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BIOSYNTHESIS OF HYDROXYPHASEOLLIN AND RELATED ISOFLAVANOIDS IN DISEASE-RESISTANT SOYBEAN HYPOCOTYLS

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Abstract—Phenylalanine-U-^{14}C and isoliquiritigenin-9-^{14}C were readily incorporated into the antifungal pterocarpan hydroxyphaseollin in soybean hypocotyls that were inoculated with incompatible strains of the phytopathogenic fungus *Phytophthora megasperma* var. *sojae*. Hydroxyphaseollin accounted for over half of the phenylalanine and isoliquiritigenin incorporated into ethyl acetate soluble compounds. Daidzein, coumestrol, and sojagol were identified as major compounds which accumulated coordinately with hydroxyphaseollin and contained significant amounts of radioactivity from the labelled isoflavanoid precursors. Hydroxyphaseollin was not present in healthy soybean plants and was not detected until *ca.* 16 hr after inoculation with the fungus. The pterocarpan then accumulated rapidly between 16 and 48 hr after inoculation, while the greatest accumulations of daidzein, coumestrol, and sojagol occurred between 48 and 72 hr after inoculation, when hydroxyphaseollin accumulation had ceased. Although soybean hypocotyls contained the anthocyanin malvin, neither this compound nor any other flavone pathway product was observed to accumulate after fungus-inoculation. The results therefore indicate that the accumulation of hydroxyphaseollin in fungus-inoculated soybean hypocotyls involves the activation of isoflavanoid biosynthesis with 'direction' of metabolic intermediates to biosynthesis of the pterocarpan.

INTRODUCTION

SOYBEAN plants containing a single, dominant resistance gene[1] to the disease caused by *Phytophthora megasperma* var. *sojae* accumulate the antifungal pterocarpan hydroxyphaseollin from 10 to 100 times faster when inoculated with the fungus than near-isogenic susceptible plants lacking the resistance gene.[2,3] Similar to certain other legume pterocarpans, hydroxyphaseollin has not been detected in unwounded, non-inoculated plants,[4] but its accumulation can be 'induced' by inoculation with microorganisms or chemical treatments.[5,6] Considerable data[2–5] indicate that rapid accumulation of hydroxyphaseollin is the basis for restriction of fungal growth in *Phytophthora*-resistant plants and that the single disease resistance gene in some manner accounts for this accelerated accumulation. We have begun an investigation concerned with understanding the molecular mechanisms underlying this expression of the soybean disease resistance gene.

It is possible that the production of hydroxyphaseollin in fungus-inoculated soybeans is due to hydrolysis of a preformed glycoside or other conjugate, but attempts to detect the pterocarpan in acid- or enzyme-hydrolyzed aqueous extracts from non-inoculated soybean hypocotyls have thus far failed. To test the alternative hypothesis that fungus stimulated accumulation of hydroxyphaseollin involves *de novo* synthesis, we have supplied ^{14}C-phenylalanine and -isoliquiritigenin to fungus-inoculated resistant soybean hypocotyls and determined their incorporation into hydroxyphaseollin.

[1] R. L. BERNARD, *Crop Sci.* **4**, 663 (1964).
[2] G. GRAY, W. L. KLARMAN and M. BRIDGE, *Can. J. Bot.* **46**, 285 (1968).
[3] N. T. KEEN, *Physiol. Plant Pathol.* **1**, 265 (1971).
[4] N. T. KEEN, J. J. SIMS, D. C. ERWIN, E. RICE and J. E. PARTRIDGE, *Phytopathol.* **61**, 1084 (1971).
[5] W. L. KLARMAN and J. B. SANFORD, *Life Sci.* **7**, 1095 (1968).
[6] M. BRIDGE and W. L. KLARMAN, *Phytopathol.* **60**, 1013 (1970).

Earlier observations[4,5] indicated that the soybean disease resistance response resulted in the accumulation of not only hydroxyphaseollin but also other fluorescing compounds which were not particularly antifungal. It was of interest to identify the nature of these compounds and to learn whether they accumulated coordinately with hydroxyphaseollin. We have therefore identified coumestrol, daidzein, and sojagol from soybean hypocotyls and followed their concentration along with hydroxyphaseollin after inoculation with *P. megasperma* var. *sojae*.

In addition to hydroxyphaseollin, resistant responding tissues accumulate a red, water soluble pigment at the fungus infection site[7,8] and soybean plants also normally contain a violet-red pigment throughout lower parts of the hypocotyl. Since hydroxyphaseollin production was found to be accompanied by the accumulation of other isoflavanoid compounds, it became of interest to know if the pigment accumulating in the resistant-responding soybean hypocotyls was an anthocyanin. We have therefore isolated malvin (malvidin 3,5-diglucoside) from soybean hypocotyls and followed its concentration during challenge by *P. megasperma* var. *sojae*.

RESULTS

Incorporation of ^{14}C-labelled Compounds into Hydroxyphaseollin

Phenylalanine-U-^{14}C and isoliquiritigenin-9-^{14}C were readily incorporated into hydroxyphaseollin (Table 1) and isotope dilution values were lower when labelled isoliquiritigenin was supplied. Total recovery of radioactivity in the isolated hydroxyphaseollin was 0·1–1·0 %.

Radiochromatograms of crude hypocotyl extracts showed that non-inoculated soybean hypocotyls contained an unidentified radioactive spot(s) at *ca.* R_f 0·05, but little or no radioactivity at higher R_fs. Fungus-inoculated hypocotyls, however, contained considerable radioactivity that moved coincidentally with hydroxyphaseollin (R_f 0·35) and daidzein (R_f 0·15).

Excised, inoculated soybean hypocotyls also incorporated phenylalanine-^{14}C into hydroxyphaseollin and other isoflavanoids (Table 2). Radioactivity was highest in daidzein after 20 min, with lower activities detected in the other compounds. Radioactivity in daidzein continued to accumulate at 1 hr but then declined. Radioactivity in hydroxyphaseollin continued to accumulate at 1 and 72 hr, but little change was noted in levels present in coumestrol and an unidentified compound during the feeding period (Table 2). In both intact plants and excised hypocotyls, hydroxyphaseollin accounted for half or more of the radioactivity metabolized from phenylalanine into ethyl acetate soluble products after 24 hr.

Accumulation of Isoflavanoids in Inoculated Hypocotyls

Coumestrol, sojagol, and daidzein were identified as compounds which accumulated coordinately with hydroxyphaseollin by spectral data and R_fs in various TLC systems (Table 3). Increases were not noted for any other flavanoid or isoflavanoid in extracts from fungus inoculated hypocotyls by TLC using various solvent systems.

As reported previously,[3] hydroxyphaseollin was not detected (<10 ppm) in healthy soybean hypocotyls or in fungus-inoculated hypocotyls until 16 hr after inoculation (Fig. 1).

[7] W. L. KLARMAN and J. W. GERDEMANN, *Phytopathol.* **53**, 863 (1968).
[8] W. L. KLARMAN and J. W. GERDEMANN, *Phytopathol.* **53**, 1317 (1968).

TABLE 1. THE INCORPORATION OF PHENYLALANINE-U-^{14}C AND ISOLIQUIRITIGENIN-9-^{14}C INTO HYDROXYPHASEOLLIN BY FUNGUS-INOCULATED SOYBEAN HYPOCOTYLS*

	Precursor			
	Phenylalanine-U-^{14}C†		Isoliquiritigenin-9-^{14}C‡	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Amount of isolated hydroxyphaseollin (μmoles/g dry wt. of tissue)	12·2	9·3	5·1	10
Total radioactivity recovered in hydroxyphaseollin (nc)	70	90	1·4	1·8
Per cent recovery of radioactivity in hydroxyphaseollin	0·7	0·9	0·11	0·15
Specific activity of isolated hydroxyphaseollin (μc/m-mole)	3·9	9·7	0·28	0·18
Isotopic dilution value§	2654	1030	71	111

* Labelled metabolites were applied to hypocotyl wounds of 7-day-old Harosoy 63 soybean seedlings at 24 hr after inoculation with *Phytophthora* and hydroxyphaseollin was isolated after 48 hr incubation.

† Specific activity supplied = 10 μc/μmole and 10 μc were supplied to 120 plants.

‡ Specific activity supplied = 0·02 μc/μmole and 1·2 μc were supplied to 120 plants.

§ Isotopic dilution value $= \dfrac{\text{Specific activity of precursor } (\mu c/\mu mole)}{\text{Specific activity of isolated hydroxyphaseollin } (\mu c/\mu mole)}$.

It then accumulated rapidly, reaching 2700 ppm at 48 hr. Daidzein was present at the time of inoculation (110 ppm), increased to *ca.* 850 ppm at 24 hr, but then remained essentially constant until 40 hr. It accumulated rapidly after 48 hr post-inoculation, reaching about 2000 ppm at 56 hr (Fig. 1). Coumestrol was below detection limits (5 ppm) until 16 hr after inoculation, increased gradually until 40 hr after inoculation and then increased sharply, reaching 700 ppm at 72 hr after inoculation. Sojagol was not detected until 56 hr after inoculation when it was present at 24 ppm (Fig. 1).

TABLE 2. RADIOACTIVITY DETECTED IN HYDROXYPHASEOLLIN AND OTHER FLUORESCING COMPOUNDS ISOLATED FROM CRUDE EXTRACT OF FUNGUS-INOCULATED,* EXCISED SOYBEAN HYPOCOTYLS SUPPLIED WITH PHENYLALANINE-U-^{14}C FOR VARIOUS PERIODS OF TIME†

		Counts/min§ Feeding period		
R_f value‡	Compound	20 min	1 hr	72 hr
0·20	Unknown	700	324	448
0·40	Daidzein	2400	6244	1050
0·49	Coumestrol	308	1022	756
0·64	Hydroxyphaseollin	126	406	2968

* Hypocotyls were challenged with *P. megasperma* var. *sojae* for 24 hr prior to feeding the labelled precursor and the excised hypocotyls were maintained at 25° after supplying radioactive phenylalanine. Data represent extract from 0·5 g dry wt. of excised hypocotyl tissue.

† Radioactivity was supplied at the rate of 3 μc/5 g fresh wt. of hypocotyl tissue.

‡ The various compounds were first separated by silica gel TLC with hexane–EtOAc–MeOH 60:40:2, then eluted and re-chromatographed with toluene–$CHCl_3$–acetone 40:25:35. The reported R_f values were those obtained by the latter separation.

§ Radioactivity counts were obtained by placing the scraped spot from the TLC plate directly into a scintillation vial.

TABLE 3. R_f VALUES OF COUMESTROL, DAIDZEIN, SOJAGOL AND HYDROXYPHASEOLLIN IN VARIOUS TLC SYSTEMS

Solvent*	R_f value × 100			
	Coumestrol	Daidzein	Sojagol	Hydroxy-phaseollin
1. Hexane–EtOAc–MeOH (60:40:2)	25	14	30	35
2. $CHCl_3$–isopropanol (90:10)	60	51	64	45
3. Toluene–$CHCl_3$–Acetone (40:25:35)	49	41	59	64
4. $CHCl_3$–Acetone–HOAc (90:10:1)	30	16	40	50
5. $CHCl_3$–Acetone–conc. NH_4OH (65:35:1)	5	00	—	35

* All v/v; all runs were made at room temp. on 0·375 mm layers of silica gel GF_{254} (Merck).

Malvin Levels in Soybean Hypocotyls

Chromatography of crude methanolic–HCl extracts from non-inoculated or fungus-inoculated soybean hypocotyls in various solvent systems with PC disclosed the presence of only one anthocyanin pigment. Visible spectra of the crude methanol extracts showed a single, symmetrical peak with a maximum at 530 nm.

The crystalline soybean anthocyanin chromatographed similarly to peonin with all solvent systems used, but was considerably bluer, suggesting a higher degree of methoxylation. The anthocyanin was identified as malvin based on co-chromatography with a purified commercial malvin sample in all paper chromatographic solvent systems and identical spectral data for the glycoside and aglycone.

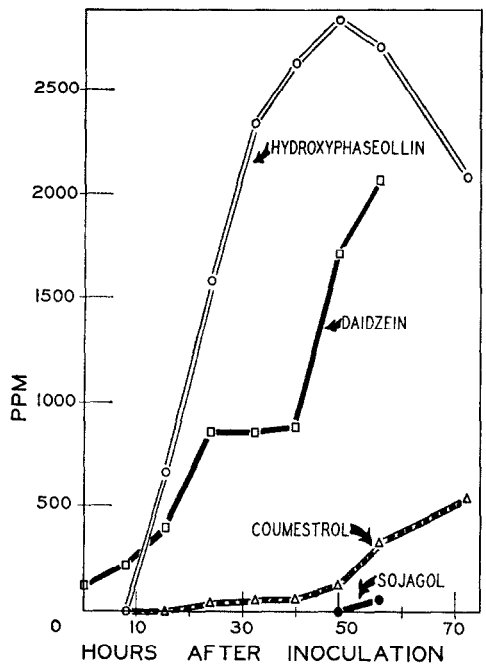

FIG. 1. CONCENTRATIONS OF ISOFLAVANOIDS AT VARIOUS INTERVALS AFTER INOCULATION OF SOYBEAN HYPOCOTYLS WITH *Phytophthora*.

Approximately 50 μg of malvin were extracted from each gram of fresh non-inoculated 7-day-old soybean hypocotyls, but levels of the pigment decreased in fungus-inoculated hypocotyls, ranging from 30 to 45 μg/g fresh wt. tissue at 48 hr after inoculation.

DISCUSSION

Similar to the related antifungal pterocarpans pisatin[9,10] and phaseollin,[11] hydroxyphaseollin was readily biosynthesized from phenylalanine-^{14}C. The feeding data strongly indicate that the production of hydroxyphaseollin following fungus inoculation or treat-

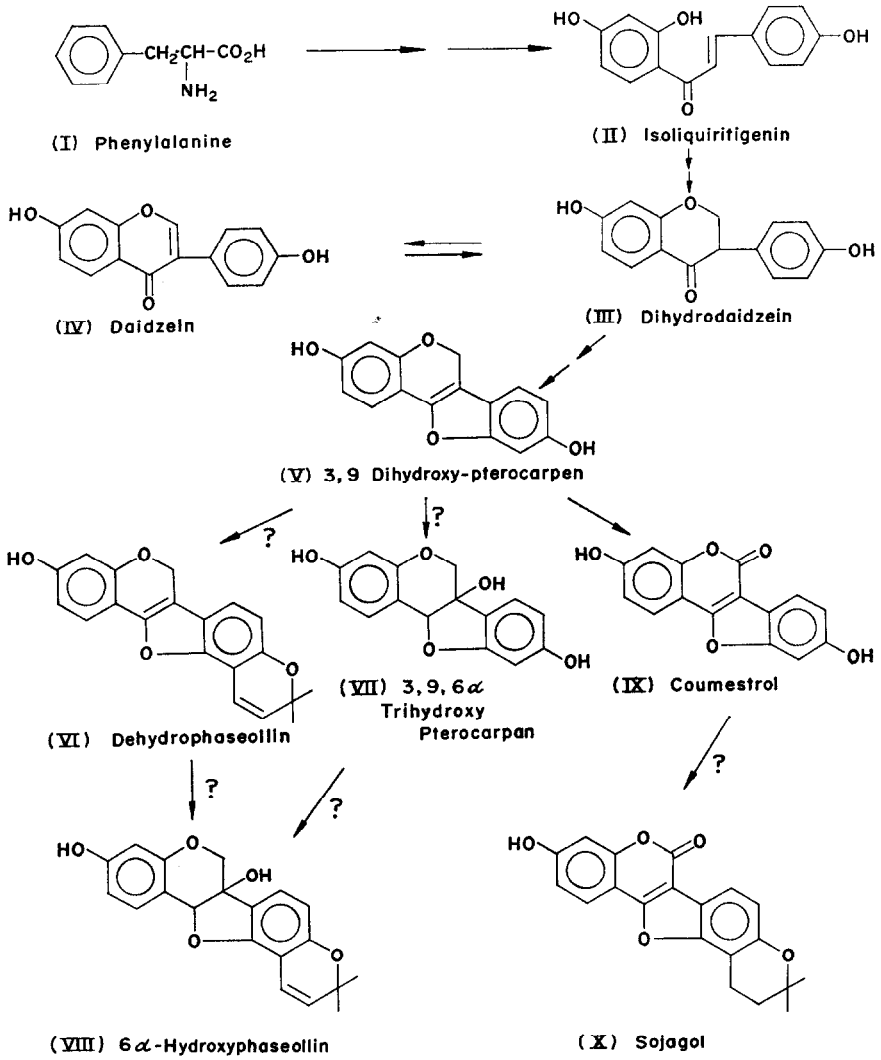

FIG. 2. PROPOSED BIOSYNTHETIC PATHWAY FOR 6a-HYDROXYPHASEOLLIN AND RELATED ISOFLAVANOIDS. THE SCHEME IS BASED IN PART ON WORK BY DEWICK *et al.*[12] WITH COUMESTROL.

[9] L. A. HADWIGER, *Phytochem.* **5**, 523 (1966).
[10] L. A. HADWIGER, *Phytopathol.* **57**, 1258 (1967).
[11] S. L. HESS, LEE A. HADWIGER and MARTIN E. SCHWOCHAU, *Phytopathol.* **61**, 79 (1971).

ment of soybean plants with certain chemicals is due to *de novo* synthesis. The incorporation of phenylalanine and isoliquiritigenin into hydroxyphaseollin and the concomitant accumulation of daidzein, coumestrol and sojagol during pterocarpan production support the proposed biosynthetic pathway for hydroxyphaseollin shown in Fig. 2. This scheme is very similar to the one suggested by Dewick *et al.*[12] for coumestrol biosynthesis and comprises a working hypothesis in the search for biosynthetic enzymes of hydroxyphaseollin.

Our data indicate that two discrete phenomena occur during the 'induction' of hydroxyphaseollin biosynthesis in fungus-inoculated hypocotyls: (1) initiation of the terminal biosynthetic steps from structure V to VIII (Fig. 2); (2) increased general capacity for isoflavanoid biosynthesis. Similar to observations for pisatin in peas[13] and phaseollin in green beans,[14,15] hydroxyphaseollin has not been detected in non-inoculated soybeans,[4,5] but accumulates to high levels after fungus inoculation. Daidzein, coumestrol, and sojagol, on the other hand, are detected in extracts from healthy soybean hypocotyls. This indicates that a specific, precise metabolic control must be exerted over the reactions from structure V to hydroxyphaseollin (VIII) in Fig. 2. The fact that daidzein, coumestrol, and sojagol only accumulate rapidly before and/or after rapid hydroxyphaseollin biosynthesis (Fig. 1), further indicates preferential biosynthesis of the pterocarpan following fungus inoculation. The magnitude of hydroxyphaseollin accumulation alone demonstrates that a very efficient accumulation mechanism is at work. Although our experiments here employed entire hypocotyls for analyses and as such involved considerable dilution of hydroxyphaseollin concentrations formed at the inoculation areas, other experiments[3] in which only the fungus-inoculated, resistant responding portion of the hypocotyl was harvested showed that in this localized area hydroxyphaseollin increased from below detection levels (10 ppm) to greater than 100,000 ppm in *ca.* 36 hr.

The 'lag-period' which occurs after inoculation of plants until hydroxyphaseollin first appears (Fig. 1) may mean that the *de novo* synthesis of an enzyme(s) operating between structure V and hydroxyphaseollin is required for pterocarpan biosynthesis. This may be the initiation step in hydroxyphaseollin accumulation. This possibility has not been successfully checked because we have thus far failed to demonstrate the production of hydroxyphaseollin with cell-free extracts obtained from fungus-inoculated soybean hypocotyls when isoliquiritigenin (II, Fig. 2), dihydrodaidzein (III), or dehydrophaseollin (VI) were supplied.

The data in Fig. 1 indicate that, in addition to specific activation of hydroxyphaseollin biosynthesis, the 'induction' of hydroxyphaseollin also involves a general stimulation of isoflavanoid biosynthesis. The accumulation of daidzein, coumestrol, and sojagol before and/or after hydroxyphaseollin accumulation indicate that fungus-challenged soybean tissues acquire a greater capacity for isoflavanoid biosynthesis. Our unpublished data also show that the biosynthetic enzymes phenylalanine-ammonia lyase[16] and chalcone-flavanone isomerase[17] increase during hydroxyphaseollin 'induction', thereby suggesting that the capacity of the tissue to biosynthesize flavanoids is in fact increased.

No literature reports were found concerning the occurrence of malvin in soybeans, but numerous investigators have noted the presence of chrysanthemin (cyanidin-3-monoglucoside) in soybean seed coats.[18–20] We found no evidence for the occurrence of antho-

[12] P. M. DEWICK, W. BARZ and H. GRISEBACH, *Phytochem.* **9,** 775 (1970).
[13] I. A. M. CRUICKSHANK and D. R. PERRIN, *Austral. J. Biol. Sci.* **16,** 111 (1963).
[14] I. A. M. CRUICKSHANK and DAWN R. PERRIN, *Life Sci.* **9,** 680 (1963).
[15] I. A. M. CRUICKSHANK and DAWN PERRIN, *Phytopathol. Z.* **70,** 209 (1971).
[16] W. L. BIEHN, J. KUĆ and E. B. WILLIAMS, *Phytopathol.* **58,** 1255 (1968).
[17] E. MAUSTAFA and EDMOND WONG, *Phytochem.* **6,** 625 (1967).

cyanins other than malvin in the soybean hypocotyl. The fact that malvin levels did not increase following inoculation of soybean hypocotyls with *P. megasperma* var. *sojae* would indicate that this anthocyanin is not the red pigment which accumulates during the resistant response.[7,8]

Although soybean hypocotyls contain the flavone pathway metabolite malvin and a compound tentatively identified as 7,4'-dihydroxyflavone, TLC showed that neither these nor any other flavone pathway metabolites increased in concentration during the accumulation of hydroxyphaseollin and the other isoflavanoids. This indicates that the observed stimulation in phenylpropanoid biosynthesis in fungus-inoculated plants is specifically directed to isoflavanoids, particularly hydroxyphaseollin. Such a conclusion is further supported by the finding that hydroxyphaseollin and daidzein accounted for a high percentage of the radioactivity occurring in ethyl acetate extracts from soybean hypocotyls which were supplied radioactive phenylalanine (Fig. 1).

The accumulation of coumestanes during disease development has been observed in a number of legumes.[21–25] Recently Rathmell and Bendall[21] reported that accumulation of the antifungal pterocarpan phaseollin in *Phaseolus vulgaris* is also accompanied by increased levels of coumestrol. These workers further showed that the flavone pathway metabolites kaempferol and quercetin, and leucoanthocyanin levels increased only slightly or decreased during rapid production of phaseollin and coumestrol. Thus, similar to *Glycine max*, *Phaseolus vulgaris* appears to invoke pterocarpan production by a specific activation of isoflavanoid biosynthesis.

The soybean disease resistant response has been considered a 'hypersensitive reaction,[26] with eventual death of the initially fungus-colonized area of the hypocotyl. We have previously shown that hydroxyphaseollin production is strictly localized in this responding tissue[3] and now know that daidzein and coumestrol also attain sizeable concentrations. Since coumestrol concentrations above 2000 ppm are known to cause tissue necrosis in alfalfa,[27] cell death in the resistant-responding soybean hypocotyls may also be due to the accumulation of isoflavanoids.

EXPERIMENTAL

Five-day-old *Phytophthora*-resistant soybean plants (Harosoy 63[1]) were grown as described previously[4] and inoculated with *P. megasperma* var. *sojae* (P174) by introducing minced mycelium into 1 cm slash wounds in the hypocotyl.

Feeding labelled precursors to soybean hypocotyls and extraction of labelled compounds. Inoculated plants were maintained at *ca.* 100% relative humidity for 24 hr and then 10 μc phenylalanine-U-^{14}C (Amersham Searle Corp., homogeneous by TLC on silica gel) dissolved in water or 2 μc isoliquiritigenin-9-^{14}C dissolved in 0·25 N Na_2CO_3 were fed to 120 plants by applying 30 μl of the solutions to the inoculated surface of each hypocotyl. After 48 hr, the hypocotyls were harvested and freeze-dried prior to extraction. In other experiments, inoculated hypocotyls were excised from the plant and placed in sterilized Petri plates containing moist filter paper. The labelled precursors were then fed as with intact plants.

[18] K. HAYASHI, in *The Chemistry of Flavanoid Compounds* (edited by T. GEISSMAN), p. 248, MacMillan, London (1962).
[19] K. YOSHIKURU and Y. YAMAGUCHI, *Chem. Abs.* **72**, 63600 (1969).
[20] Y. SASANUMA, *Bot. Mag.* **79**, 807 (1966).
[21] W. G. RATHMELL and D. S. BENDALL, *Physiol. Plant Pathol.* **1**, 351 (1971).
[22] C. M. FRANCIS and A. J. MILLINGTON, *Austral. J. Agric. Res.* **22**, 75 (1971).
[23] G. M. LOPER, *Crop Sci.* **8**, 104 (1968).
[24] R. T. SHERWOOD, A. F. OLAH, W. H. OLESON and E. E. JONES, *Phytopathol.* **60**, 684 (1970).
[25] E. WONG and G. C. M. LATCH, *Phytochem.* **10**, 466 (1971).
[26] C. W. AVERRE, Ph.D. thesis, Purdue Univ. Lafayette, Ind., U.S.A. (1963).
[27] G. M. LOPER, *Crop Sci.* **8**, 317 (1968).

Hypocotyls from intact plants and excised hypocotyls were freeze-dried and extracted with 80% EtOH. After concentration, the aqueous mixture was extracted with EtOAc. The organic fraction was dried, concentrated, and flavanoids separated on silica gel GF_{254} TLC plates. In the ^{14}C-phenylalanine experiments, solvent 1 (Table 3) was used as the developing solvent since it allowed the migration of hydroxyphaseollin (R_f 0·35) while phenylalanine remained at the origin. Solvent 5 (Table 3) was used as the developing solvent in the ^{14}C-isoliquiritigenin experiments since it allowed the migration of hydroxyphaseollin (R_f value 0·35) but not isoliquiritigenin, which remained at the origin. In both cases, the isolated hydroxyphaseollin was purified to a constant specific activity by rechromatography using solvents 1, 3 and 5 (Table 3). Radioactivity was determined by scintillation spectrometry (efficiency *ca.* 58%) and the concentration of hydroxyphaseollin was determined by its absorption at 285 nm ($\log \epsilon = 3{\cdot}92$).[28] Five × 20 cm TLC plates were scanned for radioactivity using a Vanguard counter with strip chart recorder.

Synthesis of isoliquiritigenin-9-^{14}C. Isoliquiritigenin-9-^{14}C was synthesized by cold condensation of ^{14}C-resacetophenone and 4-hydroxybenzaldehyde.[29–31] ^{14}C-Resacetophenone was synthesized by reacting resorcinol with ^{14}C-acetic anhydride, which in turn was made from sodium acetate-1-^{14}C and acetyl chloride. The labelled isoliquiritigenin was recrystallized to a constant specific activity of 0·02 mc/mmole. The product had a m.p. of 199–200° (lit. 202–204°[29]) and chromatographed as a single spot on silica gel thin layer plates developed with various solvent systems.

Identification of isoflavanoids. Daidzein was isolated from ethanol extracts of fungus-inoculated soybean hypocotyls by concentration of the solutions and extraction into ethyl acetate, followed by preparative TLC in solvents 1 and 3 (Table 3). Daidzein was identified by its UV absorption [λ_{max} (EtOH) = 208, 255, 305, 340 nm; (ethanolic NaOH) = 212, 250, 345 nm] and mass spectrum [M^+ = 254, prominent peaks at *m/e* 225, 211, 197, 183, 181, 169, 155, 153, 137 (base peak), and 118] and co-chromatography in 3 TLC systems with daidzein synthesized by the method of Bose and Dutta[32] (Table 3).

Coumestrol was purified from ethanol extracts of soybean hypocotyls by preparative TLC in solvents 1 and 3 (Table 3) and eluted with 95% EtOH. It was identified by similarity of its UV absorption [λ_{max} (EtOH) = 243, 305, 345 nm; (ethanolic NaOH) = 250, 280, 310, 385 nm] and co-chromatography in several TLC systems with authentic coumestrol.

Sojagol was originally isolated from mung beans (*Phaseolus aureus*) by Zilg and Grisebach[33] [in the original report[33] the plant was mis-identified as soybean (Prof. H. Grisebach, personal communication)]. We isolated sojagol from soybeans by the TLC procedure used for coumestrol. It was identified by similarity of UV absorption [λ_{max} (EtOH) = 257, 305, 346 nm; (ethanolic NaOH) = 275, 321, 392 nm] and mass spectral [M^+ = 336, prominent fragments at *m/e* 293, 281, 280 (base peak), 253, 252, 225, 224, and 167] data to those published by Zilg and Grisebach.[33]

Hydroxyphaseollin was isolated as described previously[4] and its structure was determined by us.[28]

Quantitation of isoflavanoids. For the determination of coumestrol,[34,35] daidzein, sojagol, and hydroxyphaseollin, a 1-g sample of freeze-dried soybean hypocotyls was ground in a blender with 40 ml of 80% EtOH, filtered, concentrated, and extracted with hexane (discarded) and then three times with EtOAc. After drying and concentration to 2·0 ml, 100 μl was spotted on a TLC plate, which was developed with solvent 1 (Table 3) for coumestrol and daidzein and solvent 5 (Table 3) for the separation of sojagol. The respective fluorescing areas were scraped from the plate, extracted with known volumes of MeOH and the flavanoids quantitated using a Turner Model III fluorometer with a primary filter of 360 nm and secondary filter of 415 nm. Standard curves were constructed using known concentrations of the respective compounds. Hydroxyphaseollin in the crude extracts was quantitated by GLC as previously described.[4]

Isolation of malvin. The anthocyanin was exhaustively extracted from soybean hypocotyls with cold 1% methanolic–HCl; after filtration, it was quantitated by direct spectrophotometric measurement at 530 nm. Paper chromatography was done in the ascending manner using Whatman No. 1 or 3 MM paper with the following solvents (all v/v): epiphase of BuOH–HOAc–H_2O (4:1:5) (BAW) (malvin R_f = 0·27); HOAc–H_2O–12 N HCl (15:82:3) (AWH) (malvin R_f = 0·48); HOAc–12 N HCl–H_2O (5:1:5) (malvin R_f = 0·65); BuOH–2 N HCl (1:1) (malvin R_f = 0·05). For purification, the anthocyanin was precipitated from concentrated methanolic HCl extracts with Et_2O and applied to a 1·5 × 10 cm column of polyvinyl pyrollidone (Polyclar AT, General Aniline) in 1% ethanolic HCl. The column was washed with 30–50 ml of the same solvent, and the anthocyanin eluted with 1% HCl in 75% aq. EtOH. The pigment was then precipitated with Et_2O, redissolved in a minimal volume of 1% HCl and crystallized as deep red needles at least 3 succesive times at 1°. A commercial sample of malvin (Aldrich) was heavily contaminated with malvidin, malvidin

[28] J. J. SIMS, N. T. KEEN and V. K. HONWAD, *Phytochem.* **11**, 827 (1972).
[29] T. A. GREISSMAN and R. O. CLINTON, *J. Chem. Soc.* **68**, 697 (1946).
[30] D. R. NADKARNI and T. S. WHEELER, *J. Chem. Soc.* 1320 (1938).
[31] E. WONG, P. I. MORTIMER and T. A. GEISSMAN, *Phytochem.* **4**, 89 (1965).
[32] J. L. BOSE and N. L. DUTTA, *J. Sci. Ind. Res.* **17B**, 266 (1958).
[33] H. ZILG and H. GRISEBACH, *Phytochem.* **7**, 1765 (1968).
[34] A. L. LIVINGSTON, E. M. BICKOFF, J. GUGGOLZ and C. R. THOMPSON, *Analyt. Chem.* **32**, 1620 (1960).
[35] A. L. LIVINGSTON, E. M. BICKOFF, J. GUGGOLZ and C. R. THOMPSON, *J. Agric. Food Chem.* **9**, 135 (1961).

monoglucoside and colorless impurities; malvin was therefore crystallized from this material by the procedure used for the soybean anthocyanin. Peonin was similarly extracted from commercial pink peony flowers and purified by Et_2O precipitation and preparative paper chromatography using BAW. The crystalline soybean pigment gave: λ_{max} = 531–533 nm in 0·01% HCl, A_{440}/A_{530} = 0·12–0·14; literature λ_{max} = 533 nm, A_{440}/A_{530} = 0·12.[36] No spectral shifts occurred with $AlCl_3$. Graded hydrolysis of the soybean anthocyanin and the commerical malvin sample (R_f 0·27 in BAW; 0·48 in AWH) in 1 N HCl at 100°[37] gave identical rates of appearance of two new spots on chromatograms which were presumed to be malvidin 3-monoglucoside (R_f 0·35 in BAW; 0·28 in AWH) and malvidin (R_f 0·50 in BAW; 0·11 in AWH), by similarity of R_fs to published data.[37,38]

Upon elution from paper chromatograms, the anthocyanidin gave a λ_{max} of 540–541 nm (literature for malvidin = 542[39]) in 0·01% methanolic HCl, with no $AlCl_3$ shift observed. GLC sugar analysis[40] of completely hydrolyzed[37] and silylated samples of the pigment showed the presence of 1·79 ± 0·09 moles glucose/mole anthocyanin chloride.

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