able effect on gastric secretion at 5 mg/kg sc in dogs; VI showed slight histamine-like activity. Compounds II–VII, XI, XIII, XVIII, and XIX exhibited histamine-like effect on blood pressure and histamine-like or no effect on nasal volume. Compounds IX, XII, and XVI caused an increase in blood pressure (dissimilar effect) and no effect on nasal volume, while XIV produced no effect on blood pressure and caused an increase in nasal volume (dissimilar effect). Compounds VIII, XVII, and XX lowered blood pressure (similar effect) and caused an increase in nasal volume (dissimilar effect). Compounds I and XV exhibited effects dissimilar to those of histamine by causing an increase in blood pressure and nasal volume.

While the data indicate that structural changes can selectively minimize certain pharmacological actions of histamine, no correlation of structural changes to selectivity of action was observed.

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Multiliter Production and Immunochemical Cross-Reactivity of Plant Tissue Culture Antigens

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Abstract □ Cells of short ragweed suspension culture were successfully propagated in a multiliter fermentor. Proteins were isolated from tissue cultures (short ragweed, cantaloupe, marigold, and Ammi) and plants (short ragweed pollen, cantaloupe fruit, and marigold flowers) by ammonium sulfate precipitation, cross-linked dextran gel filtration, and anion-exchange chromatography. Some fractions elicited allergenic responses in ragweed-sensitive patients but not in ragweed-nonsensitive control patients. Double-immunodiffusion analysis of these fractions showed their nonidentity with short ragweed antigen E.

Keyphrases □ Plant antigens—isolated from tissue cultures, allergenic activity screened □ Antigens—proteins isolated from plant tissue cultures, allergenic activity screened

Ragweed pollen is a major causative agent of allergic rhinitis. Active allergens were isolated from short ragweed pollen, and antigen E was found to be the most active (1–3). In one study, ragweed-sensitive patients reacted positively to intradermal injections of the allergens isolated from short ragweed tissue cultures (4). In the present study, short ragweed tissue cultures were grown in multiliter fermentors to determine their growth characteristics in such systems and to produce an adequate amount of cells for further clinical investigation.

The relationship between short ragweed allergic rhinitis and melon or banana sensitivity was studied (5). All patients with melon- or banana-induced oral pruritus were also sensitive to pollen. Therefore, other plant (melon and marigold flowers) and tissue culture (short ragweed, melon, marigold, and Ammi) sources were examined for their antigenic cross-reactivities with short ragweed pollen extract and antigen E.

EXPERIMENTAL

Plant Materials[1]—Short ragweed (*Ambrosia elatior* L.) pollen was purchased[2]. Cantaloupe[3] (*Cucumis melo* var. *cantalupensis* Naud.) and marigold[3] (*Tagetes patula* L.) seeds were germinated and grown in the medicinal garden of the University of Minnesota. Cantaloupe fruit and marigold flowers were harvested in August 1973.

Plant Tissue Cultures—Cantaloupe and marigold seeds were surface sterilized in 2.5% sodium hypochlorite solution for 20 min under reduced pressure and then rinsed three times with sterile distilled water. Germinated seeds were transferred into sterile 30-ml (1-oz.) square vials containing 18 ml of revised Murashige and Skoog tobacco medium (6) supplemented with 1% agar and 1.0 ppm of (2,4-dichlorophenoxy)acetic acid ($RT_{1.0}$). After incubation at 25° in the dark for 5–6 weeks, sufficient light-yellow cantaloupe callus and brownish-green marigold callus were obtained for transfer. They were designated seedling calli and were subcultured every 4 weeks.

The short ragweed and Ammi [*Ammi visnaga* (L.) Lam.] tissue cultures used were approximately 2 years old in June 1973. The short ragweed tissue culture was initiated from the leaves of short ragweed (4), and the Ammi callus culture was initiated from Ammi fruit. Both short ragweed and Ammi tussue cultures were maintained on $RT_{2.0}$ [2 ppm of (2,4-dichlorophenoxy)acetic acid] and $RT_{0.1}$ [0.1 ppm of (2,4-dichlorophenoxy)acetic acid] media, respectively.

Suspension cultures were initiated by aseptically transferring

[1] Short ragweed antigen E was provided by Dr. R. V. Pennington, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

[2] Greer Labs, Lenoir, N.C. (1972 collection).

[3] George W. Park Seed Co., Greenwood, S.C.

Table I—Growth Indexes of Plant Suspension Cultures[a]

Source	Sub-culture	Growth Period, days	Final Fresh Weight, g	Growth Index[b]	Growth Efficiency Index[c]
Short ragweed	19	18	29	4.8	2.7
Ammi	18	18	34	4.8	3.7
Cantaloupe	5	28	26	5.2	1.9
Marigold	4	18	28	5.6	3.1

[a] Conditions: Revised Murashige and Skoog tobacco medium (100 ml) in a 500-ml erlenmeyer flask at 22–23° and 80 rpm. [b] Growth index = final fresh weight of tissue/initial fresh weight of inoculum (average of five determinations). [c] Growth efficiency index = $\frac{\text{growth index at maximum growth}}{\text{volume of medium (liters)} \times \text{days required for growth}}$.

the calli from solid media to 500-ml flasks containing 100 ml of sterile liquid RT media. The flasks were placed on a rotary shaker[4] operating at 80 rpm at 25°. Preliminary studies showed that the cantaloupe suspension culture grew best in the $RT_{2.0}$ medium and that the marigold suspension culture grew best in the $RT_{0.1}$ medium. Once the suspension cultures were established, they were maintained by transferring about 5 g of inocula, with a stainless steel transfer spoon with perforations in its base, to flasks of fresh media every 2–3 weeks.

Short ragweed tissues from 14-day-old flask suspension cultures were used as inocula (~100 or 150 g in each fermentor) for three fermentors, each containing 2.5 liters of the sterilized $RT_{2.0}$ medium. The aeration was maintained at 500, 1000, or 1500 ml/min, with an agitation rate of 80 rpm, at 25–26° for 14 days. No antifoam was added to the fermentation system.

Allergen Isolation Procedure—The procedure of King *et al.* (2) and Shafiee and Staba (4) was slightly modified and used to isolate antigens from short ragweed pollen, plant tissues, and plant tissue cultures.

Short ragweed pollen (50 g) was defatted with peroxide-free anhydrous ether. The air-dried pollen was extracted with 0.005 *M* phosphate buffer (pH 7.4, 250 ml) on a gyrotory shaker[5] at 200 rpm for 24 hr at 22–23°. The buffer extract was filtered, and the filtrate pH was adjusted from 6.0 to 7.2 with 3 *N* ammonium hydroxide. The filtrate was brought to 90% saturation with ammonium sulfate at 4°. The resulting suspension was stirred overnight and centrifuged[6] at 14,350×*g* for 40 min at 4°.

The dark-brown gummy precipitate was dissolved in 0.1 *M* tromethamine–hydrochloric acid buffer (pH 7.9, 15 ml) and desalted and depigmented by passage through a cross-linked dextran gel column[7] (50 × 4 cm), previously equilibrated with 0.025 *M* tromethamine–hydrochloric acid buffer (pH 7.9). The cross-linked dextran gel column flow rate was adjusted to 60 ml/hr, and 10-ml fractions were collected at 22–23°. The presence of protein was determined by its UV absorption at 280 nm. The first peak, Fraction A, was concentrated[8], and the concentrate was exhaustively dialyzed against 0.025 *M* tromethamine–hydrochloric acid buffer (pH 7.9) and applied to a diethylaminoethylcellulose[9] column (26 × 2.7 cm), previously equilibrated with 0.025 *M* tromethamine–hydrochloric acid.

The second dextran gel column peak, Fraction B, contained yellow pigments and was discarded. The flow rate of 0.025 *M* tromethamine–hydrochloric acid buffer (pH 7.9) through the diethylaminoethylcellulose column was adjusted to 60 ml/hr, and 10-ml fractions were collected and monitored at 280 nm. The first diethylaminoethylcellulose peak, Fraction C, was collected. The column was then eluted with equal volumes of 0.05 *M* tromethamine–hydrochloric acid and 0.2 *M* sodium chloride buffer (pH 7.9) to obtain a second peak, Fraction D. Fraction D was concentrated to 30.5 ml and contained 5.0 mg of protein/ml. The protein content

[4] Model-53, New Brunswick Scientific Co., New Brunswick, N.J.
[5] Model-G-10, New Brunswick Scientific Co., New Brunswick, N.J.
[6] Sorvall SS-12, Newton, Conn.
[7] Sephadex G-25.
[8] Amicon ultrafiltration cell, model 402, Amicon Corp., Lexington, Mass., and UM-2 membrane.
[9] Exchange capacity of 0.88 mEq/g, medium mesh; Sigma Chemical Co., St. Louis, Mo.

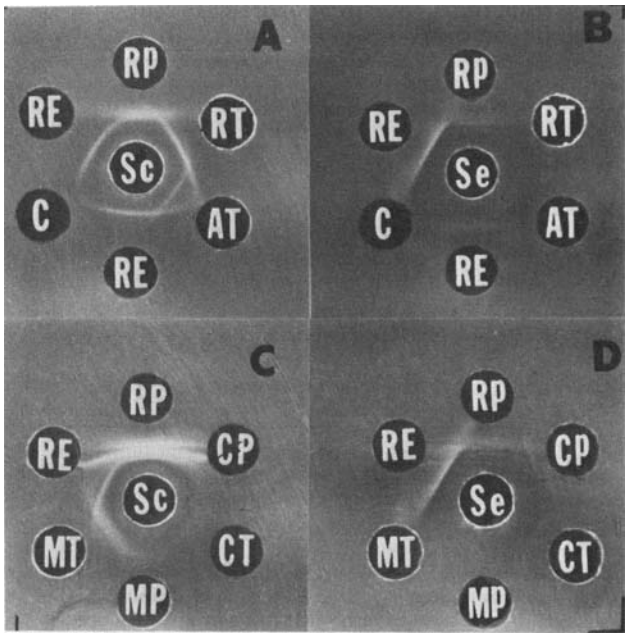

Figure 1—*Double-diffusion analysis of Fraction D tissue culture and plant extracts against short ragweed pollen serum (A and C) and antigen E serum (B and D). Key: AT, Ammi tissue culture; C, control (0.005 M phosphate buffer, pH 7.9); CP, cantaloupe plant (fruit); CT, cantaloupe tissue culture; MP, marigold plant (flower); MT, marigold tissue culture; RE, short ragweed antigen E; RP, short ragweed pollen; RT, short ragweed tissue culture; Sc, rabbit short ragweed pollen serum; and Se, rabbit antigen E serum. Plate wells were 3 mm in diameter and contained approximately 4 µl of suspension culture Fraction D (1 mg/ml) or antigen E (0.06 mg/ml).*

was determined by the method of Lowry *et al.* (7), with crystalline bovine albumin as the reference standard.

Suspension culture cells (1 kg fresh weight) of short ragweed, cantaloupe, marigold, and Ammi tissue cultures were in turn suspended in 0.005 *M* phosphate buffer (pH 7.5, 1.5 liters) and sonified at 90 w for 2 min. The sonified cell suspension was shaken overnight at 200 rpm at 22–23°, and the resulting suspension was centrifuged at 1230×*g* for 10 min. The pH of the collected supernate was adjusted to 7.0 with 3 *N* ammonium hydroxide. The supernate was concentrated by ultrafiltration[10] to about 500 ml and brought to 90% saturation with ammonium sulfate. The precipitated protein was further fractionated to Fraction D, as previously described for short ragweed pollen.

Peeled cantaloupe fruit and marigold flowers (1 kg fresh weight) were homogenized with an appropriate amount of 0.005 *M* phosphate buffer (pH 7.4) to prepare a slurry, and Fraction D was prepared as described for the tissue culture.

Skin Testing—Concentrated Fraction D proteins (anionic proteins) for skin testing were sterilized by passing through sterile filters[11] (0.22-µm pore size). The sterility of the filtered solutions was substantiated by plating on both blood agar and fluid thioglycolate medium and examining the plates for growth after 72 hr of incubation. The allergenic activity of the samples was determined by direct skin testing in ragweed-sensitive and ragweed-nonsensitive persons. A series of 10-fold sequential dilutions of sterile sample fractions in sterile buffered saline allergy diluent[12] was made approximately 12 hr before skin testing. Patients were injected intradermally on the forearm with a 0.02-ml volume of the diluted solutions, and the wheal and flare reactions were read within 20 min after injection. Ragweed-nonsensitive persons served as controls.

Immunodiffusion Analysis—The rabbit anti-short-ragweed-pollen serum and rabbit anti-antigen-E serum used were as pre-

[10] Diaflo.
[11] Millipore Corp., Bedford, Mass.
[12] Hollister-Stier Lab, Downers Grove, Ill.

Table II—Multiliter Growth of Short Ragweed Suspension Cultures

Experiment[a]	Inoculum, g	Aeration, ml/min	Final Weight, g	Growth Index[b]
Fermentor A				
1	100	500	263	2.6
2	100	500	205	2.1
3	100	500	250	2.5
Fermentor B				
1	100	1000	246	2.5
2	100	1000	260	2.6
3	100	1000	252	2.5
Fermentor C				
1	150	1500	640	4.3
2	150	1500	645	4.3
3	150	1500	600	4.0

[a] Experimental conditions: 2.5 liters of $RT_{2.0}$ medium in each fermentor for a 14-day growth period at 25–26° and 80 rpm agitation. [b] Growth index = final fresh weight of tissue/initial fresh weight of inoculum.

viously reported (8). Ouchterlony double-diffusion tests were carried out in 60-mm plastic petri dishes, each containing 5 ml of 1% Noble agar[13]/0.1% sodium azide (9). Antiserum was placed in the center well (3-mm diameter), and antigens were place in the outer wells. After 48–72 hr, precipitin lines were observed and photographed (10).

Disk Electrophoresis[14]—The electrophoretic system used was 7% acrylamide gel (pH 8.9) with a bromophenol blue tracking dye (11). The sample (0.1 ml containing about 0.05 mg of protein) was mixed with 0.1 ml of 20% sucrose, and the mixture was introduced into the gel tubes (7.7 × 0.7 cm). The gels were run at 4 mamp/tube at room temperature until the blue tracking dye had migrated from 2 to 5 mm from the bottom of the gel.

RESULTS AND DISCUSSION

Tissue Culture—Tissue cultures were successfully grown in both solid and liquid revised Murashige and Skoog tobacco medium. Experimental trials suggested that (2,4-dichlorophenoxy)acetic acid in the concentration of 1 ppm was most effective in promoting static culture growth; 0.1 ppm was best for the suspension culture growth of marigold and Ammi. Short ragweed and cantaloupe suspension cultures required 2.0 ppm of (2,4-dichlorophenoxy)acetic acid for growth. The suspension cultures were uniform in appearance, except for cantaloupe suspension culture which aggregated. Among the suspension cultures studied, Ammi had the highest growth efficiency index and cantaloupe the lowest, 3.7 and 1.9, respectively (Table I).

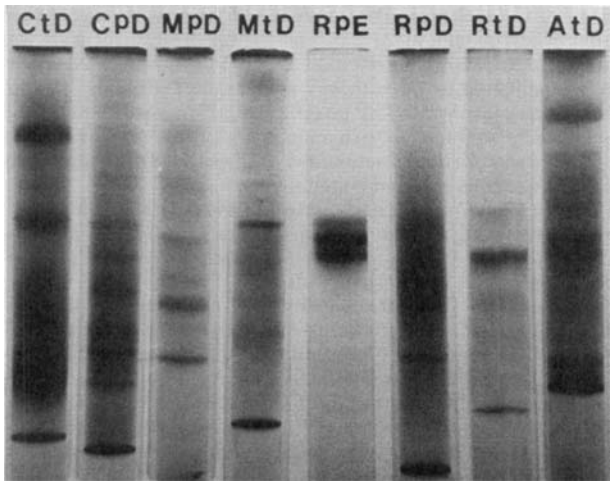

Figure 2—*Polyacrylamide disk electrophoretic patterns of Fraction D from tissue culture and plants. Tracking dye is shown at the top of each disk; disks are aligned from one electrophoretic determination. Key; ctD, cantaloupe tissue culture; CPD, cantaloupe fruit; MPD, marigold flower; MtD, marigold tissue culture; RPE, short ragweed antigen E; RPD, short ragweed pollen; RtD, short ragweed tissue culture; and AtD, Ammi tissue culture.*

[13] Difco Labs, Detroit, Mich.

[14] The reagents and apparatus were purchased from Canalco, Co., Rockville, Md.

Table III—Lowry Protein Content (Milligrams per Gram Fresh Weight) of Plant Tissue and Tissue Cultures

Material	Phosphate Buffer Extract	Dextran Gel Fraction A	Diethylaminoethylcellulose Fraction D
Plant tissue			
Short ragweed pollen	—[a]	—[b]	3.04
Cantaloupe fruit	4.23	0.63	0.21
Marigold flower	9.02	0.93	0.14
Tissue culture			
Short ragweed	2.19	0.57	0.06
Cantaloupe	1.99	0.86	0.07
Marigold	1.45	0.47	0.06
Ammi	2.05	0.21	0.05

[a] 10.53 g of extract/50 g of pollen. [b] 1.15 g of extract/50 g of pollen.

An important factor influencing the growth index is the amount of inoculum. A 50% increase in inoculum (Table II, Experiment C) resulted in a 2.5 increase in the harvested amount. The aeration rate at 500 and 1000 ml/min did not appear to change the growth rate significantly, but the 1500-ml/min rate may have. It is unknown if the growth increase observed resulted from the inoculum size or the aeration rate. The agitation rate was adjusted to a slow value (80 rpm) to avoid shearing the plant cells. Because low agitation and aeration rates were used, no serious foaming problem was encountered.

Short ragweed suspension culture was grown in multiliter fermentors for 14 days. The growth indexes ranged from 2.1 to 4.3 (Table II).

Isolation of Antigens and Skin Testing—The protein content of plant tissues and plant tissue culture fractions is shown in Table III. Fraction D portions of all tissue cultures contained approximately the same amount of protein. In general, the Fraction D protein content of tissue cultures was lower in quantity as compared to that of natural plant sources. Cantaloupe tissue culture had the highest soluble protein content among all tissue cultures examined, whereas marigold had the lowest.

Skin testing of ragweed pollen, plant, and tissue culture Fraction D was performed on three ragweed-sensitive and four ragweed-nonsensitive persons. The results, expressed in allergenic specific activities, are shown in Table IV. All fractions elicited sensitization reactions on two ragweed-sensitive persons (S.R. and T.M.). The third ragweed-sensitive person (K.S.) responded only

Table IV—Allergenic Activities of Tissue Culture and Plant Extracts, Fraction D, in Ragweed-Sensitive Persons

Material	Specific Activity[a], units/mg		
	Subject S.R.	Subject T.M.	Subject K.S.
Plant extracts			
Ragweed pollen	5×10^{10}	5×10^{7}	5×10^{4}
Marigold flower	5×10^{8}	5×10^{3}	(—)
Cantaloupe fruit	5×10^{9}	5×10^{4}	(—)
Tissue culture extracts			
Ragweed	5×10^{8}	5×10^{2}	(—)
Marigold	5×10^{6}	5×10^{2}	(—)
Cantaloupe	5×10^{8}	5×10^{3}	(—)
Ammi	5×10^{8}	5×10^{5}	5×10^{2}

[a] Specific activity is the reciprocal of the minimum quantity of intradermally injected protein fraction that elicits a minimum reaction in ragweed-sensitive persons (12). Four ragweed-nonsensitive individuals were tested and reacted negatively to both plant and tissue culture extracts.

to ragweed pollen and Ammi tissue culture. The response to the latter was low (5×10^2 units/mg of specific activity). Plant extract fractions generally elicited a stronger allergenic reaction than tissue culture fractions.

Of all of the fractions tested, the pollen extract consistently gave the highest allergenic activity. Ragweed-sensitive patients (S.R. and T.M.) reacted consistently to the plant materials and tissue cultures tested, whereas ragweed-nonsensitive individuals did not.

Immunodiffusion and Electrophoresis—Immunodiffusion analysis was performed to observe if Fraction D from plants and tissue cultures cross-reacted with short ragweed pollen and ragweed antigen E. No precipitin lines were observed between plant and tissue culture Fraction D and anti-antigen E serum (Fig. 1). None of the fractions tested, except ragweed pollen, contained allergens that have a total identity or partial identity with antigen E. Several fractions from short ragweed tissue cultures, Ammi tissue culture, marigold tissue culture, and marigold plant (weak) did have precipitin lines against anti-short-ragweed-pollen serum. It is possible that those fractions contained either other ragweed allergens, such as antigen K, Ra_3, or common proteins, *e.g.*, enzymes. It is presently unknown if the hypersensitivity reactions observed are specific reactions to Fraction D or a general hypersensitivity toward proteins that is peculiarly demonstrated by ragweed-sensitive patients. There also is some evidence that Fraction C from both plants and tissue cultures may contain allergens.

Polyacrylamide gel electrophoresis was used to determine if the plant and tissue culture allergenic fractions contained proteins with similar electrophoretic characteristics as antigen E. All plant and tissue culture fractions had a protein band in the antigen E region (Fig. 2). Ammi tissue culture, cantaloupe tisue culture, and short ragweed tissue culture had strong bands. The proteins in this region are responsible for the high allergenic activity in skin testing. However, further work is required to examine the proteins and allergenic properties of the protein band observed.

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Single-Tablet Enantiomeric Purity Assay of Amphetamine by Rotation Enhancement

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Abstract □ Enhancement of the optical rotation of dextroamphetamine by production of an optically active chromophore through reaction with 1-fluoro-2,4-dinitrobenzene to give α-methyl-*N*-(2,4-dinitrophenyl)-β-phenylethylamine is reported. This reaction forms the basis of an assay for both the content and optical purity of dextroamphetamine sulfate at single-dose levels (5 mg). Results of assays of standard solutions and of commercial tablets demonstrate the suitability of the method for the determination of enantiomeric purity and amphetamine content.

Keyphrases □ Amphetamine—colorimetric analysis, optical purity assay, commercial tablets □ Colorimetry—analysis, amphetamine, commercial tablets

The USP XIX (1) isomeric purity[1] test for dextroamphetamine sulfate (I) requires separation of the amphetamine from dosage excipients and conversion to *N*-acetylamphetamine, followed by isolation, purification, and determination of the specific rotation of the derivative. This method suffers from low sensitivity; 130 mg of the drug is specified for the test, although amphetamine sulfate is commonly formulated in 5- or 10-mg dosage units. Some very sensitive tests (2, 3), which depend on physical examination of crystalline derivatives, readily distinguish *d*- from *dl*- and *l*-amphetamines at milligram levels, but they cannot be used for the determination of enantiomeric purity[1].

Although several NMR methods for the determination of enantiomeric composition were reported re-

[1] Isomeric purity, also called enantiomeric purity, is a measure of the percent of one enantiomer. Optical purity is the percent of the specific rotation of the pure enantiomer. The relationship between them is given in Eqs. 2 and 3.