lower one third of the chromatogram. Resolution was greatly improved by pre-running the chromatograms. When this was done, the Rf values of charged compounds decreased and those of uncharged compounds increased. The system proved unsuited for two-dimensional separations except when used in the first dimension after pre-running. Then, with WMBC or BAW for the second dimension, excellent separations were obtained.

**Electrophoremetry**

A high voltage electrophorator by Gilson (Model D-HVE) was used. Samples were run on 15.0 by 56.5 cm. sheets of Whatman #3 filter paper in pyridine-acetic acid-water(1:3.4:409) at 4000 volts. Standard flavins were run for three-fourths hour. Riboflavin, lumichrome, the flavin nucleotides, and most material from extracts were run for one hour.

**Fluorimetry**

The fluorimeter was constructed by Mr. N. E. Busch of the Department of Biophysics at Iowa State University. The light source was a Hanovia lamp. A filter produced
a beam for excitation consisting of the 360 nm. band of the mercury emission spectrum. A photocell by Pacific Photometric Instruments was connected to a Model 417 High Speed Picoammeter by Keithley Instruments. Concentrations was determined by referring to the standard curve shown in Figure 1. This method was used for the determination of the concentrations of flavins in corn exposed to sunlight.

**Spectrophotometry**

Determinations of all other concentrations of flavins were made using a Cary (Model 1501) recording spectrophotometer. Determinations done by fluorimetry were found to give values which agreed to within ±2.0% to those determined with the spectrophotometer.

**Chemical test**

A solution of 24 mg. of periodic acid in 24 ml. of methanol was used to spray the area of chromatograms containing flavin until it was visibly wet.

Photolysis on chromatograms and in methanolic solution was carried out according to the procedure of Treadwell, Cairns, and Metzler (124), except time of exposure was varied.

Saturated aqueous solutions of silver nitrate and acetate were applied from capillary tubes to compounds adsorbed on paper. Acidic hydrolysis was with N HCl at 100°C for 2.5 hours (according to the procedure of Whitby (139)).
Figure 1. Standard curves for fluorimetry
Alkaline hydrolysis was with 0.01 N NaOH at room temperature for one hour.
RESULTS

Isolations of Free Flavins

For the isolation of flavins from plants, use of resin and of talc columns followed by thin-layer chromatograms gave the best results. In the following discussion the color of a compound refers to the color of its fluorescence in ultraviolet light. After passing the extract from one coleoptile (2.7 cm. long; 0.063 g., fresh wt.) through both resin and talc columns, varying amounts of the resulting sample were spotted on thin-layer chromatograms and allowed to migrate in AKMB. The limit of detection was such that when 1/40 of the total sample (approximately 1.5 ng.) was applied to the chromatogram, riboflavin could be seen after migration. However, the spot was extremely faint and no flavin was detected when 1/100 of the sample (approximately 0.6 ng.) was spotted. When 1/10 of the sample was spotted, a readily detectable yellow spot was observed.

Materials giving riboflavin only

Corn The only yellow compound observed in extracts from coleoptiles, shoots and whole seedlings of corn was one having the same chromatographic behavior in BAW and in AKMB as authentic riboflavin. Samples from phenolic extraction confirmed the results for coleoptiles.
<table>
<thead>
<tr>
<th>Name of plant</th>
<th>Variety (source)</th>
<th>Part</th>
<th>Presence of Ribo-flavin</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta vulgaris (Beet)</td>
<td>(local)</td>
<td>root</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Daucus carota (Carrot)</td>
<td>(Hy-Vee)</td>
<td>root</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Saccharomyces cerevisae (Yeast)</td>
<td></td>
<td>whole cell</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Spinacea oleracea (Spinach)</td>
<td>(Libby)</td>
<td>leaf</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Magnolia soulangeana (Japanese Magnolia)</td>
<td></td>
<td>leaf</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gossypium herbaceum (Cotton)</td>
<td>Coker-413-68</td>
<td>seed</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Solanum tuberosum (Irish potato)</td>
<td>(Hy-Vee)</td>
<td>tuber</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ipomoae batatas (Sweet potato)</td>
<td>(Hy-Vee)</td>
<td>tuber</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Zea mays (Corn)</td>
<td>Snowcross</td>
<td>etiolated coleoptile</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>shoots</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>seedling</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Triticum aestivum (Wheat)</td>
<td>Gage</td>
<td>etiolated shoot</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Avena sativa (Oats)</td>
<td>Multiline-E-69</td>
<td>etiolated shoot</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Clinford</td>
<td>etiolated shoot</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Coix lacryma-jobi (Job's tears)</td>
<td></td>
<td>etiolated shoot</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>
Beets, carrots, sweet potato, yeast, wheat, Irish potato, cotton, and magnolia Extracts from these plants gave, as the only compound having a yellow fluorescence, a compound having the same chromatographic migration in BAW and AKMB as authentic riboflavin. The content of riboflavin in leaves of magnolia seemed to be relatively high; that in dormant seeds of cotton, relatively low.

Materials giving flavins or flavin-like compounds of unknown structure

Job's tears Samples from etiolated shoots of Job's tears gave relatively high concentrations of free riboflavin and of two yellow compounds of unknown structure, T-1 and T-2. On electrophoresis, only one spot, whose migration corresponded to that of authentic riboflavin, was obtained from a mixture of the three compounds. The chromatographic migrations of T-1 and T-2 are shown in Figure 2.

When photolysis, on chromatograms after one dimension in BAW, was attempted for 20 and for 60 minutes, no decomposition was detected for T-1 or T-2. When allowed to migrate in BAW, sprayed with methanolic solution of periodic acid and then allowed to migrate in a second dimension in BAW, T-1 and T-2 each gave a compound with a migration corresponding to that of authentic formylmethylflavin.
Oats  Riboflavin and a second compound, X, which migrated below riboflavin in BAW, WMBC and AKMB were detected (see Figure 2). The presence of the second compound in oat seedlings was confirmed by use of phenolic extraction. Compound X remained at the origin in PKMB and migrated near the solvent front in W. It moved 8.1 cm. toward the positive electrode during electrophoresis.

The amount of X in preparations relative to riboflavin varied greatly. Largest relative yields were obtained when samples were shielded from all light during growth and isolation.

When X was photolyzed on chromatograms for 20 minutes after one-dimension in AKMB, Compound "S" (124) was the only product. Some X remained unphotolyzed. Photolysis for five, seven and ten minutes in a partially degassed methanolic solution of X gave a compound which migrated the same as authentic lumichrome. No formylmethylflavin and Compound "A" (124) were apparent.

Treatment with a solution of dithionite caused loss of fluorescence followed by its rapid return in the presence of oxygen. Treatment with solutions of silver acetate and nitrate cause a loss of fluorescence. When X was sprayed with a methanolic solution of periodic acid and then allowed to migrate in BAW, a compound with a migration similar to authentic formylmethylflavin was formed. Some tailing always
Figure 2. Migration on thin-layer chromatograms of standard flavins, and unknowns extracted from plants; for composition of solvent systems see Table 2
occurred and the spot repeatedly had a slightly lower migration than authentic formylmethylflavin.

Hydrolysis of X with acid gave a compound having the same chromatographic migration in AKMB, BAW and WMBC and the same electrophoretic migration as authentic riboflavin. Spraying the product of hydrolysis with a solution of periodic acid gave the same results as obtained with the original X. Alkaline hydrolysis gave a compound with the same migration in BAW and AKMB as authentic riboflavin.

Spinach On electrophoretograms yellow compounds migrated 14.5 and 10.5 cm. toward the positive electrode. Riboflavin was obscured. When the extract was allowed to migrate on chromatograms in W, two spots were observed near the solvent front; in BAW a spot migrated the same as authentic flavin mononucleotide and another the same as authentic flavin adenine dinucleotide. The amount of free riboflavin was low relative to that of the other two compounds.

Sweet potato For early samples, a yellow band migrated 1.4 cm. toward the negative electrode during electrophoresis. A dull yellow band migrated 4.1 cm. toward the positive electrode; two larger, bright yellow bands migrated 6.6 and 8.1 cm. The fluorescence of the bright yellow compounds greatly increased after about eight seconds exposure to ultraviolet light.
When the three yellow compounds which migrated toward the positive electrode were treated with a solution of sodium dithionite, their fluorescence disappeared, but then returned after a few seconds. When these compounds were treated with solutions of silver nitrate and acetate, their fluorescences did not disappear. Elution from the paper electrophoretograms was not accomplished with cold water, hot water, phenol, diethyl ether, acetone, methanol, 0.5 N NaOH, or N HCl.

Later samples from sweet potato gave only one yellow compound that migrated toward the positive electrode. When material from two kilograms of tubers was allowed to migrate on ten electrophoretograms, the yellow bands cut out, blended and treated for hydrolysis, no free riboflavin was obtained. In AKMB a blue compound and a dull reddish compound were observed. These compounds were not observed when clean paper was treated similarly. The blue compound retained the same fluorescence in the presence and absence of acetic acid and had the same migration in AKMB as the major blue compound from oats. Much of the yellow material remained on the homogenized paper.

The three yellow compounds which migrated toward the positive electrode were not detected when extracts which had passed through the resin were allowed to migrate on chromatograms in BAW and PKMB. When such samples were allowed
Table 4. Data on electrophoresis of flavins and flavin-like compounds

<table>
<thead>
<tr>
<th>Flavin or plant source</th>
<th>Distance of migration in cm.</th>
<th>Electrode toward which migrated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(_{a})</td>
<td>(_{b})</td>
</tr>
<tr>
<td>Formylmethylflavin (streaking)</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Lumiflavin</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>Hydroxylethylflavin</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>3-Acetic acid riboflavin</td>
<td>9.5</td>
<td>+</td>
</tr>
<tr>
<td>Riboflavinyl glucoside</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Glyceryl flavin</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Lumichrome</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Flavin mononucleotide</td>
<td>7.6</td>
<td>10.9</td>
</tr>
<tr>
<td>Flavin adenine dinucleotide 12.0</td>
<td>14.7</td>
<td>+</td>
</tr>
<tr>
<td>Compound B</td>
<td>13.3</td>
<td>+</td>
</tr>
<tr>
<td>Spinach</td>
<td>10.5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>14.0</td>
<td>+</td>
</tr>
<tr>
<td>Sweet potato</td>
<td>7.9</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6.7</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td>+</td>
</tr>
<tr>
<td>Oats</td>
<td>8.1</td>
<td>+</td>
</tr>
<tr>
<td>Job's tears</td>
<td>1.5</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\text{For 3/4 hour.}\)

\(^b\text{For 1 hour.}\)
to migrate in $W$, the upper part of the chromatogram was obscured by blue material. Electrophoresis showed the compounds to be in the washings from talc columns.

Isolations of Lumichrome

Detection of flavins

When yeast and shoots of etiolated corn and oat seedlings were exposed to light, free riboflavin and a compound which is most probably lumichrome were detected. These flavins were found in extracts from yeast, corn and oats after exposures to 1100 foot-candles for six hours. They were also found in extracts from 550 coleoptiles from corn seedlings which had been exposed to sunlight for eight hours on August 14, and in extracts from approximately 7.25 g. (fresh wt.) of corn shoots exposed to sunlight with a maximum intensity of 6000 foot-candles for six hours on December 4. Lumichrome remained detectable in samples from corn which were exposed to the eight hours of sunlight and then placed in the dark for four hours before homogenization.

The concentrations of flavins were determined for samples exposed to the six hour period of sunlight. Figure 3 contains a photograph of a preparative-layer chromatogram of a sample which was exposed to sunlight. The lower band is riboflavin. The upper band contains the compound formed during exposure to light. Authentic lumichrome, which was spotted in a gap
Figure 3. Preparative-layer chromatogram of extract showing riboflavin and compound produced on exposure of etiolated seedlings of corn to sunlight
in the band of sample, is in the wedge-shaped area at the center of the upper band. A trace of blue material occurred at the origin. The data on the concentrations of riboflavin and lumichrome from the samples which exposed to sunlight for six hours is given in Table 5. The averaged concentration of free riboflavin was $11.8 \pm 0.5$ mg./kg. of dried tissue. For lumichrome the averaged concentration was $0.32 \pm 0.02$ mg./kg. Thus $2.5 \pm 0.1\%$ of the total flavin was converted to lumichrome (on the basis of milligrams of flavin per kilogram of dried tissue).

Unexposed samples of each of the plant materials were analyzed also. Riboflavin was the only flavin detected. Samples of leaves harvested in mid-summer from corn growing

<table>
<thead>
<tr>
<th>Compounds from samples</th>
<th>Fresh weight$^{-1}$</th>
<th>Dry weight$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>riboflavin</td>
<td>1.05</td>
<td>12.4</td>
</tr>
<tr>
<td>lumichrome</td>
<td>0.0257</td>
<td>0.302</td>
</tr>
<tr>
<td>Sample b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>riboflavin</td>
<td>0.964</td>
<td>11.3</td>
</tr>
<tr>
<td>lumichrome</td>
<td>0.0291</td>
<td>0.342</td>
</tr>
</tbody>
</table>

$^a$In mg. of flavin/kg. of tissue.
in the field contained no detectable lumichrome or other flavin except free riboflavin.

**Evidence for lumichrome**

The compound detected after exposure to light migrated the same as authentic lumichrome in BAW, AKMB, ZAW, MB, CEA and AW on one-dimensional chromatograms. Its position corresponded to that of lumichrome on two-dimensional chromatograms migrated in MB-W and CEA-AW. After migration in systems containing acetic acid the fluorescence of the compound was yellow; upon evaporation of the acetic acid the fluorescence became blue. This behavior is characteristic of lumichrome. The absorption spectrum of the compound showed no peak in the visible and had a peak in the ultraviolet (at 358 nm.) which corresponded to that of lumichrome. During migration in n-propanol-1% ammonia(2:1) a second spot formed. This compound is thought to be lumichrome hydrate, a derivative of lumichrome which is formed reversibly under alkaline conditions (55).

A compound having the same fluorescent characteristics and chromatographic migration in AKMB, WMBC and BAW as authentic lumichrome was obtained from Multiline-E-69 and Clinford oats, and from yeast. When samples from oats migrated in BAW this compound was somewhat obscured by a faint bluish-white fluorescence on either side of it.
The compound was distinctly evident, however, when the sample was heavily spotted as a short band.

**In vitro photolysis of riboflavin**

Aqueous solutions of pure riboflavin were photolyzed for periods of time similar to those used in the exposure of the seedlings. Photolysis for six hours with the same light source as use in the exposure of the seedling, but at a distance such as to give an intensity of 20 foot-candles, gave the following results. Aerobic solutions gave Compound "A" as the major product, moderate amounts of lumichrome and a trace of formylmethylflavin. Anaerobic solutions gave lumichrome as the major product and only traces of Compound "A", formylmethylflavin, and lumiflavin. These conditions of exposure seemed to give far more photolysis than was indicated under the *in vivo* conditions. Under the anaerobic conditions most of the riboflavin was converted to lumichrome as indicated by visual observation of fluorescence.
DISCUSSION

Methods

Some of the weaknesses of earlier methods for isolation of flavins from tissues were discussed in the review of literature. In the present work harsh conditions have been avoided, and removal of most impurities has been accomplished. The R-15 resin removed the flavins from salts, remaining proteins and some pigments, talc removed them from most nonflavin pigments, and one- and two-dimensional thin-layer chromatograms separated the flavins.

The work on the limits of detection for flavins from corn suggests that the methods could be used in the study of flavins in individual coleoptiles (and other small amounts of tissue). Corn coleoptiles seem better suited for such studies than those of oats because of their relatively large size and lack of many of the non-flavin pigments which oats contain. For such small samples, extracts could be applied to talc without prior use of resin.

The new solvent systems for thin-layer chromatography allow the separation of labile flavins from riboflavin without decomposition. The system WMBC is especially useful in that it contains no acid and yet gives good resolution of the more polar flavins, such as the glycosides. PKMB and W are of particular value in testing for the possibility of charged
groups on compounds of unknown structures.

The methods of isolation should be of use in studying compounds other than flavins. Often, distinct spots which were not flavins were observed on chromatograms of samples which had passed through the resin (many of these are described in Methods). Thus from oats, the blue compound, which is probably scopoletin glucoside (145) and the flavone reported by Zenk (145), were detected. Many of the solvent systems used in this work for thin-layer chromatography have already been applied in studies of other compounds. Several were used in the isolation of the above compounds from oats, and others have been used in separating mixtures of water-soluble vitamins (35, 49) and members of the vitamin B complex (47). Use of many of the other solvent systems may be of value in similar separations.

All the chemical tests employed in this work have been used before, except the one involving complexing with silver ions (5). The quenching of fluorescence by the silver salts seems to be more specific for flavins than does the quenching and sequent color return given by dithionite.

Isolations of Free Flavins

The materials listed in Table 3 were selected to test the developed methods on a wide range of plant materials and to examine those materials for free flavins other than
riboflavin. Thus seeds, etiolated shoots, whole seedlings, leaves, tubers, roots and free cells were used. Roots of beet and carrot were selected primarily to test isolation from highly pigmented tissues. The isolations were excellent in both cases.

All tissues examined contained free riboflavin. Yagi (140) points out that phosphatases released during homogenization convert riboflavin mononucleotide and riboflavin adenine dinucleotide to the mononucleotide and to riboflavin. Homogenization in solutions of saturated ammonium sulfate should eliminate this possibility, and the riboflavin detected probably occurs free in the cell.

The free riboflavin could be an intermediate in the synthesis of the nucleotides, for transport to parts of the plant not able to synthesize their requirement of flavin, or excess flavin which, in the intact cell, may have been in crystalline form. Conceivably, non-protein bound flavin in a special environment could meet the requirements of orientation for such responses as induction of polarity (70) and displacement of chloroplasts (96, 147).

The studies of the plant materials showed that free flavins other than riboflavin do indeed exist in plants. Two compounds which are apparently derivatives of riboflavin were detected in samples from Job's tears and another such compound in samples from oats. Two compounds which are probably the
nucleotides were detected in samples from spinach. Three compounds which were detected only on electrophoretograms after use of the resin, and which are probably not flavins, were obtained from sweet potato. Discussion of the data relating to these compounds is given below.

Compounds T-1 and T-2 from Job's tears were resistant to photolysis, but flavin mononucleotide, FX, and riboflavinyl glucoside were more resistant to photolysis than was riboflavin. T-1, and T-2, gave a compound with the chromatographic behavior of formylmethylflavin after treatment with periodate. Their chromatographic and electrophoretic migration indicates that T-1 and T-2 are not charged. Thus they are not FX. Their chromatographic behavior in BAW did not correspond to that of riboflavinyl glucoside or galactoside; in AKMB it did not correspond to nekoflavin. Since Compound YG-4 from yeast migration above riboflavin in chromatographic systems in which the polar nucleotides were below riboflavin (98), T-1 and T-2 are probably not this compound. Present knowledge of flavins suggests that T-1 and T-2 are sugar derivatives of riboflavin. Treatment with periodic acid indicates that attachment is not to the second or third carbon of the side chain.

The second flavin-like compound isolated from oats is probably Compound FX (144, 145). Evidence for this conclusion are its fluorescence, its general chromatographic behavior,
its presence in phenolic extractions (as well as in elutes from resin and talc), and its lability. Evidence for its being a flavin are its behavior in the presence of dithionite, and silver ions, its hydrolysis to a compound with the same migration in AKMB and BAW as riboflavin, and its products of photolysis. Its chromatographic and electrophoretic behavior indicate that it has a negative charge. Although the product of periodic cleavage did not have exactly the same migration as formylmethylflavin, the nonflavin cleavage product could have caused the slight difference in migration. If the product was formylmethylflavin, then the second and third carbons of the side chain are probably not the site of attachment of the derivative. Hydrolysis of FX by mildly alkaline conditions indicates that the derivative is not bound by a glycosidic bond. The chromatographic migration, and periodic and hydrolytic cleavage products indicate that FX is not a product of the photolysis of riboflavin. Its migration in AKMB indicates that it is not nekoflavin (66). Its migration in BAW is similar to that of U₁ from the urine of rats, but U₁ is not affected by high pH (99). Chromatographic data by Shen (99) indicate that it is neither U₂ from the urine of rats nor 2', 5'-phosphate cyclic flavin. Since chromatographic migration indicated that Compound YG-4 (from yeast) was uncharged (98), it is probably not that compound. Zenk (144) reported that FX contained no phosphate
groups. FX could be a derivative of riboflavin containing a charged sugar, an amino acid, auxin, or some other organic or inorganic anion.

During electrophoresis, the migration of the two yellow fluorescent compounds from leaves of spinach was 10.5 and 14.0 cm. That of riboflavin mononucleotide and adenine dinucleotide was 11.2 and 14.9 cm. respectively. Thus the compounds from spinach are probably the nucleotides. Their chromatographic migration in BAW supports this conclusion. Samples for electrophoretic determination were applied after use of the resin only, and thus interfering substances in the extracts probably retarded migration. Green leaves may be richer in the nucleotides than etiolated ones (4).

Studies of the yellow compounds from sweet potato were hindered because these compounds could be neither eluted readily from electrophoretograms nor detected on thin-layer chromatograms. Study was further handicapped by variation in the number of compounds detected in samples. This is an excellent example of why tissues of a known variety of plants kept under controlled conditions should be used.

The compounds gave reversible bleaching in the presence of dithionite as do flavins but so did a blue compound from oats. The lack of riboflavin after hydrolysis of the yellow compound from later samples suggests that this compound was not a flavin. Furthermore, flavins are known to
form complexes with silver ions (5). Riboflavin and its glucoside did this as indicated by their loss of fluorescence after the addition of solutions of silver acetate and nitrate. The three yellow compounds from sweet potato did not. Finally, these compounds did not bind to talc.

Flavins of unknown structure are now being shown to occur widely, but their functions are unknown. Possibly functions of these compounds are intermediates in the biosynthesis of flavin nucleotides (134, 135), polysaccharides (48, 109), or in other biosynthetic pathways, and transport carriers which might increase the solubility of riboflavin or of the group attached to the flavin. Another interesting possibility is that the flavin molecule functions in the oxidation of the group attached to it. Finally, the compounds may function as photo-receptors. Zenk (145) has suggested that FX has such a role. The failure to detect FX in corn and wheat in the present work does not rule out the possibility of it being the receptor in these plants. It could be present in extremely low concentrations and/or be largely in a bound form.

Isolation of Lumichrome

The data indicate the in vivo formation of lumichrome. The evidence for the formation of lumichrome includes the same chromatographic migration of the isolated compound as that
of authentic lumichrome, the change in fluorescence from yellow in the presence of acetic acid to blue in its absence, the formation of a second spot when migrated in systems containing ammonia, and the absorption spectrum.

The formation of the lumichrome was dependent upon light. Lumichrome was not found in samples grown in the dark. Moreover lumichrome is known to be a product of the photolysis of riboflavin (124). Thus its formation in tissue probably comes from the photolysis of riboflavin.

The question then arises as to why lumichrome is the only photo-product found in vivo. During short term photolysis, many other photo-products are formed (124). The formation of lumichrome in vivo could be by an enzyme stimulated by light rather than by direct photolysis. Riboflavin hydrolase could be activated or stimulated by light. Or, bound flavin could be in a special chemical environment such that it would produce only lumichrome during photolysis. However, direct photolysis is known to be influenced by factors such as pH, presence of buffers and organic quenchers, duration and intensity of illumination, and concentration of oxygen. Compounds and processes in the cellular fluid could influence these factors.

Many of the photo-products of riboflavin are themselves photo-labile (124). Therefore one would expect a different pattern of products from relatively long, low intensity
exposures. The experiments with aqueous solution of pure riboflavin support the idea. Photolysis for six hours with white light of an intensity of 20 foot-candles gave simpler results than are obtained during shorter photolyses. Aerobic solutions gave Compound "A" (124) and lumichrome as major photo-products. Degassed solutions gave lumichrome as the major product.

These results might be interpreted as evidence that the cells of corn and oats were anaerobic. However, O'Brien and Thimann (77) have shown that coleoptiles of oats and wheat are well equipped for exchange of gases. The coleoptiles have stomata in both the inner and outer epidermis, the parenchyma of both the tip and the shaft have numerous air spaces, and plastids are distributed throughout the tissues. Moreover, oxygen is needed for growth of coleoptiles (10, 92), for cytoplasmic streaming (112), and for transport and exit of auxin (18). Of course the enclosed leaves were included in most of the analyses, but the amount of photolysis in them can be questioned. They were shielded by the surrounding coleoptiles and were much more pigmented with non-flavins than the coleoptiles. The results indicate that the flavins were in a special chemical environment or that any Compound "A" produced was rapidly bound or degraded. Most of the lumichrome does not seem to be rapidly bound or degraded.

Although the data obtained are not directly concerned
with photo-responses of plants, they should be considered in relation to those responses.

First, there is the problem of intensity of illumination. The intensity of sunlight on the December day of exposure is approximately $5 \times 10^7$ times greater than the threshold for phototropism in *Avena* and that of the fluorescent tubes, about $1 \times 10^7$ times greater (94). (The extreme sensitivity of the response of *Avena*, which has a threshold of approximately $1 \times 10^{-4}$ foot-candles, can be appreciated by the fact that starlight is $8 \times 10^{-5}$ foot-candles (94)).

Enough lumichrome was produced to give 10 ml. of $8.0 \times 10^{-8}$ M lumichrome or $4.8 \times 10^{17}$ molecules for the total sample. This means that $1 \times 10^{15} - 1 \times 10^{16}$ molecules were formed per coleoptile. If the production of lumichrome were linear with respect to intensity, then $5 \times 10^{15}/5 \times 10^7$ or $1 \times 10^{8}$ molecules of lumichrome would be produced at the intensity for the threshold of phototropism. This at least shows that riboflavin could be photochemically active *in vivo* at intensities of the phototropic response.

The production of lumichrome itself could be active in photo-responses. Lumichrome is a known inhibitor of flavoenzymes, and Carlile (15) has shown that the action of lumichrome in photosensitive tissue to be greater in light than in darkness. Moreover, Yeoh and Raghavan (142) have observed an, as yet unexplained, stimulation, in the presence
of inhibitors, of two-dimensional growth in gametophytes of ferns. Lumichrome was more active than was riboflavin in this stimulation. In the present work, the production of lumichrome was detected only in non-green tissues. Etiolated plants are known to be more sensitive phototropically than are green plants.

If the production of lumichrome has a role in photoprocesses it is likely a specific one. The low concentration of lumichrome per cell and the detection of lumichrome in samples from seedlings which remained in the dark after illumination indicates interaction and inhibition of flavoenzymes in general is not the mechanism. Lumichrome could inhibit specific steps in respiration, cytoplasmic streaming, or the binding, transformation, or transport of auxin. On the other hand, lumichrome could stimulate or activate one of the above processes. Also, photolytic cleavage of riboflavin could free a protein of a flavin which was bound to it and thus produce an active (or inactive) enzyme. Or, some fragment freed from an enzyme might take part in the physiological process. Another possibility is that lumichrome is not the direct product of the photochemical reaction. Some intermediate compound could be the active compound. The intermediate compound could be enzymically or photochemically degraded to lumichrome. To know whether riboflavin hydrase were present in the tissues studied and whether it degrades
Compound "A" would be interesting. Knowing whether this or other enzymes of flavin metabolism are photo-stimulated would also be of interest. Finally, the possibility that lumichrome and the photolysis of riboflavin have nothing to do with photo-processes must be admitted.
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