DOE ER 4967/3

PROGRESS REPORT

I. RELEVANT PUBLISHED WORK

Two papers represent the sort of enzymology that we have carried out in organismal material.

Rubin, J.L. & R.A. Jensen. 1979. Enzymology of L-tyrosine biosynthesis in mung bean. Plant Physiol. 64:727-734.

Byng, G., R. Whitaker, C. Flick, & R.A. Jensen. 1980. Enzymology of L-tyrosine biosynthesis in corn (Zea mays) Phytochemistry (in press)

The aromatic pathway in Euglena represents the opposite extreme from E. coli, L-arogenate being an obligatory precursor for both L-phenylalanine and L-tyrosine. Our studies in cyanobacteria, Euglena, and plants provide a basis eventually for consideration of the endosymbiotic hypothesis of chloroplast evolution in photosynthetic eukaryotes. Euglena possesses the experimental advantages of being able to grow as a heterotroph in the dark and of being represented by mutant lines cured of chloroplasts.

Byng, G.S., R.J. Whitaker, C.L. Shapiro, R.A Jensen. 1981. The aromatic amino acid pathway branches at L-arogenate in Euglena gracilis. Molec. Cell Biol. (in press).

A definitive study was done by Dr. Zamir to establish the proof-of-structure for L-(8S)-arogenate. Preparation of an adequate, high-quality sample represented a huge expense for us largely because we were unaware of the sometimes presence of a second related amino acid (see later section on spiro-arogenate).

Zamir, L.O., R.A. Jensen, B.H. Arison, A.W. Douglas, G. Albers, & J.R. Bowen. 1980. Structure of arogenate (pretyrosine), an amino acid intermediate of aromatic biosynthesis. J. Am. Chem. Soc. 102:4499-4504.

The assay for arogenate dehydratase is technically difficult, and the following technique was worked out.

Shapiro, C.L., R.A. Jensen, K.A. Wilson, & J.R. Bowen. 1981. An assay for activity of arogenate dehydratase based upon the selective oxidation of arogenate. Anal. Biochem. 110:27-30.

The following paper describes production of somatic hybrid plants between cultivated tobacco and sexually incompatible wild species of Nicotiana endowed with disease resistance. Our report describes a cellular genetic approach for incorporation of genetic variability into sexually propagated crops. Since most crop species are propagated sexually and since sexual hybridization has already proven useful in crop improvement, somatic hybridization may prove extremely useful for crop improvement.

Evans, D.A., R.A. Jensen, & C.E. Flick. 1981. Incorporation of disease resistance into sexually incompatible somatic hybrids of the Genus Nicotiana. Science (in press).

DISCLAIMER

The following pages are an exact representation of what is in the original document folder.

p-Fluorophenylalanine was used to generate variant lines in N. tabacum, some of which are excellent candidates for regulatory mutants.

Flick, C.E., R.A. Jensen, & D.E. Evans. 1981. Isolation, protoplast culture, and plant regeneration of PFP-resistant variants of N. tabacum Su/Su. Zeit. Pflanzenphysiol. (in press)

II. PRELIMINARY RESULTS & UNPUBLISHED DATA

A. Spiro-arogenate, a new amino acid.

Spiro-arogenate is the lactam derivative of L-arogenate. It is a non-enzymatically converted to L-phenylalanine at acidic pH, exactly like L-arogenate. It is not a substrate for arogenate-utilizing enzymes. Neurospora crassa, the organism used to accumulate L-arogenate actually forms a mixture of these compounds. The biological significance of spiro-arogenate, if any, is thus far unknown. Since we assayed initial arogenate levels after its conversion to phenylalanine, we now appreciate the basis for frequent unexplained losses in apparent yield during purification. It is of practical importance to recognize and separate these compounds.

Quantitation of Arogenate and Spiro-Arogenate in Culture Supernatants.

Separation of arogenate and spiro-arogenate. Arogenate and spiro-arogenate can be separated on silica gel thin-layer (silica gel 60 F-254, 0.25 mm, EM Reagents) using ethanol: $CHCl_3$: NH_4OH, (5:1:1) as the developing solvent. Under these conditions arogenate has an R_f of 0.25 -0.30 and spiro-arogenate R_f = 0.55.

Because the compounds are found in low concentrations (0.05 mM) the supernatants were concentrated as follows: 200 µl of each culture supernatant was lyophilized and redissolved in 50 µl of methanol: 1 mM sodium phosphate buffer, pH=8, (1:1). 8 µl of the methanol: buffer extract was spotted and developed as described above. After development the plate was dried and exposed to formic acid vapors for 10 minutes. Once acid-conversion to phenylalanine was complete, the plate was air-dried until no trace of formic acid remained.

The compounds of interest were removed from the silica by thoroughly mixing the scraped spots with 75 µl of acetone: H_2O, 1:1. Capped plastic centrifuge tubes (200 µl) facilitated this procedure.

Dansylation: After centrifuging the acetone:H_2O extracts for 1 minute, 50 µl of the supernatant was removed and placed in a 6 x 50mm culture tube. To each tube was added 2 µl 3.4 M K_2CO_3 buffer, pH 10.2, 1 µl 5% w/w dansyl chloride in acetone, and 5 µl ^{3}H-dansyl chloride; the mixture was incubated at 37°C for 35 minutes. The samples were then removed and chilled on ice, and 3 µl of cold dansyl phenylalanine were added to each sample. The samples were dried under vacuum. Dansyl-phenylalanine was extracted into 25 µl of acetone and chilled on ice.

TLC: 10 µl of the acetone extract was spotted on 5 x 5 cm polyamide plates (Cheng-Chin) and developed in H_2O: NH_4OH, 4:1. The plates were developed in the second dimension with benzene: acetic acid: pyridine, 50:5:1. The dansyl-phenylalanine spot was visualized under short wave UV light and cut from the plate The radioactivity was monitored by counting the dpm's in 10 ml Aquasol. The concentration of arogenate and spiro-arogenate

was determined by comparing the measured dpm to a standard curve for phenylalanine standards which were treated in an identical manner.

B. Preparation of ^{14}C-L-Arogenate

This is used in certain enzyme assays and is available for pending tracer studies. The first step requires the preparation of ^{14}C-chorismate, obtained from accumulation supernatants of Aerobacter aerogenes 62-1 in medium containing 1 mCi of ^{14}C-glucose (230 mCi/mM). About 30 mg of ^{14}C-chorismate (399,454 dpm per mg) was obtained).

The second step employs the immobilization of enzyme in a crude extract of cyanobacterium (Synechocystis sp) on a solid support of phenoxyacetyl cellulose. Prephenate aminotransferase was stably bound under conditions where other enzymes were not active to compete for prephenate or to utilize L-arogenate. The yield of L-arogenate product from prephenate, leucine, and pyridoxal-5'-phosphate was 45-50%.

Thirdly, about 18 mg of radioactive chorismate was added to 10 ml of a buffered solution (50 mM phosphate; pH 7.8) consisting of 75 mM L-leucine (98.4 mg), and 0.04 mM pyridoxal-5'-phosphate (0.1 mg). This mixture was then applied to a column containing immobilized crude extract from Synechocystis sp. The column has previously been shown to contain chorismate mutase and prephenate aminotransferase activity. A peristaltic pump was used to continuously recycle the mixture through the column at 0.5 ml per min. After 16 hr at about 25°C, all the fluid in the pump circuit (about 20 ml) was collected in 5-ml fractions (while additional buffer was added to the top of the column).

C. Improved Assay for Arogenate Dehydratase.

An assay superior to our previous one (see Appendix E) has been developed for arogenate dehydratase. It is more sensitive, less vulnerable to interfering compounds, and less tedious.

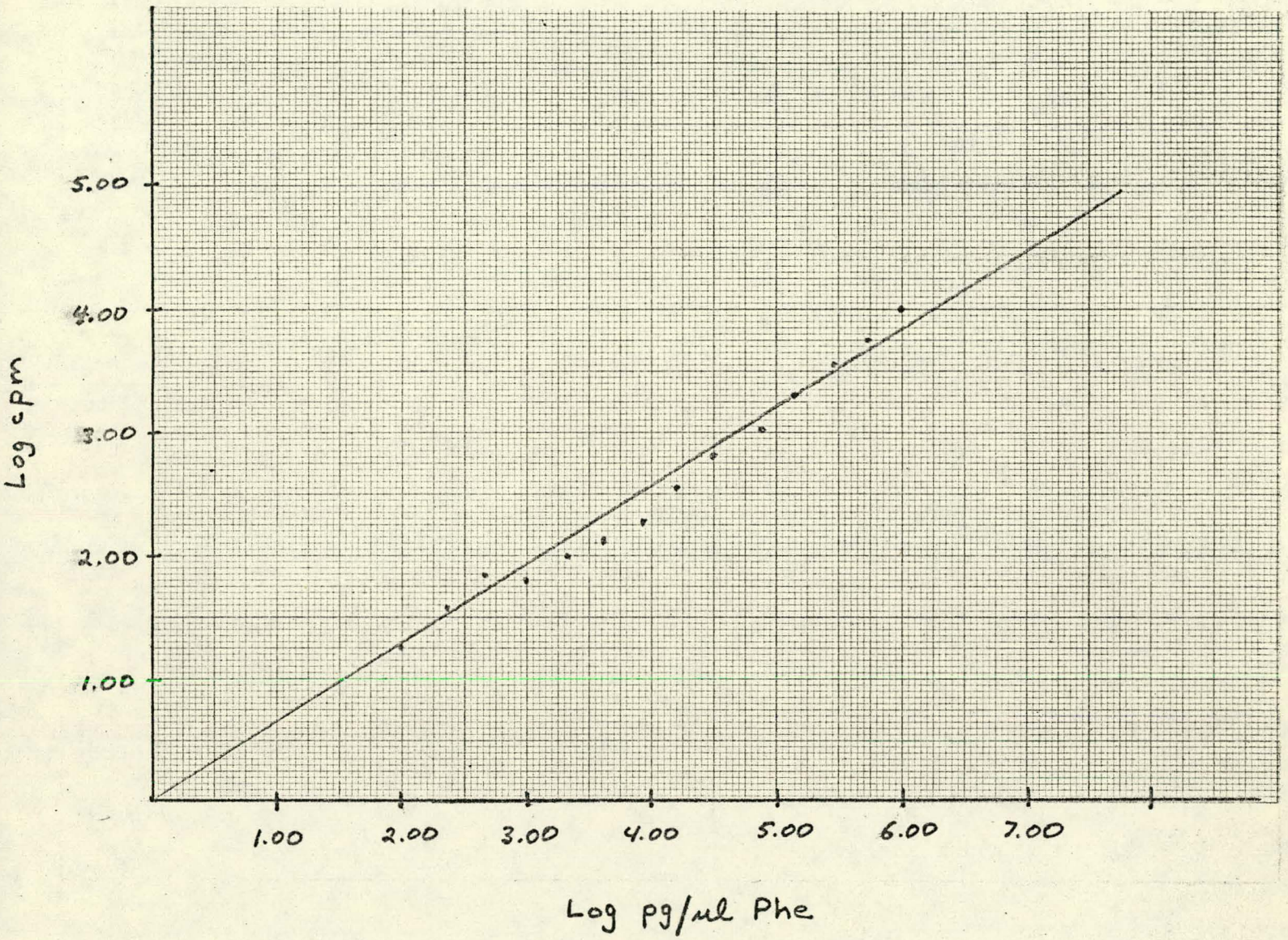
Dansylation. A 25-µl reaction mixture containing 1.44 mM arogenate; 50 mM KPO_4 buffer, pH 7.0; and enzyme is incubated at 37°C for 20 min. in sealed 6 x 50 mm tubes. Following incubation the tubes are transferred to ice and 2 µl of 3.4 M K_2CO_3, pH 10.2, is added and the mixture vortexed. Control tubes containing arogenate and KPO_4 buffer are simultaneously incubated and enzyme is added after the addition of K_2CO_3.

Ten µl of a 9:1 (v/v) mixture of ^{3}H-dansylchloride (1 mCi/1.0 ml New England Nuclear):5% dansylchloride in acetone is added to each and the tubes are then vortexed, sealed, and incubated at 37°C for 30 min. Following incubation the tubes are transferred to ice and 5 µl cold dansyl-phenylalanine is added to each sample. The samples are then lyophilized and the residue redissolved in 100 µl of methanol and vortexed.

TLC. Six µl from each sample is spotted in the corner of a 5 x 5 cm polyamide plate. The plate is then developed in the ascending direction in a 1:4 (v/v) $NH_4OH:H_2O$ solvent, dried, and developed in the second direction in a 50:5:1 (v/v/v) benzene:glacial acetic acid: pyridine solvent.

After the development the plates are dried and the dansyl-phenylalanine spot is cut from each plate and placed into a liquid scintillation vial. 100 µl of 88% formic acid followed by 500 µl of H_2O is added in order to dissolve the polyamide. 15 ml of Aquasol (New England Nuclear) is added and the vials are sealed, mixed and counted.

A standard curve is constructed with H^3 dansyl-phenylalanine in order to quantitate the amount of phenylalanine formed in the reaction mixture.



L-phenylalanine was dansylated with H^3. Dansyl chloride as described and a standard curve constructed relating Log picograms/ul Phe to the Log cpm

D. Cell Biology of Nicotiana sylvestris

(i) Callus culture

Callus cultures have been successfully established by the following method. Leaf disks are cut from leaf surfaces sterilized in 0.5% Na hypochlorite. Disks are placed on Murashige and Skoog medium (1) containing 0.5 mg/l, of 2,4-dichlorophenoxyacetic acid (2,4-D) and 1% agar. In about four weeks callus tissue growing around the edge of the leaf disk can be transferred to fresh culture medium and maintained with periodic transfers every 3-4 weeks. Browning of callus can be inhibited by the inclusion of 210 μM L-cysteine or 1% polyvinylpyrollidone-40 in the culture medium.

(ii) Suspension culture

Suspension cultures have been successfully established as follows. A piece of callus tissue is placed in B5 medium (2) containing 1.0 mg/l of 2,4-D and shaken at 25-50 rpm in a 60 mm petri plate. Fresh culture medium is added at 3 day intervals twice. Cells are diluted 1/4 to 1/2 with fresh medium and placed on a shaker at 150 rpm. Suspension cultures are subcultured every 4-5 days. Less frequent subculturing will result in changes of ploidy.

(iii) Production and maintenance of haploid plants.

Haploid plants have been established in our laboratory. Flower buds are surface sterilized in 0.5% Na hypochlorite for 20 min. Only the first set of flower buds produced by each plant are suitable for anther cultures. Anthers are removed and cultured on Nitsch's H medium (3). Only those anthers in which the microspores are uninucleate or in mitosis are suitable for culture. Flower buds 15-20 mm long generally contain microspores at these stages (corresponds to stage 3 buds as defined by Nitsch and Nitsch in ref. 3). Five to ten percent of the anthers produced plantlets of which about 70% are haploid. The production of plantlets from cultured anthers appears to be only minimally affected by the inclusion of activated charcoal in the medium (4) or a pretreatment of the buds at 4°C (5). When IAA was included in the culture medium as suggested by Nitsch and Nitsch (3), we were unable to isolate any plantlets from anthers. Plantlets were detached from the anther as soon as they had fully emerged and transferred to MS medium with no hormones. Plants are maintained on agar by rooting of shoots every 3-4 weeks. We have maintained the haploid chromosome number under such a continual rooting routine for over 7 months.

(iv) Chromosome counts

Chromosomes are routinely counted in root tips. Root tips are incubated from 2-4 hours in 1 mg/ml colchicine in B5 medium. Colchicine arrests mitosis at metaphase when the chromosomes are most condensed and hence easiest to count. The root tips are fixed overnight in ethanol-acetic acid (3:1) at room temperature. The tips may be stored for several weeks in fixative. The tissue is hydrolyzed with 1 N HCl for 4-6 hours at room temperature and stained in modified carbol fuchsin (6).

(v) Shoot cultures

Shoots can easily be regenerated from haploid or diploid plant material or callus cultures by incubating explants on MS medium containing 1 μM benzylaminopurine. Cultures are transferred every 3-4 weeks to fresh medium. After 1-2 transfers shoots may be rooted. Diploid leaf explants consistently generate all diploid shoots whereas haploid explants produce only about 30% haploid shoots. The remaining 70% of the shoots are diploid;

no aneuploid or higher ploidy shoots have been found. We are currently selecting a haploid line which will produce all haploid shoots.

(vi) Rooting of shoots

Shoots are rooted in 1/2 strength MS medium containing 5 mg/ml 3-aminopyridine (3-aminopyridine stimulates and enhances rooting). A clean-cut shoot will root in 7-10 days. Roots produced in the presence of 3-aminopyridine have a higher mitotic index than those produced in the absence of this substance, and are, therefore, more suitable for chromosome counts.

(vii) Protoplast Isolation (diploid)

Leaf mesophyll protoplasts have been successfully isolated from diploid plants. Essential to this process is the cultivation of plants under proper conditions to yield large numbers of viable protoplasts. It has been determined that in this laboratory plants of Nicotiana sylvestris grown under lighting conditions of 3500 LUX with a photoperiod of 16/8 hours are most suitable for protoplast isolation. In addition, plants are watered daily and receive fertilizer weekly. Leaves that have expanded to between 5-8 cm have been found to be suitable for protoplast isolation. Flowering plants do not seem to yield viable protoplasts. Leaves are surface sterilized by dipping in 95% ethanol for 1 min and by soaking in 0.5% Na hypochloride for 5 min. Sterilant is removed by rinsing three times with sterile distilled water. Due to the difficulty in removing the epidermis from Nicotiana sylvestris, leaves are sliced into strips approximately 1 mm in width. This procedure produces a minimum amount of cell damage and provides excellent access of the protoplasting enzymes to the mesophyll tissue. Once the tissue has been sliced the tissue is then plasmolyzed by immersion in the protoplast isolation medium of Van Slogteren (7) for 4 hours. After the plasmolys period medium is removed and replaced by protoplast isolation medium plus the digestion mixture of Bourgin (8). Enzymatic release of protoplasts is allowed to proceed for 18 hours at 27°C in the dark. The mixture is shaken gently about 15 minutes before harvesting in order to release protoplasts. Debris is removed by filtration of the protoplasts suspension through a 43-micron stainless steel sieve. Protoplasts are subsequently harvested by centrifugation at 100 x g for 4 min in a swinging bucket rotor. In this medium protoplasts float to the surface and extraneous cellular debris pellets. The protoplast layer is carefully removed from the tube with a pasteur pipette and washed 3 times in the glucose-based medium of Van Slogteren (7). Finally protoplasts are resuspended in the sucrose-based medium of Kao (6) and plated at a density of 7×10^4 protoplasts per ml. Incubation is carried out in thin layers of medium or agar solidified medium in the dark at 27°C for 4 days. The first protoplast divisions occur after 24 hours of culture. Approximately 2-3 weeks are required for the formation of visible-size colonies that can be physically manipulated for transfer to regeneration medium.

(viii) Protoplast Culture

The methods of Nagy and Maliga (9) are followed. After 3 weeks of culture, calli are incorporated into RM medium (10) which contains 1 mg/L benzylaminopurine and vitamin B_1 and 0.6% agar. In 3 weeks calli are individually subcultured on the same medium and shoots regenerate in about 4 weeks. Shoots may be rooted on Nitsch's medium T (3).

An alternate method of diploid N. sylvestris protoplast isolation and culture has been reported by Bourgin (11).

(ix) Isolation and Culture of Haploid N. Sylvestris Protoplasts

Batchelor and Street (12) have reported that the isolation and culture of haploid N. sylvestris protoplasts is somewhat more difficult than diploid protoplasts, but have defined conditions which permit cell wall regeneration, cell division, callus growth and plant regeneration. Bourgin (8) also reported the isolation and culture of haploid N. sylvestris protoplasts. More recently, Durand (13) has decribed the cultivation conditions of haploid protoplasts of N. sylvestris from haploid in vitro grown shoots.

Work is presently in progress to adapt these experimental parameters to our laboratory. In addition, Caboche (14) has recently reported the cultivation conditions for obtaining excellent plating efficiencies of haploid N. tabacum protoplasts plated at very low cell densities (400 protoplasts per ml). His preliminary findings suggest that these conditions may also apply to N. sylvestris. The ability to manipulate protoplasts through mutant selection procedures at very low densities will prove extremely valuable in selecting mutants without interference from the cross-feeding phenomenon.

(1) Murashige, T., and Skoog, F. Physiol. Plant 15:151 (1968).
(2) Gamborg, O.L., Miller, R.A., and Ojima, K. Exp. Cell. Res. 50:151 (1968).
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(6) Kao, K.N., A nuclear staining method for plant protoplasts. In: Gamborg, O.L. and Wetter, L.R. (eds): Plant Tissue Culture Methods Saskatoon, Saskatchewan: Nat. Res. Council of Canada. pp 60 (1975).
(7) Van Slogteren, G.M.S., K. Planque and J. Lekkerkerk. Plant Sci. Lett. 20:35 (1980).
(8) Bourgin, J.P. C. Missonier, and Y. Chupeau. C.R. Acad. Sci Paris 282:1853 (1976).
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(10) Linsmaier, E.M., and Skoog, R. Physiol. Plant 18:100 (1965)
(11) Bourgin, J.P., Missonier, C., and Chupeau, Y. C.R. Acad. Sci. Paris. 282:1853 (1976).
(12) Batchelor, S.M., and Street, H.E. Isolation and culture of leaf mesophyll protoplasts of haploid Nicotiana sylvestris. Abstracts of the Fourth International Congress of Plant Tissue and Cell Culture. Calgary, Canada 61: (1978).
(13) Durand, J. Z. Pflanzenphysiol. 93:283 (1979).
(14) Caboche, M. Planta 149:7 (1980).

E. Screen of Sensitivity to Analogue-Inhibition of Growth in N. tabacum Su/Su

We compared the measurements of dry weight and mitotic index to evaluate growth inhibition, and concluded that for our purposes collection of mitotic index data is too tedious. Suspension cultures are maintained through subculture every 4 days by diluting 5 ml cells into 50 ml fresh MS medium with 4.5 μM 2,4-D and 2g/l casin hydrolysate (1MSC). To test for sensitivity to amino acid analogues, the antimetabolites are added to 40 ml 1MSC to 0,50,200, and 500 μM contrations. These cultures are inoculated with 10 ml of 4-day old suspension cultured cells.

Mitotic indices are determined as described previously by Evans and Reed (1980). Samples are taken every 12 hours for determining growth curves by mitotic index. In routine screening of analogues mitotic index is determined only at 24 hours after subculture (when M.I. is at its maximum).

For determination of dry weight growth curves, cultures are inoculated as described for determination of mitotic index. 5-ml samples are collected by harvesting onto preweighed 47-mm diameter Miracloth filters. Filters are dried at room temperature for 24 hours, before determining the dry weight.

Suspension cultured cells are filtered through a 200-micron stainless steel filter before plating to remove the large cell aggregates. The remaining cells are in aggregates of 2-30 cells. Cells are plated in pour plates by mixing 5 ml filtered cells, with 5 ml 1MS medium with 2% agar in a 100-mm petri plate. Resistant variants are visible after 7 days growth, and resistant colonies may be transferred to fresh culture medium 3 weeks after plating.

ANALOGUE	INHIBITION					
	50 µM		200 µM		500 µM	
	D.W.[a]	M.I.[b]	D.W.	M.I.	D.W.	M.I.
D-Tyrosine	0	35	0	17	0	27
D-Phenylalanine	0	0	0	26	0	17
3-Amino-L-tyrosine	0	20	10	89	55	78
DL-α-Methyltyrosine	0	2	19	17	0	35
p-Amino-DL-phenylalanine	0	15	7	22	0	0
Chloroacetyl-DL-phenylalanine	8	0	6	19	7	62
L-3-Methylhistidine	20	25	77	100	82	100
DL-6-Fluorotryptophan	38	84	72	91	80	96
α-Methyl-DL-methionine	21	22	25	27	0	31
Hydroxy-L-proline	7	44	3	82	75	100
Chloro-DL-acetylalanine	7	35	16	55	28	65
Chloro-DL-acetylvaline	0	22	0	17	10	62
N-Methyl-L-leucine	0	0	0	0	0	0
DL-Methionine sulfoxide	0	0	0	0	10	0
DL-Methionine sulfone	0	11	3	13	7	39
N-Acetyl-L-glutamate	0	0	0	0	0	0
Meta-DL-Fluorophenylalanine	26	13	4	92	83	81
DL-4-Azaleucine	35	64	76	91	90	96
L-Canavanine	77	83	75	100	85	85
Meta-DL-Fluorotyrosine	65	4	75	26	85	44
N-Chloroacetyl-L-leucine	0	0	0	0	26	0
N-Chloroacetyl-L-leucine	25	60	50	75	84	72
N-Acetyl-L-phenylalanine	39	14	24	14	27	8
DL-Ortho-Fluorophenylalanine	12	0	-	27	47	95
N-Acetyl-L-tyrosine	21	28	32	40	45	32
5-Methyl-DL-tryptophan	56	85	50	73	49	75
L-Ethionine	86	93	92	95	89	100
N-Chloroacetylglycine	0	0	0	0	0	0
L-Phenylalanine	0	14	4	27	47	13
β-2-Thienyl-DL-alanine	64	13	94	13	77	74
1-Methyl-L-histidine	4	0	4	0	0	0
L-Tyrosine	3	0	1	0	23	0
L-Tryptophan	2	0	17	0	19	6

[a]Inhibition determined by dry weight on four-day old suspensions.

[b]Inhibition determined by mitotic index at 24 hours.

F. Extraction of Enzymes from Plant Tissues

The isolation of active enzyme from plant tissue is complicated by the presence of phenolic compounds which are physically separated from enzymes of intact cells. At cell disruption phenolics released from vacuolar compartments react reversibly or irreversibly with proteins. In general it is necessary to remove phenolics as swiftly as possible, at the same time avoiding oxidation of phenolics. The procedure described below is used in our laboratory and calls for adsorbents (XAD-4 and PVPP) to remove phenolics, high concentrations of reducing agents and nitrogen powders of plant material in order to reduce or prevent oxidation of phenolics.

The method that we currently use for extraction of active DAHP synthase from mung bean seedlings is as follows. Four-day old seedlings are ground in liquid nitrogen in a Waring Blender. The resulting frozen powder may be stored at -40°C for at least one month without loss of activity. The frozen powder is combined with wet XAD-4, wet polyvinylpolypyrollidone (PVPP), and extraction buffer (1:1:1:1, w/w/w/w) and stirred until thawed. The extraction buffer consists of 10 mM Pipes buffer (pH 7.2) with 50 mM sodium ascorbate, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The slurry is filtered through cheesecloth and miracloth and then centrifuged briefly at 62,000 x g at 4°C. The resulting clear, pale yellow supernatant may be applied directly to a Sephacryl S-200 or S-300 column, or desalted on G-25 (equilibrated with extraction buffer lacking Na ascorbate). This is suitable for subsequent fractionations steps on chromatography columns.

G. Regulatory Mutant of N. tabacum Su/Su

A mutant selected for resistance to PFP was selected for enzymological study because high levels of both phenylalanine and tyrosine were assayed in culture supernatants. Mutant PFP-33A was found to possess a chorismate mutase that was less sensitive to allosteric effectors as shown below.

Effector [0.5 mM]	Wild Type % Inhibition	Wild Type Activation Factor	Mutant PFP-33A % Inhibition	Mutant PFP-33A Activation Factor
None				
L-Phenylalanine (PHE)	37.2		10.7	
L-Tyrosine (Tyr)	34.2		14.4	
L-Tryptophan (Trp)		1.9		1.1
Phe + Tyr	44.0		17.8	
Phe + Trp		1.7		1.1
Tyr + Trp		1.9		1.0
Phe + Tyr + Trp		1.0	2.4	
L-Arogenate	14.7		4.1	

Data above are from crude extracts. Apparently three isozymes of chorismate mutase are present, a complicating aspect requiring more detailed studies.

H. Enzymological Results so far in Nicotiana sylvestris.

Thus far our enzyme assays have been restricted to diploid leaves of N. sylvestris.

(i) Chorismate mutase. Three forms were separated by DE-52 chromatography. Only one form was sufficiently stable to allow study of regulatory

properties. Phenylalanine, tyrosine and arogenate were inhibitory while tryptophan was an activator whose activating effect dominated in combination with phenylalanine or tyrosine. Crude extract behaved very similarly to the partially purified preparation in inhibition studies.

(ii) Aromatic aminotransferases. Three aromatic aminotransferases were also separated by DE-52 chromatography. One exhibited an accentuated preference for prephenate as substrate. The remaining two were fairly reactive with 4-hydroxyphenylpyruvate.

(iii) Arogenate dehydrogenase. This enzyme was NADP-linked and strongly inhibited by L-tyrosine.

Prephenate dehydratase and prephenate dehydrogenase were not found. shikimate/NADP dehydrogenase was present in relatively high activity.

I. DAHP synthase.

One of our prime objectives is to characterize tobacco DAHP synthase since it is a crucial and key catalyst of the pathway. Activity in N. sylvestris leaves and in N. tabaccum suspension cultures was unsatisfactory in our first assay attempts. Mung bean seedlings provide extracts exhibiting activities that exceed those of most other plant materials for aromatic biosynthetic enzymes. DAHP synthase is being studied in mung bean as a model system to help resolve the numerous assay difficulties found in plants.

A comprehensive battery of experiments in crude extracts showed that activity was unstable in phosphate or tris buffers, while stability was excellent in PIPES buffer. Secondly, 0.38 M NaF was important to negate (about 80%) very active phosphatases present. Thirdly Co^{++} OR Mn^{++} but not Mg^{++} were metal activitors. Apparently separate Co^{++}-activated and Mn^{++}-activated forms of DAHP synthase are present since both differences in stability (Fig. A) and regulation (Table A) are found when crude extract was assayed in the presence of 2 mM Mn^{++} or 0.5 mM Co^{++}.

Fig. A. Stability of Mn^{++}-activated and Co^{++}-activated DAHP synthase in mung bean crude extract at 4°C.

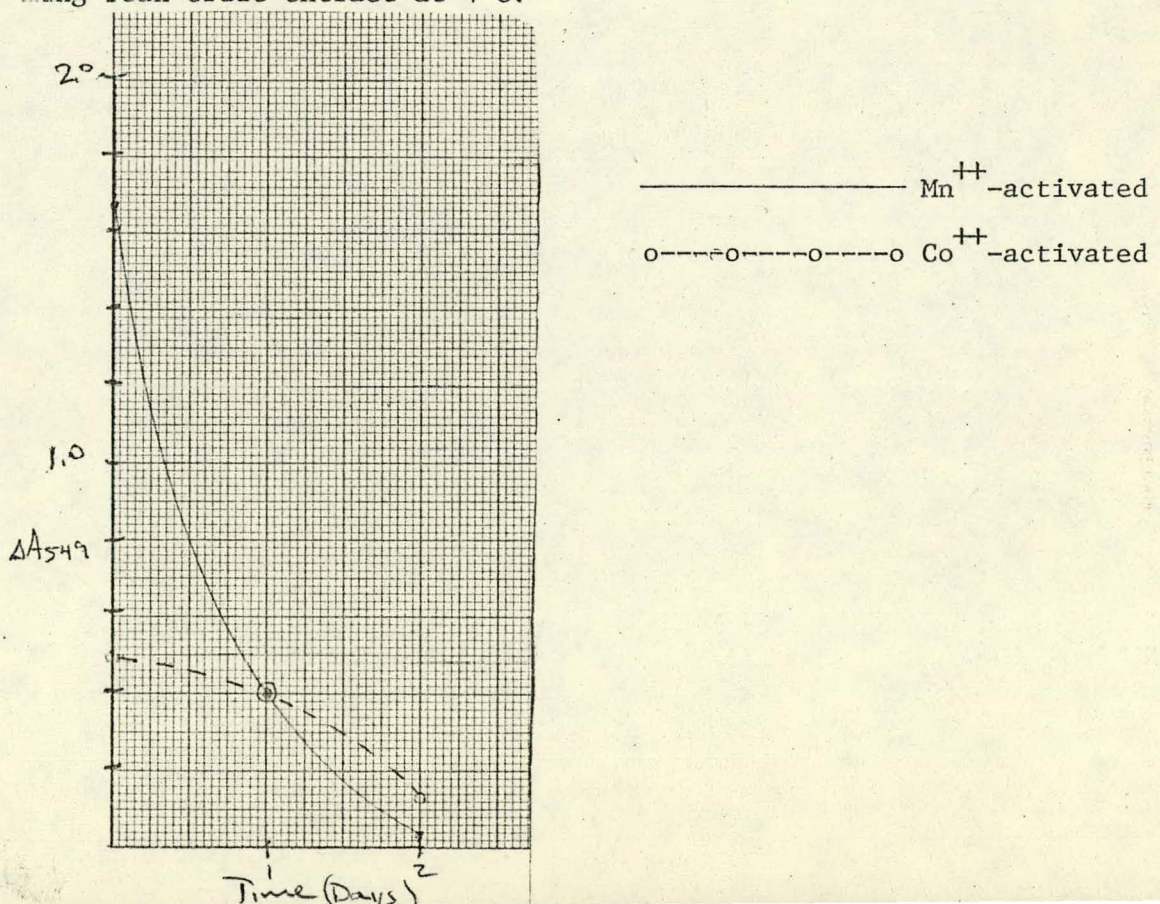


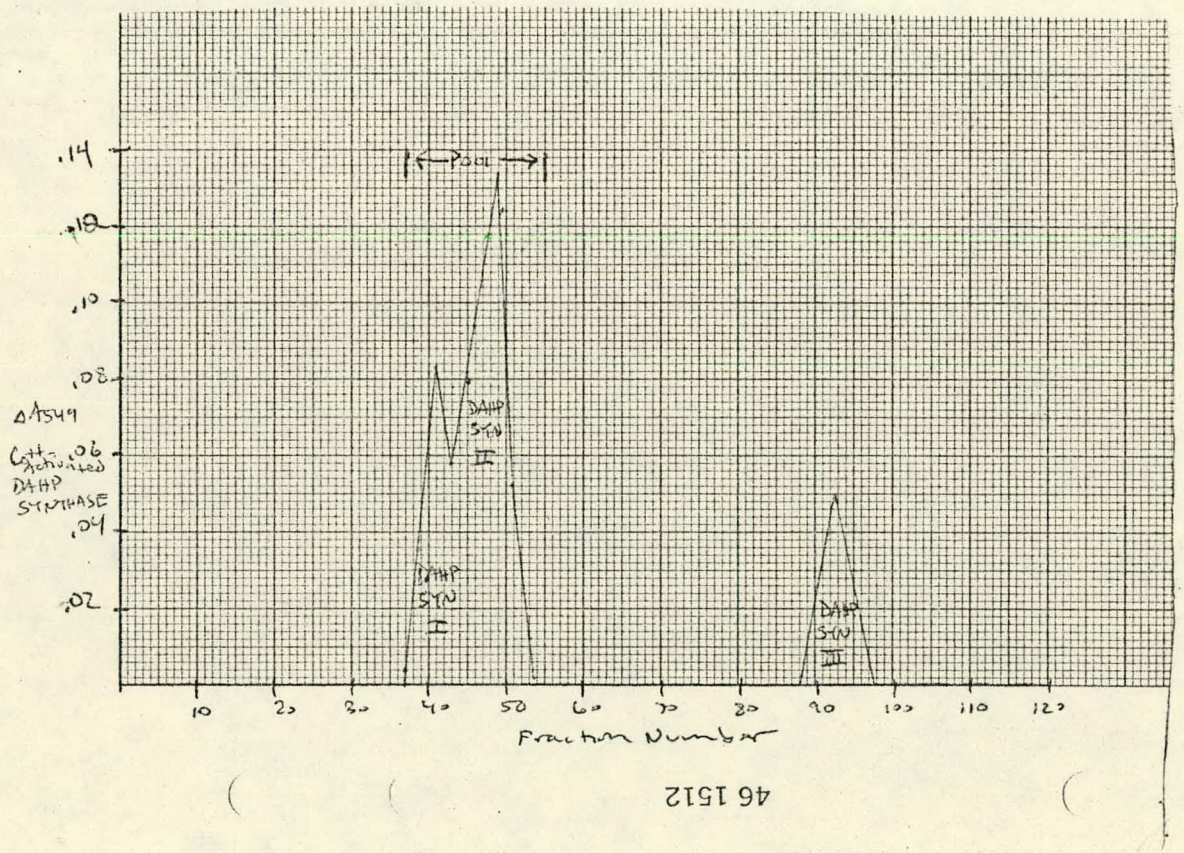
Table B. Regulation of Mn^{++}-activated and Co^{++}-activated DAHP synthase in Mung Bean Crude Extract.

1 mM Effector	Mn^{++}-Activated		Co^{++}-Activated	
	% Inhibition	Activation Factor	%Inhibition	Activation Factor
None				
Phenylalanine				
Tyrosine				
Tryptophan		1.87		1.21
Chorismate	11.7		7.3	
Prephenate	55.1		8.4	
* Arogenate	62.6		52.7	

*present at 0.69 mM

Gel filtration and ion-exchange chromatography fractionations have been carried out. Gel-filtration of crude extract on Sepharacryl S-200 yielded 3 peaks of DAHP synthase (I,II, III) as drawn in Fig. C. Species I & II are very large (>250,000 daltons). This suggests the possibility of multi-functional proteins. Thus far, we have ruled out shikimate dehydrogenase, prephenate aminotransferase, and arogenate/NADP dehydrogenase as co-eluting proteins.

Fig. C. Separation of DAHP Synthase Isozymes by Gel Filtration on Sephacryl S-300



Further purification by ion-exchange chromatography resolved species I and II (Fig. D). DAHP synthase I is probably synonymous with the unstable, Mn^{++}-activated species of crude extracts since it loses activity during both purification steps. DAHP synthase II is fairly stable and is preferentially activated by Co^{++}. The third isozyme (DAHP synthase III) has a molecular wt. of 16,200 (Fig. C), but has not yet been purified further.

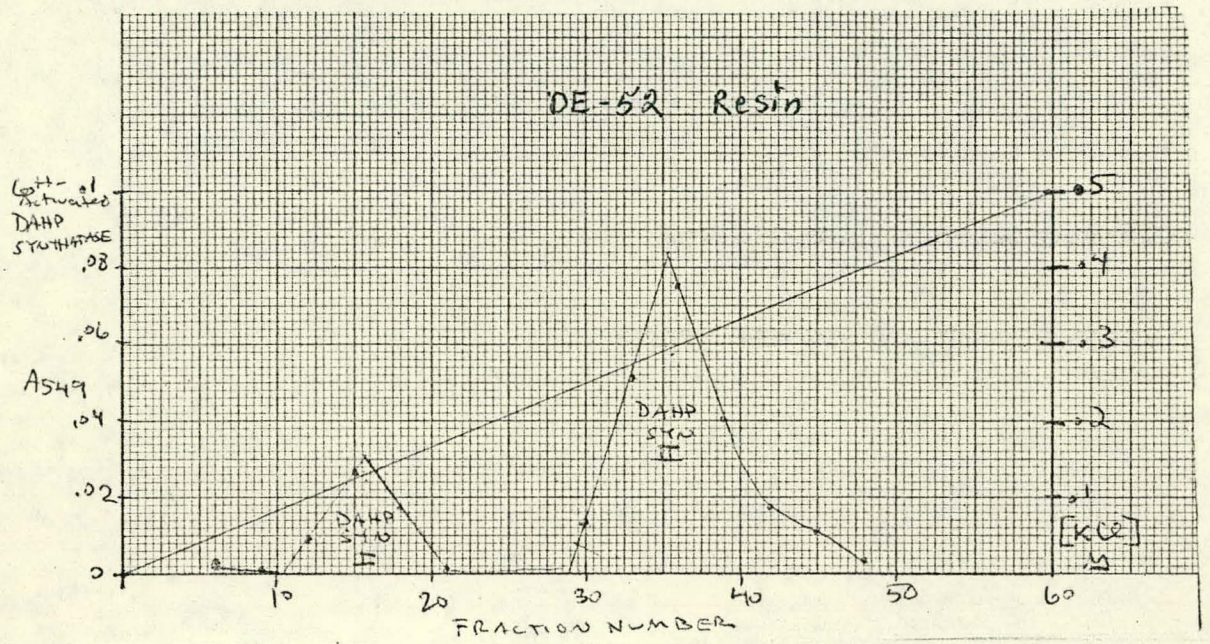


FIG. D. Separation of species I & II (from Fig. C) by Ion-Exchange Chromatography.

General problem with phsophatase, proteases and phenolics probably explain explain the limited scope of most studies on plant DAHP synthases thus far (which can be summarized in the following table).

Table C. DAHP Synthase in Higher Plants

Plant material[a]	Metal Activator	PH Optiumum	Form of Enzyme	Ref.
A) Sweet potato roots	Mg^{++}	7.2-8.0	No data	1
B) Mung bean shoots	Mg^{++}	7.2-7.9	No data	2
C) Cauliflower florets	Mn^{++}	6.4-6.9	> 1 form detected by gel filtration	3
D) Pea cotyledons	Mn^{++} or Co^{++}	7.6 (2 isozymes); 7.0 (1 isozyme)	Three	4
E) Tea shoots	Mn^{++}	No data	Soluble and particulate forms	5
F) Corn shoots	Mn^{++}	7.4-7.6	One	6
G) Pea seedling leaves	Co^{++}	No data	No data	7

[a] Purification steps from top to bottom in the listing were: (A), none; (B), $(NH_4)_2SO_4$ fractionation, (C), protamine sulfate, $(NH_4)_2SO_4$ fractionation, Ion-exchange chromatography, isoelectric foucsing centrifugation; (D), continual $(NH_4)_2SO_4$ solubilization; (E) differential centrifugation; (F), tryptophan agarose affinity chromatography, (G), Sephacryl S-200.

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