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AUXIN-KINETIN INTERACTION REGULATING THE SCOPOLETIN AND SCOPOLIN LEVELS IN TOBACCO TISSUE CULTURES

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Tobacco tissue cultured *in vitro* has been observed to release a strongly ultraviolet fluorescent material into the agar medium. The intensity of the fluorescence varies with the composition of the medium and the age and other conditions of the cultures. It is especially striking in the presence of high concentrations of 3-indoleacetic acid (IAA). A major fraction of the fluorescent material has been isolated and identified as scopoletin (6-methoxy-7-hydroxycourmarin; 6-methoxy-7-hydroxy-1,2-benzopyrone). Influences of auxins and kinetin on its formation, its liberation from a glycosidic bound form, and its release into the medium will be reported.

Materials and Methods.—Cultures of tobacco tissue (pith sections, stem segments, and especially subcultures of callus), Nicotiana tabacum, variety Wis. #38 were grown in 125-ml Erlenmeyer flasks with 50 ml. of modified White's nutrient agar medium as described by Miller and Skoog[1] or Jablonski and Skoog[2] for periods of 1 to 60 days in different experiments and with various growth substances added to the culture medium. The cultures were kept behind glass doors in cabinets at a room temperature of about 25°C and in the presence of continuous, weak, diffuse light from fluorescent daylight tubes in ceiling fixtures.

Extraction of Scopoletin.—a. *Free scopoletin in the medium:* The fluorescent material was extracted from the medium with freshly redistilled chloroform. Best's[3] procedure for the extraction of scopoletin from tobacco tissues was employed. All operations were carried out in dim light to minimize photoinactivation of the fluorescent substance.

In the preparation of samples large enough for investigations of chemical properties, six liters of agar medium (from 150 flasks) were melted in an Arnold steamer, pooled, and made up to seven liters with distilled H_2O. The pH was adjusted to 2.0 with 1 *N* HCl. The material was allowed to cool to room temperature for one hour in a dark room and was then extracted with three 1-liter portions of chloroform. The chloroform extracts were pooled and the fluorescent material taken up in dilute KOH solution (2 successive 200-ml portions of 0.005 *N* KOH). These alkaline portions were combined, acidified to pH 3 with HCl, and extracted with two 200-ml volumes of chloroform. The chloroform layer was freed from traces of water with anhydrous neutral $CaCl_2$ powder and passed through a 2- × 30-cm alumina column (Bio-Rad Laboratories, 100–200 mesh, activity grade 1). The fluorescent material was retained in the upper one cm of the column. The column was washed with *ca.* 50 ml of chloroform and then with *ca.* 50 ml 9:1 v/v solvent

mixture of petroleum ether and butanol. The latter was used to separate out two bands with a weak blue and yellow fluorescence respectively. The upper one cm of the column was removed and its remaining fluorescent material was eluted with 0.005 *N* KOH. The KOH filtrate was acidified to pH 3, extracted with chloroform which was then dried with neutral $CaCl_2$, filtered, and evaporated to dryness under vacuum at room temperature. A residue of *ca.* five mg total dry weight was obtained.

For quantitative comparisons of amounts of substance released into the medium, the agar from 10 flasks (about 500 cc) was pooled, made up to 600 ml, and adjusted to pH 2. In these cases, one ml 0.005 *N* H_2SO_4 was added to each flask before the agar was melted in the Arnold steamer. Duplicate 100-ml samples of the liquefied agar were transferred to separatory funnels and extracted with four successive 100-ml. volumes of chloroform. The pooled chloroform extract was dried with neutral $CaCl_2$, filtered and chromatographed on a neutral alumina column (0.7 × 20 cm). The column was washed first with chloroform and then with a 9:1 v/v petroleum ether–butanol mixture. The top one cm of the column was removed and eluted with successive portions of pH-10 borate buffer to make 100 ml of eluate. The concentration of scopoletin in 20 ml aliquots was determined from the intensity of the fluorescence in a Coleman photofluorimeter, Model 12, with filters B_1 (365 mμ) and PC-1. The values obtained were in the range from 2×10^{-3} to 1×10^{-2} μg/ml as determined by interpolation on a standard linear concentration curve obtained with a sample of pure chemical† dissolved in pH-10 buffer.

b. *Free scopoletin in tissues:* For determinations of free scopoletin in tissues, samples of approximately one gm fresh weight were dropped into boiling, pH-2 citrate-phosphate buffer (*ca.* 20 ml) for 2 min, ground in a Potter-Elvehjem homogenizer, and centrifuged. The supernatant was decanted and the residue washed with successive 30-ml portions of buffer until all fluorescent material was removed (usually 3 times). The buffer solutions were pooled and made up to 150 ml, and the scopoletin was determined quantitatively as above. The bound scopoletin present in the tissue was retained in the aqueous supernatant from which free scopoletin had been extracted with chloroform.

c. *Extraction from the tissue, hydrolysis, and estimation of the bound form of scopoletin:* In analysis of tissue samples for bound scopoletin, ordinarily the aqueous supernatant remaining after chloroform extraction of free scopoletin, as above, was acidified with 6 *N* HCl (1 volume to 5 of the tissue extract) and refluxed for one hour. The solution was then adjusted to pH 2 with NaOH, and the liberated scopoletin was extracted and estimated as above.

To prepare a sample of the bound form, 300 gm fresh weight of tobacco callus tissue which had been cultured for 40 days on modified White's basal medium with 2.0 mg/l IAA and 0.20 mg/l kinetin were plunged into 300 ml of hot ethanol, kept boiling for 3 min, and homogenized in a Waring blender. The homogenate with Cellite added was filtered through a Gooch filter. The filtrate was concentrated to 10 ml under vacuum at 30°C. A precipitate formed in this process was filtered off, and the concentrate was chromatographed, deposited as a line on a 40- × 40- × 0.3-cm sheet of Eaton Dikeman #320 filter paper, and developed with distilled water. The fluorescent band at approximately R_f 0.80 was eluted with 80 per cent ethanol, concentrated to 2 ml, and rechromatographed with *n*-butanol:acetic acid:water (4:1:2 v/v) as developer on a sheet of Whatman #42 filter paper. The fluorescent material (R_f 0.50) was eluted with 50 per cent ethanol, evaporated to dryness under vacuum, and its properties investigated.

Ultraviolet adsorption spectra were measured with a Beckman Model D. U. spectrophotometer. Fluorescence was measured in accordance with Goodwin and Kavanagh[4] except that as above a Coleman photofluorimeter was employed.

Characterization of the Fluorescent Substance in the Medium.—About five mg of the fluorescent material was extracted from agar medium on which tobacco callus tissue had been grown and was obtained in relatively pure state by chromatography procedures as described above.

Spectral and chromatographic properties: As may be seen from Figure 1 (upper curve), the absorption spectrum of the extracted material closely matches that of a standard solution of scopoletin throughout the tested range (from 220 to 360 mμ) with maxima at 229, 255, 298, and 343 mμ and with minima at 250, 269, and 308 mμ.

Such minor differences as occur may be readily accounted for by concentration effects and by impurities present in the extract.

The fluorescence of the extracted material in ultraviolet light has a maximum intensity above pH 8 and is relatively weak below pH 6. The change in intensity with pH is very nearly the same as for the standard scopoletin sample, as is shown by the curve in Figure 2, and matches Goodwin's and Kavanagh's previously published curves for scopoletin.[4]

Chromatographic properties of the material extracted from the medium also have been compared with those of the scopoletin sample as follows: The two substances were co-chromatographed as separate spots and as combined single spots on Whatman #1 paper with various solvent systems as indicated in Table 1. In all cases,

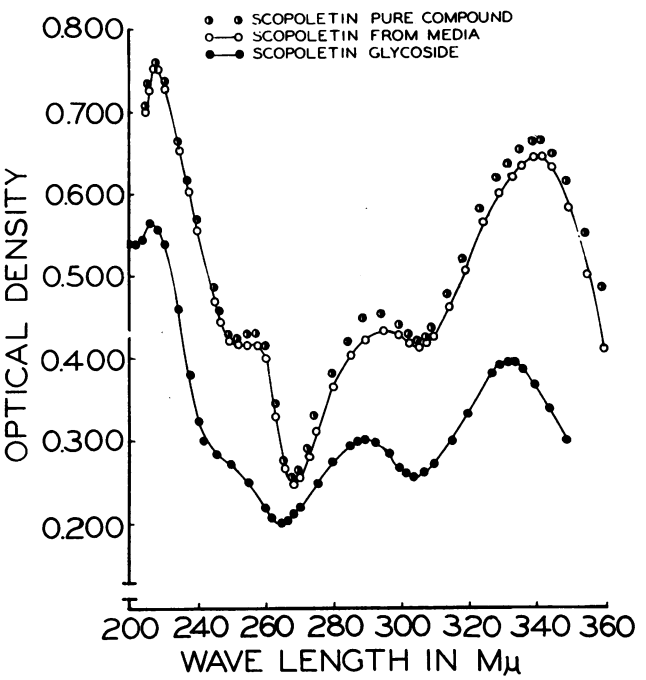

FIG. 1.—Absorption spectra of scopoletin (5×10^{-6} *M*) and of fluorescent materials from tobacco callus tissue cultures.

the substances run together as a single compound with the R_f values listed in the column headed scopoletin. It is concluded, therefore, that the fluorescent substance extracted from the medium is in fact scopoletin.

Free scopoletin in the tissue: The callus tissue itself tends to contain relatively low concentrations of free scopoletin. Typical amounts are represented by the curves in Figs. 4, 5, and 6, i. e., on the order of 5 μg/gm. of fresh growing tissue.

Characterization of a Bound Form of Scopoletin in the Tissue.—Most of the scopoletin in the tissue is present in a bound form which has been estimated in terms of the scopoletin set free by acid hydrolysis. This bound form apparently is retained entirely within the cells; at least, it could not be detected in the medium. The

properties of one sample of the material extracted from 300 gm. fresh weight of callus and prepared as described above have been investigated.

As shown in Figure 1, the UV absorption spectrum of this bound form (lower curve) resembles that of free scopoletin but shows slight shifts in the positions of maxima from 229, 298, and 343 to 225, 290, and 334 mμ respectively and of minima from 260 and 308 to 265 and 305 mμ respectively. A possible difference between the curves exists in the 250–260 mμ region where the bound form for some reason gives at most a hint of the shelf exhibited by the free scopoletin.

The fluorescence intensity–pH curve of the bound form, as shown in Figure 2, is strikingly different from that of free scopoletin in being much higher in strong acid, in being practically constant between pH 1 and 11, and in falling rapidly around pH 12. This curve is similar if not identical to that of the scopoletin glycoside reported by Goodwin and Pollock[5] (Fig. 3, p. 518 of ref. 5) to be present in *Avena* roots.

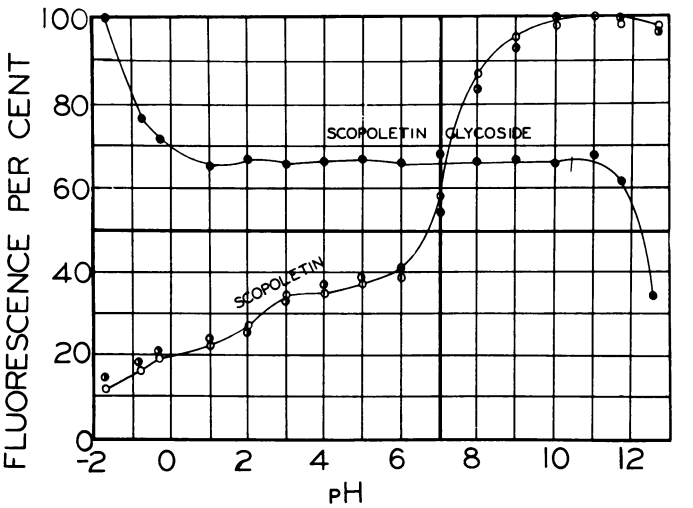

FIG. 2.—Effect of pH on the intensity of fluorescence of a standard scopoletin sample (lower curve), of material (scopoletin) leached by tobacco callus tissue into the medium (half-filled circles), and of a bound form of scopoletin (scopolin) obtained in water extracts of the tissue (upper curve).

Also the R_f values obtained in paper chromatograms with different solvent mixtures are quite different for scopoletin and this substance, as may be seen by comparing columns 1 and 2 in Table 1.

Evidence that the fluorescent material in the bound form actually is scopoletin was obtained by co-chromatographing its hydrolysate with the pure substance. As shown in Figure 3, the hydrolysate (H) gives a fluorescent compound with the identical Rf value as scopoletin. Also the pH-fluorescence curve of the eluate of this spot is identical with that of scopoletin. Two additional spots are obtained in chromatograms of the hydrolysate by spraying with alkaline 3,5-dinitrosalicylate—spots not obtained in chromatograms of the untreated material. In co-chromatograms with sugars (Fig. 3), these spots correspond to those of glucose and fructose with Rf values 0.25 and 0.31 respectively in butanol : acetic acid : water (4:1:2). These results strongly suggest that the substance is a glycoside and that sucrose

TABLE 1

R_F VALUES OF SCOPOLETIN AND ITS ABUNDANT GLYCOSIDE, OBTAINED FROM TOBACCO CALLUS TISSUE, FOR VARIOUS SOLVENT MIXTURES AT *CA.* 28°C

Solvent mixture developer	R_F Scopoletin	R_F Scopoletin glycoside
Water	0.45	0.85
Acetic acid 10%	0.57	0.84
n-Butanol:acetic acid:water (4:1:2)	0.85	0.50
n-Butanol:ammonia:water (4:1:5)	0.45	0.55
n-Butanol:water (4:1)	0.85	0.40
Ethyl acetate:2 *N* HCl (1:1)	0.95	0.10
Amyl alcohol:acetic acid:water (4:1:5)	0.88	0.30
Isopropanol:ammonia:water (10:1:1)	0.75	0.80

might be the glucone. It is of interest to note the close agreement between these findings and the results reported by Goodwin and Kavanagh[6] for one of the glycosides they isolated from *Avena* roots. However, the possibility that the composition

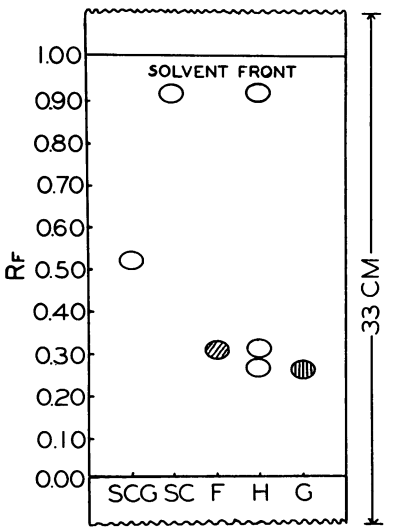

FIG. 3.—Chromatogram of scopoletin (SC), scopoletin glycoside (SCG), SCG-hydrolysate (H), fructose (F), and glucose (G). The developer was *n*-butanol:acetic acid:water (4:2:1 v/v) on Whatman No. 1 filter paper.

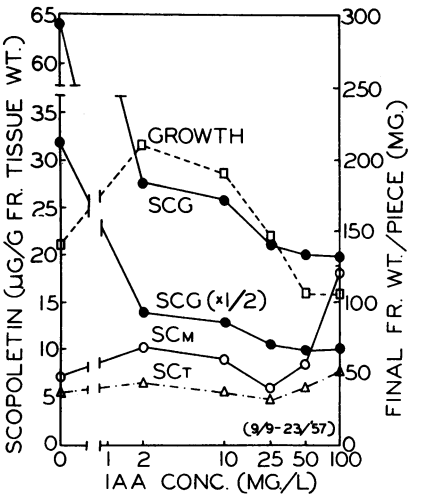

FIG. 4.—Effects of IAA concentration on growth of tobacco callus, on the levels of free and bound scopoletin in the tissue, and on the content of scopoletin leached into the medium. SC_T, scopoletin in tissue; SC_M, scopoletin in medium; SCG (× 1/2), scopoletin glycoside in tissue (half scale); growth, average final fresh weight of callus. Cultures harvested after 14 days growth. Kinetin concentration 0.2 mg/l.

may involve fructose and glucose separately, or even some other sugars, is of course not excluded.‡

Factors Influencing the Appearance of Scopoletin in the Medium.—Effects of auxins: As mentioned above, this investigation started from the observation that in the

presence of IAA a strong, bluish U.V. fluorescence would appear in the agar medium on which tobacco tissue was grown. With relatively high IAA concentrations (0.10 mg/l or more) this fluorescence became noticeable within four hours after the cultures had been started, even though only three pieces of tissue with a total volume of *ca.* 0.15 ml were placed on the surface of a 50-ml volume of agar. With lower IAA concentrations, longer times were required for the fluorescence to appear clearly, but even in control cultures without added IAA, it was definitely visible after two days. The rate of release of scopoletin has not been studied quantitatively, but the influence of the added IAA concentration on the amounts of scopoletin present in the medium of 1- to 4-week-old cultures has been determined in a series of 12 experiments performed within a fifteen-month period. Quantitative determinations based on measurements of the fluorescent material in extracts of the medium and of tissues in relation to the growth of the tissues have been made in five experiments with serial concentrations of IAA. Results of one typical experiment are presented in Figure 4. It may be seen that the amount of scopoletin released into the medium (curve SC_M) first increases with the concentration of IAA and then falls gradually, more or less in parallel with the effectiveness of the IAA concentration in promoting growth, until a toxic level of IAA is reached. From this point on, there is a marked rise in scopoletin content in the medium, which is correlated with a decrease in final weight of the tissue pieces, especially in the presence of the higher IAA concentrations. Very similar results have been obtained with α-naphthaleneacetic acid (NAA) substituted for IAA (Fig. 5A). With 2,4-dichlorophenoxyacetic acid (2,4-D), the rise and gradual fall of the scopoletin curve in the low dosage range have not been found, but, as shown in Figure 5B, the release of scopoletin by the high concentration is striking. It appears, therefore, that the release of scopoletin into the medium is a general effect of high auxin levels on the tobacco tissue.

The levels of free scopoletin in the tissue (curves SC_T, Figs. 4, 5A, and 5B), are relatively low in healthy tissue (about 5 μg/gm) and increase with the IAA concentration or decrease with the kinetin concentration (Fig. 6), as reflected also in each case by changes in the amounts of scopoletin in the medium (curves SC_M).

The level of scopoletin glycoside in the tissue is also greatly affected by IAA treatments. As shown in Figure 4 by curve SCG (or SCG ($\times 1/2$), plotted on half the scale of the others), in the presence of low, growth-promoting concentrations of IAA, the glycoside level drops sharply to about a third or fourth the value for controls and then continues to fall gradually with higher IAA concentrations, which give marked increases in the level of free scopoletin in the medium. In the cited experiment, the sharp drop in glycoside level is from 65 to 25 μg/gm. scopoletin equivalents, and in repeated experiments, drops from the 80–60 μg/gm range to the 30–20 μg range have been found consistently.

Although auxin treatments enhance breakdown of the bound form of scopoletin in the tissue and the appearance of its free form in the medium, the causal relationship between the two processes is far from a simple one. In the low IAA concentration range, more glycoside is broken down than is accounted for by the sum of free scopoletin present in the tissue and leached into the medium. In higher IAA concentration range, on the other hand, the free scopoletin level in the medium goes up several fold without much change in the content of the bound form in the tissue. Limitations in analytic procedures may be partly responsible for this. In view of

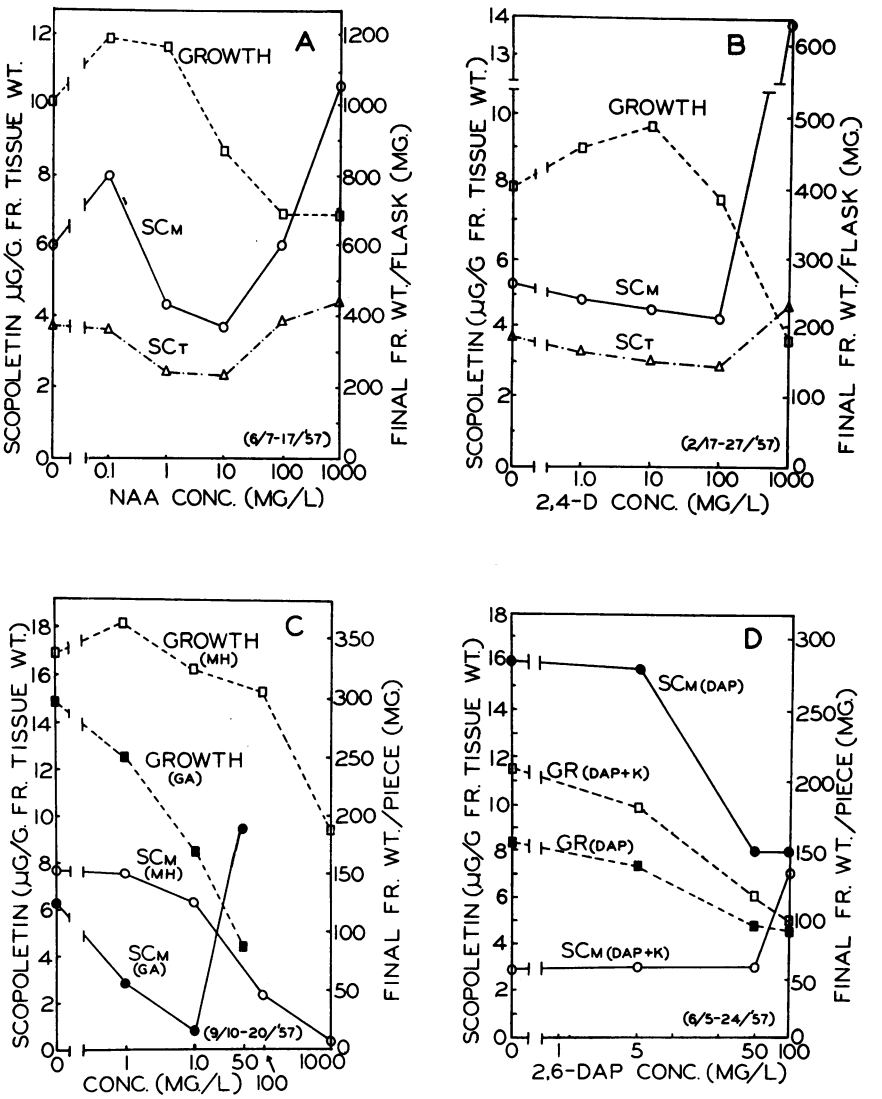

FIG. 5.—Effects of concentration of various substances on growth of tobacco callus and on the content of scopoletin released into the medium. Note that in Figs. 5A and B growth refers to average final fresh weight per *piece* but in Figs. 5C and D to the fresh weight per *flask*. Symbols as in Fig. 4.

A. Treatments with naphthaleneacetic acid (NAA) for a 10-day growth period.

B. Treatments with 2,4-dichlorophenoxyacetic acid (2,4-D) for a 10-day growth period.

C. Treatments with maleic hydrazide (MH) or with gibberellic acid (GA) for a 10-day growth period.

D. Treatments with 2,6-diaminopurine with and without 0.50 mg/l of kinetin (DAP and DAP + K respectively) for a 19-day growth period. IAA, 2.0 mg/l, added in all treatments.

the relative abundance of glycoside, marked shifts in free scopoletin levels might well occur without perceptibly changing the level of the bound form. Only one auxin, IAA, has been tested for an effect specifically on glycoside breakdown, but the

similar action of other auxins in releasing scopoletin into the medium suggests that they all are effective in the same manner as IAA.

Effects of kinetin: In view of the striking interaction of kinetin and IAA in the growth and differentiation of tobacco cultures (see Skoog and Miller[8]), effects of kinetin on the scopoletin status of the tissue was next studied. Results of an experiment in which the kinetin level was varied and that of IAA kept constant at 2.0 mg/l are presented in Figure 6. It may be seen that the cultures with IAA and without kinetin, as expected, show a high free-scopoletin level in the medium (18 μg/gm of tissue) and a low tissue-glycoside level (25 μg/gm as compared with 73 μg/gm of tissue in controls without IAA). With increasing kinetin concentrations, growth increases

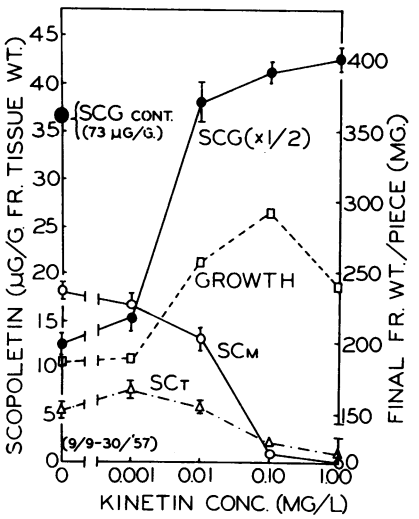

FIG. 6.—Effects of kinetin concentration on the growth of tobacco callus, on the levels of free and bound scopoletin in the tissue, and on the content of scopoletin released into the medium. Symbols as in Fig. 4. Symbols with bars represent means of duplicate determinations differing by the length of the bars. IAA 2.0 mg/l present in all treatments except SCG control. Cultures harvested after a 21-day growth period.

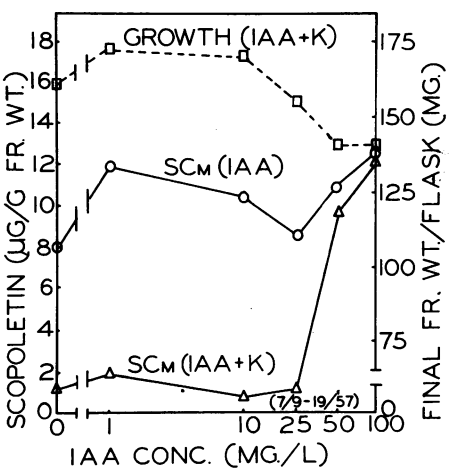

FIG. 7.—Auxin-kinetin interaction in growth of callus and in the release of scopoletin into the medium. Symbols same as in Figs. 4 and 6. Kinetin concentration 0.5 mg/l. Cultures harvested after a 10-day growth period.

to a maximum fresh-weight yield at 0.10 mg/l. Associated with this are a marked lowering of the free scopoletin level in the tissue, a drastic drop practically to zero in the scopoletin content of the medium, and a spectacular rise in scopoletin glycoside in the tissue. Even the relatively minute kinetin concentrations (0.001 and 0.010 mg/l) are effective in raising the glycoside content, and the higher concentrations apparently raise it above the level found in the control without added IAA. In Figure 6, the low kinetin concentrations had only a slight effect in preventing the release of scopoletin into the medium, but in another experiment, 0.001 and 0.010 mg/l kinetin caused decreases of 50 per cent and 90 per cent respectively. Although the absolute values for each determination varied from one experiment to the next, all essential results of this experiment have been confirmed repeatedly.

The maintenance of a high scopoletin glycoside level in the tissue represents a new, remarkably potent biological activity of kinetin, which is strikingly correlated with its activity in promoting growth and differentiation of the callus and which is not exerted in killed or physiologically damaged tissue.

The capacity of a moderate kinetin level (0.5 mg/l) to prevent increasing concentrations of IAA from causing scopoletin to be released into the medium is illustrated by the results shown in Figure 7. Note that in the presence of kinetin, concentrations of IAA up to 10 mg/l increase the yield of tissue but that higher concentrations tend to inhibit growth. In fact, the 50- and 100-mg/l concentrations are highly toxic to the tissue. The free scopoletin in the medium in the absence of kinetin (curve SC_M (IAA)) changes more or less in accordance with earlier results. The effect of kinetin in lowering the scopoletin content in the medium (curve SC_M (IAA + K)) is very striking. It should be noted especially that as little as 0.5 mg/l of kinetin counteracts completely the effects of IAA concentrations up to 25 mg/l but fails entirely to act against the toxic 50- and 100-mg/l IAA concentrations. In other experiments, higher kinetin concentrations, up to 5 mg/l , were used. These were equally ineffective in preventing the release of scopoletin in the presence of toxic IAA levels. This failure may well be due to a generally toxic action of the high IAA levels which perhaps is totally unrelated to the action of this auxin either in promoting growth or in splitting scopoletin glycosides.

That the effect of kinetin in preventing leaching of scopoletin is not merely a consequence of its growth-promoting action is suggested by results of an experiment in which cultures with 2.0 mg/l IAA and 0.5 mg/l kinetin were treated with increasing concentrations of 2,6-diaminopurine (DAP). This substance tends to stop both the division and the enlargement of cells. As shown in Figure 5D, the presence of kinetin markedly reduces the scopoletin content in the medium even when growth of the tissue is almost completely inhibited by the DAP. Results obtained with the highly toxic (100 mg/l) DAP concentration are of course questionable and may be disregarded.

It is of interest that extracts of plant materials which give kinetin-like effects on growth of callus tissue (i. e., possess kinin activity) also exert effects similar to that of kinetin in lowering the scopoletin content in the culture medium. Effects of coconut milk and of a copra extract are shown in Table 2. A comparable effect has been obtained with a beet leaf concentrate with kinin activity. Several other substances with different biological activities which have been tested, if they were effective at all, have tended to increase the scopoletin content in the medium. The

TABLE 2

EFFECTS OF COCONUT MILK OR COPRA MEAL EXTRACT ON THE GROWTH OF TOBACCO CALLUS AND ON THE CONTENT OF SCOPOLETIN RELEASED INTO THE NUTRIENT AGAR

Experiment	Treatments: Material	Concentration,* %	Average final dry weight mg/flask	Scopoletin in medium, μg/gm dry tissue
I (30 days: 11/19/56 to 12/19/56)	Coconut milk	0	49	93
		15	89	19
		45	44	15
II (26 days: 12/15/56 to 1/10/57)	Copra meal extract	0	61	83
		15	100	16.5
		30	55	17

* Concentration of coconut milk is volume per cent. The bottom two figures in the concentration column refer to extracts obtained from amounts of copra meal equivalent to 15 and 30 per cent respectively by weight of medium.

TABLE 3

EFFECT OF PHENYLALANINE ON THE RELEASE OF SCOPOLETIN FROM TOBACCO CALLUS INTO THE CULTURE MEDIUM

Experiment	Treatments, mg/l IAA	Phenylalanine	Average final dry weight mg/flask	Scopoletin in medium μg/gm fresh tissue
I (61 days: 12/14/56 to 2/13/57)	0	0	121	40
	0	50	73	91
	0	100	26	208
	0	150	18	300
	0	300	18	833
II (56 days: 6/11/57 to 8/6/57)	2	0	176	82
	2	50	103	161
	2	100	51	230
	2	150	23	310
	2	300	23	880

effects of gibberellic acid and of maleic hydrazide are shown in Figure 5C. It is of interest that the effect of gibberellic acid in this as in several other physiological respects in part resembles, but still is clearly distinct from, an effect of auxins.

In preliminary experiments, phenylalanine, generally considered to be a precursor of phenolic compounds, seemed to be highly active in releasing scopoletin into the medium (Table 3). The apparent failure of tyrosine, which also serves as a source of phenolic substances, to influence the scopoletin content in the medium in a similar experiment is perhaps misleading.

Effects of Scopcletin on Growth of Tobacco Tissues.—One interpretation of the observed interrelationships between effects of IAA and kinetin on scopoletin levels and on growth might be that auxin serves to release scopoletin, which then in turn reacts with kinetin to promote growth. Both promoting and inhibiting effects of scopoletin on growth have been reported by Andreae *et al.*[10] and Goodwin *et al.*[12, 13] To test this simple notion, tobacco callus and excised pith tissues were cultured on media with and without kinetin and with serial concentrations of either scopoletin or IAA. As shown in Figure 8, the lowest concentration (5 mg/l) of scopoletin appears to be about equally effective as IAA in promoting growth (increase in fresh weight) of the callus in a ten-day period. The action of the two substances differs in that scopoletin, regardless of the concentration employed, is unable to sustain growth over long periods or in successive transfers to new medium. Cytologic examination of sections and squash preparations of the callus by Dr. N. K. Das has shown that the effect of scopoletin like that of IAA was mainly on enlargement of the cells. In fact, the action of scopoletin in this case is the same as would be expected from a low IAA concentration in combination with the employed level of kinetin (see Fig. 9). That scopoletin does not act merely as a weak auxin, however, is clear from the results

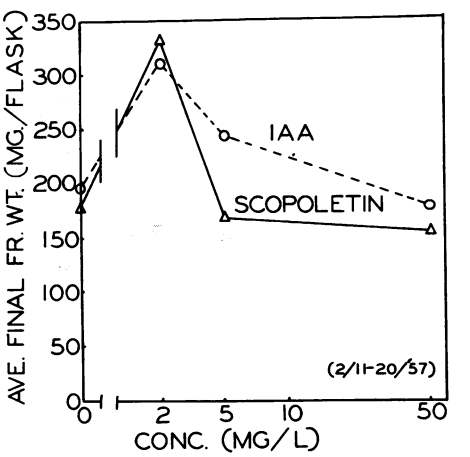

FIG. 8.—Effects of concentration of scopoletin and of IAA on the growth of tobacco callus. Average initial fresh weights *ca.* 150 mg/flask. Growth period, 9 days.

obtained with pith tissue, illustrated in Figure 10. The pith tissue, in contrast with callus, does not produce sufficient auxin to grow without an exogenous auxin supply, and in this case, scopoletin has no visible effect on growth either in the absence or presence of kinetin. These results permit the conclusion that scopoletin only promotes the growth of tissue the auxin content of which is above the required limit for growth. Furthermore, as scopoletin did promote the growth of callus but not of pith tissue its action in the former case would not be due to auxin that might be present as an impurity in the sample. The results rather are in agreement with Andreae's[9] finding that scopoletin inhibits indoleacetic acid oxidase and with the suggestion by Andreae and Andreae[10] that its growth-promoting action (when it occurs) is due to its sparing action on endogenously produced auxin.

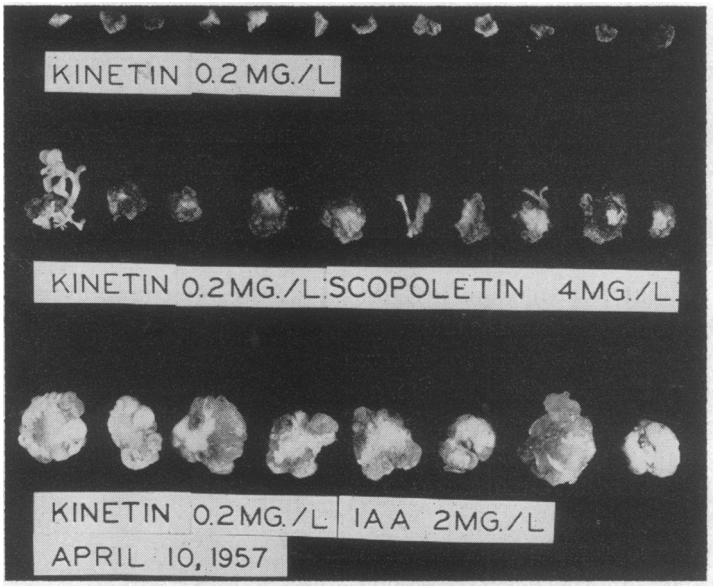

Fig. 9.—Effects of treatments with kinetin, kinetin and scopoletin or kinetin and IAA on size and form of tobacco callus after a 45-day growth period.

Discussion.—Although the exact manner in which auxin and kinetin interact to regulate the scopoletin-scopoletin glycoside levels in the tissue still remains to be determined, work to be presented elsewhere will show it to be closely correlated with these substances in regulating growth of the tissue (Sargent and Skoog[7]) and to involve a highly quantitative coordination of the metabolism of scopoletin and its family of glycosides and perhaps other bound forms (Sargent and Skoog[11]).

Many earlier observations also have pointed to relationships between growth, auxin action, and the contents or metabolism of phenolic substances. Scopoletin was reported by Andreae to stimulate or inhibit the growth of cress and pea roots as as a function of its concentration, and it has been reported by Goodwin and Taves[12] to be a naturally occurring inhibitor of root growth. It also modifies the pattern of differentiation (Avers and Goodwin[13]). Andreae and Andreae first showed that scopoletin acts as a competitive inhibitor of IAA oxidase. On the other hand, an influence of "auxin," 2,4-D , on the scopoletin content of tissues has been reported by

Fults and Johnson.[14] They showed that tobacco plants sprayed with 2,4-D "accumulate" scopoletin and considered this as a factor in the lethal action of the herbicide. Best[15] has shown, furthermore, that following "topping" of tobacco plants, scopoletin accumulates in the root system. It is of interest that bacterial or virus infections which may raise the free auxin levels in plants also appear to cause "accumulation" of scopoletin.

In work with tobacco pith cultures in this laboratory, one of the striking early changes in composition associated with IAA induction of cell enlargement was found to be an increase in the chlorogenic acid content (Wells[16]), and Tryon[17] found the capacities of strains of tobacco callus cultures to form buds to be strinkingly correlated with their scopoletin content.

The present results strongly suggest that increases in free scopoletin which have

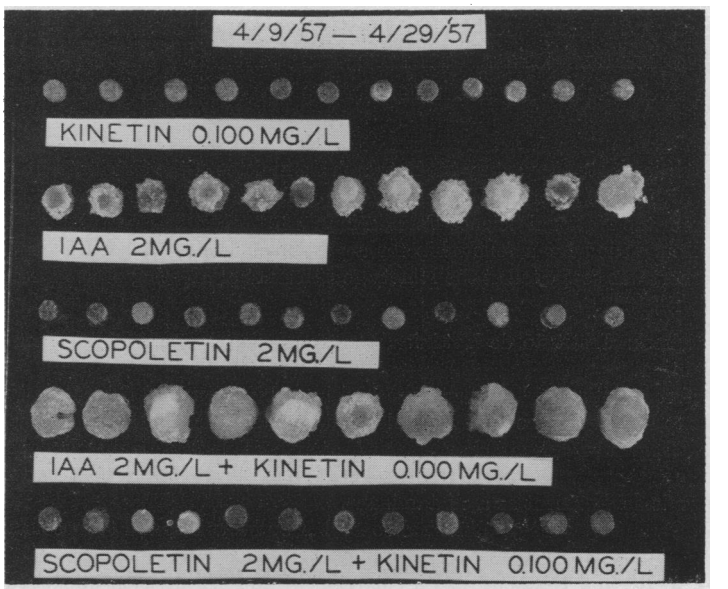

FIG. 10.—Effects of treatments with kinetin, scopoletin, IAA, or pairs of these substances on size and form of tobacco pith disks after a 20-day growth period.

been reported to occur following auxin treatments are brought about through an effect of IAA on the breakdown of bound scopoletin already present in the tissues. It should be noted, however, that a large glycoside loss in the tissues is achieved already with the lowest growth-promoting concentration of IAA, only a small portion of which is accounted for by free scopoletin. Presumably, the glycoside is converted to various cell-wall materials which are increased following treatments with growth-promoting levels of IAA and kinetin. Although exact relationships of such a conversion with reference to growth would not be revealed by the present long-term measurements, the early rise and gradual decrease in free scopoletin associated with increasing, growth-promoting concentrations of IAA would be in agreement with this. The final rapid rise in free scopoletin and the failure of kinetin to counteract it would be logically expected in the presence of high, toxic levels of IAA which stop cell-wall formation and other normal cell functions.

It would appear that relative auxin-kinetin levels regulate the relative rates of formation of different cell-wall components, which in turn may determine the size, form, and organization of cells. The nature of this regulation and its significance in growth and differentiation of the tissue cultures will be further investigated.

Summary.—Tobacco tissue cultured *in vitro* releases into the medium a fluorescent material the main portion of which has been identified as scopoletin. The amount of scopoletin released varies with the concentration of auxin in the medium and increases markedly with high, toxic levels of auxin.

It has been shown that the scopoletin derives from a glycoside (scopolin).

Kinetin added to the medium prevents the release of scopoletin by indoleacetic acid (IAA) and permits the maintenance of high glucoside levels even in the presence of high nontoxic levels of IAA. With toxic IAA levels, treatment with kinetin becomes ineffective. It is suggested that the auxin-kinetin levels regulate the scopoletin–scopoletin glycoside equilibria in the tissues and the conversion of these substances into cell-wall materials. This process is considered as a part of the system in which auxin and kinetin interact in regulating growth and organ formation in plant tissues.

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† Samples of scopoletin used as standards had been kindly supplied by Drs. W. A. Andreae and R. J. Best to Dr. Katharine Tryon Bradley in this Laboratory.

‡ New data (Sargent and Skoog[7]) have shown that glucose alone from scopolin is the glucone and suggest that the spot corresponding to fructose here and in the cited work on *Avena* may have been due to chlorogenic acid present as a contaminant.

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*NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY: ABNORMAL SPLITTING OF ETHYL GROUPS DUE TO MOLECULAR ASYMMETRY**,†

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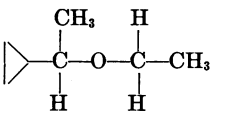
Nuclear magnetic resonance (n. m. r.) spectroscopy provides an excellent means for qualitative identification of ethyl groups by use of the familiar three-four pattern of spin-spin splitting.[1] It has been observed previously[2] that the methylene protons of systems of the type R—CH_2—$CR_1R_2R_3$ (where R_1 can be the same as R or different) may be magnetically nonequivalent and display AB rather than A_2-type spectra.[3] We now wish to report several examples of this type of behavior with ethyl groups, particularly ethoxy groups, knowledge of which could be important to anyone using n. m. r. for organic qualitative analysis.

A typical example is cyclopropylmethylcarbinyl ethyl ether (I). The spectrum of the ethyl CH_2 group of I is nearly identical to that of the CH_2 groups acetaldehyde diethyl acetal (II) shown in Figure 1 and appears not as a simple quartet



I

but as the very complicated AB part of an ABX_3-type spectrum with $\nu_A - \nu_B = 9.0$ cps and $J_{AB} = 9.4$ cps. The nonequivalence of the CH_2 hydrogens is due to the asymmetric center at the carbinyl group of the cyclopropylmethylcarbinyl moiety, which acts to favor one of the possible rotational conformations about the C—O bonds over the others.[2] Similar behavior has been noted with acetaldehyde