colleges. The number of publications does not make much difference in the salary check. But it is true that if new information is not published it does perish. A sustained effort should be made to see that the topics chosen and the quality of work done are appropriate for publication and that the information gathered is actually published and not allowed to gather dust in some forgotten notebook. Preparation of the publication is a necessary part of productive scholarship and must not be neglected. The student should participate by preparing a summary of his work in form readily worked into the finished paper, even though the professor may have to weave the substance of several such reports into a single publication. The teacher may not receive any financial reward for publication of the paper, but the student often discovers later that having his name appear as coauthor on a report in a standard scientific publication helps him in getting a position. The publicity benefits the college as it comes to be known for its scientific activities. The Collegiate Division of the Tennesse Academy of Science and some other scientific society meetings provide opportunities for the undergraduate to present the results of his work orally and provide valuable experience in explaining and defending his conclusions.

ACKNOWLEDGEMENTS

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EFFECTS OF COUMARIN UPON PLANT GROWTH AND DEVELOPMENT

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ABSTRACT

Coumarin, the lactone of *cs-o*-hydroxy cinnamic acid, is widely distributed in plants, and is especially abundant in tonka bean, *Dipteryx odorata,* and the sweet clovers *Melilotus alba* and *M. officinalis.* It inhibits growth of bacteria, and has a fairly low toxicity to higher animals and man. Its cytological effect is that of a spindle poison, producing C-mitosis. It inhibits longitudinal growth of roots, but augments lateral growth, producing swellings. It inhibits germination of a wide variety of seeds.

Tracer experiments have demonstrated that it is biosynthesized via the shikimic acid pathway, with phenylalanine and *trans*-cinnamic acid as intermediates. One pathway then involves *cis*-cinnamic acid and *o*-coumarinic acid, an alternate route consisting of *o*-coumaric acid, *o*-coumaryl-β-glucoside, *o*-coumarinyl-β-glucoside and coumarinic acid.

Coumarin has a great number of physiological effects upon plants, including decrease in the permeability of tissues to water, inhibition of a number of enzymes, interactions with auxin and other plant hormones, and decrease in the biosynthesis of cell wall materials. Despite numerous investigations of its effects upon plant growth and development, it appears that its primary effect is not known.

INTRODUCTION

While coumarin the lactone of *cis-o*-hydroxy cinnamic acid (*o*-coumarinic acid), has been the subject of numerous review articles, including those of Sethna and Shah (1945), Dean (1952), Reppel (1954) and

Bose (1958). The present article is prompted by the fact that numerous studies have been made within more recent years. Mayer and Poljakoff-Mayber (1961) have concerned themselves with coumarin in relation to seed germination and plant growth, and Soine (1964) has reviewed its physiological and pharmacological actions.

The ultraviolet absortion spectrum of coumarin has maxima at 270 and 312 nm (Goodwin and Pollock, 1954; Soine, 1964). Its infrared spectrum has a peak at 1715-1745 cm^{-1} due to the α pyrone structure, and another at 1625-1640 cm^{-1} owing to the conjugated aromatic double bond (Soine, 1964). While many coumarin derivatives are fluorescent, coumarin itself is not (Goodwin and Kavanaugh, 1950). A number of analytical methods for coumarn are sumarized by San Antonio (1951) and Haskins and Gorz (1961), while procedures for its detection by paper chromatography are given by Swain (1953).

Coumarin was first isolated from the tonka bean, *Dipteryx* (*Coumarouna*) *odorata* Willd., by Vogel in 1820. In addition to coumarin, the beans also contain a glucoside coumarin precursor, and a β-glucosidase which plays a role in coumarin biosynthesis (Lutzmann, 1940). The tonka bean, native to northern South America, remains the principal commercial source of coumarin. In processing, the beans are soaked in rum, and coumarin crystallizes out on the surface of the beans. It is used for a variety of purposes, including the manufacture of "vanilla extract" and the flavoring of tobacco (Ruisanchez Harvey, 1943).

Coumarin is fairly widely distributed in nature. It has been reported from the fungus *Lentinus* (Späth, 1937), from uredospores of wheat rust (Staples and Wynn, 1965), from rice plants infected with blast, *Piricularia oryzae* (Tamari and Kaji, 1959), and from several species of the fern. *Adiantum* (Späth, 1937). Wehmer (1929) noted its occurrence in over 50 species of higher plants, and this number has been increased to 80 (Späth, 1937), including many representatives of the Gramineae, Orchidaceae, Leguminosae and Compositae. It has been especially studied in the sweet clovers, *Melilotus alba* and *M. officinalis*, in sweet grass, *Hierochloe odorata*, and in woodruff, *Asperula odorata*.

Antibacterial Action and Toxicity to Animals

Broderson and Kjaer (1946) examined the effect of coumarin upon the growth of 25 species of bacteria of the genera *Staphylococcus, Sarcina. Enterococcus, Streptococcus, Diplococcus, Neisseria, Bacillus, Corynebacterium, Escherichia, Bacterium, Eberthella, Salmonella, Pseudomonas* and *Vibrio*. All were inhibited by coumarin in a concentration somewhat below 1:1,000. The lethal dose for a 20 g mouse by intraperitoneal injection was 7 mg.

Hazelton, *et al.* (1956) studied both acute and chronic toxicity of coumarin to rats and dogs. Using male albino rats, the acute oral LD$_{50}$ ranged from 0.29 to 0.52 g/kilo. When 1,000 ppm of coumarin was incorporated into the food of rats for a period of 90 days, growth was reduced, but no deaths and no histo-

pathology resulted. When the dose was increased to 2,500 ppm for 90 days, liver damage occurred. With dogs, liver damage also resulted from the feeding of 100 mg/kilo/day for 18-19 days. These workers emphasized the desirability of chronic feeding studies extending over even longer periods.

Five grams of coumarin will kill a sheep, and 40 g will kill a horse or cow. A dose of 4 g will produce symptoms of illness and weakness in man (Sethna and Shah, 1945).

Cytological Effects

It was first shown by Cornman (1946, 1947) that coumarin has a colchicine-like effect on mitosis. In roots of onion or lily to which a saturated solution of coumarin is applied, the spindle is destroyed, mitosis is arrested at metaphase, and tetraploid nuclei or binucleate cells are formed. Entry of interphase nuclei into mitosis is suppressed. Östergren (1948) reported that coumarin produced chromosome agglutination (stickiness) as well as bridges and breaks. Quercioli (1954, 1955), D'Amato (1954) and D'Amato and D'Amato-Avanzi (1954) are of the opinion that the primary effect of coumarin is upon the interphase nucleus, since many more chromosomal than chromatid aberrations result from its use. On the contrary, Östergren and Wakonig (1954) consider that the dividing nucleus is more sensitive to the action of the coumarin in producing chromosome breaks.

Sharma and Bal (1953) regard the use of coumarin as a technique useful in karyotype analysis, because of the great degree of metaphase contraction which it induces.

According to Svensson (1971, 1972) the effect of coumarin is exerted upon the microtubules of the mitotic apparatus, and the spindle is frequently oriented at right angles to the long axis of the cell. In roots, as will be seen later, this leads to decreased longitudinal growth and increased lateral growth.

Root Growth and Development

Because of large variations among different species of plants in their sensitivity to coumarin, Audus and Quastel (1947) have recommended its use as a selective phytocidal agent. In experiments with root growth of peas and cress. Audus (1948) was able to demonstrate complete reversbility of growth inhibition by coumarin, the degrees of recovery being determined almost entirely by the length of exposure, relatively independently of the coumarin concentration. While growth of the primary root was inhibited by coumarin, lateral root formation was stimulated (Audus, 1949). Moewus (1949) reported inhibition of growth of cress roots by coumarin in concentrations of 10^{-3} — 10^{-6}, and no stimulation of growth at any concentration.

Goodwin and collaborators have carried out an extended series of studies concerned with coumarin and its effects upon root growth. In Victory oats, the growth of roots in 10^{-3}M or 10^{-4}M coumarin in comparison with untreated controls indicated increasing in-

hibitory effects of coumarin with time up to 6-18 hours following application of the chemical (Goodwin and Taves, 1950). In roots of timothy (*Phleum pratense*), 3 x 10^{-5}M coumarin slowed longitudinal growth and induced pronounced swelling in the zone of cell elongation, through increase in the cross sectional area of the cells. While root hair formation and vascular differentiation were not interfered with, polarity of the root was destroyed and recovery from growth inhibition was not observed (Pollock, *et al.*, 1954). In a continuation of studies with timothy roots, Avers and Goodwin (1956) found that 3 x 10^{-4} to —3 x 10^{-5} coumarin induced a reduction in the rate of cell division, caused an increase in thickness of epidermal cells, and inhibited vacuolization of meristematic cells. Elongation was not inhibited in the apical 400μ segment of the root, the most pronounced effects being exerted at some distance back of the root tip.

San Antonio (1952) studied effects of exogenously applied coumarin upon growth of roots of *Melilotus alba* which have a coumarin contect of 74μ g/g fresh weight. Nevertheless, addition of 5 ppm of coumarin inhibited root growth 45-60 per cent, and induced the formation of subterminal swellings.

In roots of wheat, Burström (1954) found that the effect of coumarin resembled an all-or-none reaction. Thus, roots elongated fully in 10^{-5}M coumarin, but did not grow at all in a 10^{-4}M concentration. Loss of cell wall elasticity occurred prior to inhibition of elongation. Burström concluded that coumarin inhibits tensibility of the cell walls, but does not influence subsequent growth through intussusception.

The inhibition of elongation of Grand Rapids lettuce roots induced by coumarin can be reversed by cycocel or CCC (Khan and Tolbert, 1966).

Svensson (1971, 1972) found that the coumarin concentration-growth curve has an "all-or-none" appearance in both wheat and maize. Transverse cell divisions are inhibited in all tissues of the roots, while longitudinal divisions inside the stele are enhanced. Thus cell length is decreased and cell diameter increased. The effect of coumarin on longitudinal growth is diminished by kinetin, and on lateral growth is diminished by gibberellic acid. These effects are regarded, however, as indirect, and neither indole acetic acid, gibbrellic acid nor kinetin is required for these results to be produced.

Seed Germination

Sigmund (1914) found that coumarin in a concentration of M/50 inhibited germination of wheat and seeds of certain other plants. Coumarin is present in the seed of *Melilotus alba, M. officinalis* and many other plants (Nutile, 1945) and occurs in *Trigonella arabica* seed in a concentration sufficiently high as to inhibit germination (Lerner, *et al.*, 1959). According to Toole, *et al.* (1955), coumarin in low concentrations actually stimulates germination of *Lepidium virginicum* seed, and Valio (1973) has recently shown that in the seed of *Dipteryx* (*Coumarouna*) *odorata*, the embryos

of which have a coumarin content of 6 per cent, endogenous coumarin does not inhibit their germination. McCalla, *et al.* (1948) reported that germination and growth of maize was reduced by a 1:100 aqueous extract of sweet clover hay and that 0.062 g of coumarin/100 ml of water reduced germination to zero. In a concentration of 1:10,000, only 2 per cent germination of *Lepidium sativum* seeds was obtained (Kuhn, *et al.*, 1943). It has been suspected that coumarin is the natural germination inhibitor in lettuce seed (Nutile, 1945; Evenari, 1949), but the evidence that this is the case is not conclusive.

In experiments upon germination of lettuce seeds in the presence of various coumarin derivatives, Mayer and Evenari (1952) found that almost any substitution in the structure of the coumarin molecule resulted in loss of activity. Veldstra and Havinga (1943-1945) had earlier commented on the physiological activity of unsaturated lactones, and Mayer and Evenari (1952) likewise regarded the lactone structure and the double bond in the lactone ring as essential for activity. Coumarin is considerably more effective as an inhibitor of germination than the related compounds, *cis*-cinnamic acid, *trans*-cinnamic acid, *o*-coumaric acid, or melilotic acid (Mayer and Evenari, 1953).

In experiments with non-light-sensitive lettuce seed, Mayer (1953) noted that the amounts of coumarin recovered from seed were much lower than those applied or those disappearing from the external solution. Thus, large amounts of coumarin were inactivated by the seed. He theorized that inactivation involves reaction with some essential metabolite, the exhaustion of which leads to suppression of germination.

In experiments with dark germination of Grand Rapids lettuce seed, it was found that gibberellic acid reversed the inhibitory action of coumarin (Mayer, 1959). Stated otherwise, the action of gibberellic acid in promoting germination of lettuce seed is inhibited by coumarin (Harada and Koizumi, 1971). Berrie, *et al.* (1968) have pointed out the structural similarity of coumarin to the moiety of gibberellic acid containing the lactone bridge, but were unable to demonstrate competitive inhibition. In experiments with germination of *Lepidium* and *Sinapis* seeds, Libbert (1957) found that the inhibitory effects of indoleacetic acid and coumarin were additive, thus concluding that coumarin is not an auxin antagonist. Inhibition of germination of Grand Rapid lettuce seed by coumarin is reversed by a combination of red light and cycocel (CCC) or kinetin, but does not occur in far-red light or in darkness (Khan and Tolbert, 1965, 1966).

Coumarin modifies the sensitivity of lettuce seed to thiourea (Poljakoff-Mayber, *et al.*, 1958).

According to Misra and Patnaik (1959), coumarin in low concentrations is stimulatory to germination and growth of rice. The seed of various plants vary widely in their sensitivity to inhibition of germination by coumarin. The concentrations required to produce 50 per cent inhibition of germination range from 6.6 x 10^{-3} for wheat to 3.3 x 10^{-5} for flax (Mayer and Poljakoff-Mayber, 1963).

BIOSYNTHESIS

Experiments with acetate-^{14}C indicate that it is not a precursor of the ring system of coumarin (Weygand and Wendt, 1959; Brown, et al., 1960; Griesebach and Ollis, 1961). There is widespread agreement that the early steps in coumarin biosynthesis proceed via the shikimic acid pathway (Kosuge and Conn, 1959; Weygand and Wendt, 1959; Weygand, et al., 1960; Neish, 1960; Brown, et al., 1960; Griesebach and Ollis, 1961; Brown, 1962a, 1963a, 1963b).

Kosuge and Conn (1958) performed tracer experiments using $^{14}CO_2$ and Melilotus alba, and Brown (1962a) carried out similar studies with Hierochloe odorata. In sweet clover, the specific activity of coumaric reached a maximum after twenty-four hours, and was much higher in the tops than in the roots of the plants. In sweet grass, total radioactivity in o-coumarinc acid peaked after four days and that of coumarin after eight to seventeen days, indicating o-coumaric acid as an intermediate. Experiments by Weygand and Wendt (1959) and Weygand, et al. (1960) in which glucose-1-^{14}C was applied to root cultures of Melilotus officinalis, and by Brown (1962b, 1963a) with glucose-U-^{14}C and Lavandula officinalis indicated in each case a significant degree of incorporation of the metabolite.

A far more immediate precursor is l-phenylalanine, formed by the shikimic acid pathway (Weygand and Wendt, 1959; Brown, 1960; Brown, et al., 1960; Neish, 1960; Weygand, et al., 1960; Griesebach and Ollis, 1961; Brown, 1962a, 1962b, 1963a). Weygand and Wendt (1959) found that phenylalanine-U-^{14}C was incorporated bodily into coumarin. Brown (1960) reported that phenylalanine was sixty times as effective as tyrosine as a coumaric precursor in Hierochloe. Phenylalanine, shikimic acid, cinnamic acid and o-coumaric acid were the best of the coumarin precursors tested (Brown, et al., 1960; Griesebach and Ollis, 1961).

Through the activity of the enzyme phenylalanine ammonia lyase, phenylalanine is deaminated to produce trans-cinnamic acid. Kosuge and Conn (1959) found that trans-cinnamic acid was the most effective coumarin precursor of several which were tested, and Brown (1960) reported it to be seventy times as efficient in this regard as P-coumaric acid.

From trans-cinnamic acid, there are two distinct pathways for coumarin biosynthesis. The more direct of these involves isomerization to produce cis-cinnamic acid, o-hydroxylation to produce o-coumarinic acid, and lactonization to form coumarin (Stoker and Bellis, 1962; Pridham, 1965). Brown (1963a) reported that o-coumarinic acid was 150-200 times as effective as p-coumarinic acid as a coumarin precursor.

Alternatively, trans-cinnamic acid may undergo o-hydroxylation to form o-coumaric acid (o-hydroxy cinnamic acid) (Stoker and Bellis, 1962; Brown, 1963b; Stoker, 1964; Pridham, 1965; Edwards and Stoker, 1967). Experiments of Weygand, et al. (1960) clearly excluded p-hydroxylation as a mechanism involved in the formation of the lactone ring. o-Coumaric acid is next converted to trans-o-coumaryl-β-glucoside

(Brown, 1963b; Stoker, 1964).

The glucoside of coumaric acid, which has a trans-configuration, is then isomerized, and converted to the glucoside of coumarinic acid, which has a cis-configuration. This step has been the subject of detailed investigation as well as considerable controversy. Lutzmann (1940) was able to bring about this reaction in vitro through application of ultraviolet light. Kahnt (1962) observed that Melilotus alba plants grown in weak light contained the trans-glucoside, but the cis-isomer was present in plants grown in strong sunlight. He was able to bring about the trans→cis isomerization both in vitro and in vivo by sunlight. Haskins and Gorz (1961) suggested that the reaction might be non-enzymatic but Stoker (1964) claimed that it proceeded in darkness in M. alba, and could be induced by a heat-labile plant extract, indicating the participation of an isomerase. It subsequently appeared that these results were attributable to an artifact, and that the isomerization is non-enzymatic, and catalyzed by light (Haskins, et al., 1964; Edwards and Stoker, 1967).

A great deal of the "coumarin" present in sweet clover is in the bound or glucoside form (Haskins and Gorz, 1961). In the ^{14}C experiments of Brown, et al. (1960) and Brown (1962b), o-coumaryl glucoside was the intermediate which accumulated the greatest amount of radioactivity. It had been known since 1940 that tonka beans contain a β-glucosidase (Lutzmann, 1940). Kosuge and Conn (1961) were able to extract this enzyme from sweet clover, and purified it 40-fold. It hydrolyzes the cis- but not the trans-isomer (Kosuge, 1961). Upon hydrolysis of coumarinyl glucoside, the free coumarinic acid lactonizes spontaneously to form coumarin (Brown, 1963a, 1963b). In sweet clover, coumarin may be further metabolized to produce melilotic acid and its β-glucoside (Kosuge and Conn, 1959; Stoker and Bellis, 1962).

PHYSIOLOGICAL EFFECTS

Effect upon Permeability to Water

Guttenberg and Beythien (1951) performed deplasmolysis experiments using epidermal cells of Rhoeo which had been plasmolyzed by solutions of mannitol, finding that coumarin decreased permeability of the cells to water. The same result was obtained with potato tuber tissue, the effect being accentuated if the original permeability had been high, so that a large weight loss occurred in the preceding plasmolysis (Guttenberg and Meinl, 1954).

Blaim (1960), studying imbibition by grains of wheat, found that water uptake by the embryo was inhibited by coumarin, while that by the endosperm was unaffected. He suggested that this may explain the action of coumarin in inhibiting seed germination. San Antonio (1952) reported, however, that coumarin does not interfere with imbibition of water by seeds of sweet clover, a plant which has a high coumarin content.

Effects upon Enzymes

In studies with germinating lettuce seeds, coumarin was found to inhibit the activities of invertase, phytase and phenolase. The normal increases in activity of

lipase and dehydrogenases did not occur. In vitro experiments indicated inhibition of proteinases and A T Pase (Mayer and Poljakoff-Mayber, 1961). These workers (Mayer and Poljakoff-Mayber, 1963) observed no effect of coumarin on glycolysis, cytochrome c oxidase, or catalase. However, oxidative phosphorylation and respiratory metabolism were affected. It was suggested that coumarin uncouples ATP formation from respiration (Mayer and Poljakoff-Mayber, 1963; Berrie, et al., 1968). Mayer and Poljakoff-Mayber (1963) commented that "From the results brought about above it does not appear that any clear cut conclusion can yet be drawn about the mechanism by which coumarin inhibits germination". Likewise, Berrie, et al. (1968) regard as hazardous any statement regarding the primary effect of coumarin.

In experiments performed in vitro with commercial purified enzymes, 10^{-3}M coumarin was found to inhibit α-amylase activity 27-62 per cent and β-amplase activity 35-48 per cent (Broda, 1966). Khan (1969) reported inhibition of α-amylase synthesis in barley seedlings by coumarin, and reversal of inhibition by kinetin or benzyladenine.

Poljakoff-Mayber (1953) reported no effect of coumarin on peroxidase activity in vitro, while Goren and Tomer (1971) found increased peroxidase activity in cucumber radicles following application of coumarin, and an even greater increase in IAA oxidase activity.

Of special interest are the recent findings of Hara, et al. (1973) concerning the effects of coumarin on the biosynthesis of plant cell wall materials, especially cellulose. In plumular hook segments of mung bean, a dosage of 100 ppm coumarin did not affect incorporation of ^{14}C-glucose into the cytoplasm, but inhibited its incorporation into the cell wall by 30 per cent. Upon analysis of the cell wall constituents, it was found that coumarin had no effect upon glucose incorporation into pectin or hemicellulose fractions, while incorporation into cellulose was inhibited by 70 per cent.

Auxin Effects

Thimann and Bonner (1949) found that curvature of slit pea stem segments is inhibited by coumarin in concentrations of 10^{-4}M or above. While growth of Avena coleoptile sections is inhibited by high concentrations of coumarin, low concentrations were observed to be stimulatory. The conclusion of these workers that growth inhibition by coumarin is due to reaction with an -SH group, and can be counteracted by 1,2-dimercaptoethanol (BAL), was not supported by subsequent investigators (Mayer and Evenari, 1952).

Even though both basipetal and acropetal movement of coumarin occurs in the Avena coleoptile (Gantzer, 1960), coumarin is inactive in the coleoptile curvature test (San Antonio, 1952).

Neumann (1959, 1960) found the effects of IAA and coumarin to be additive in certain concentrations, but not in others. Pretreatment of tissues with coumarin stimulated subsequent growth in IAA, while IAA pretreatment had no effect upon subsequent growth in coumarin. The growth promoting effect of coumarin was shown to be independent of endogenous IAA. It was concluded that while the two compounds have dif-

ferent sites of action, coumarin behaves essentially like an auxin. The coumarin-induced growth response is inhibited by iodoacetate or 2,4-dinitrophenol (Knypl, 1964).

Many auxin responses are correlated with the production of ethylene. The red light-induced opening of the hypocotyl hook of etiolated bean seedlings is inhibited by coumarin (Morgan and Powell, 1970). These workers demonstrated correlations between the concentration dependence and time sequence of hook closure and ethylene production. Exogenous ethylene was shown to duplicate the inhibitory action of coumarin in this system, suggesting that many reactions of coumarin may be mediated by ethylene produced in response to coumarin treatment.

Miscellaneous Effects

Coumarin has been found to inhibit the sprouting of potato tubers (Moewus and Schader, 1951). It stimulates expansion of leaf discs of Chenopodium album floated on a solution of glucose and potassium nitrate (Miller and Meyer, 1951). It inhibits longitudinal growth and stimulates cambial activity in Beta vulgaris (Winter, 1954).

It inhibits germination of spores of the ferns Gymnogramme calomelanos (Sossountzov, 1961) and Anemia phyllitidis, inhibition in the latter case being reversible by gibberellic acid (Schraudolf, 1967).

Coumarin increases the DNA content of Pisum sativum roots (DeGreef, 1964). In isolated mesophyll cells of Calystegia sepium and several other plants, it causes the cells to swell and round up, after which they divide and grow normally (Harada, et al., 1971, 1972). This effect is not produced by a number of related compounds, nor is it counteracted by a number of plant hormones. In lettuce seedlings, Harada and Koizumi (1971) found that the positive geotropism and negative phototropism of roots and the negative geotropism of hypocotyls were all inhibited by coumarin.

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MOBILE ENVIRONMENTAL EDUCATION LABORATORY PROJECT: DEVELOPMENT, IMPLEMENTATION, AND EVALUATION

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ABSTRACT

The Kingsport City School System and the Tennessee Valley Authority cooperated in conducting an innovative demonstration project in environmental education during the 1972-73 school year. Directed toward the planning, development, implementation and evaluation of a mobile unit approach to conducting environmental studies, the project included the development of instructional materials for students, pre-service and in-service training