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Biosynthesis of the Macrocyclic Diterpene Casbene in Castor Bean (*Ricinus communis* L.) Seedlings[1]

CHANGES IN ENZYME LEVELS INDUCED BY FUNGAL INFECTION AND INTRACELLULAR LOCALIZATION OF THE PATHWAY

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ABSTRACT

Casbene is a macrocyclic diterpene hydrocarbon that is produced in young castor bean (*Ricinus communis* L.) seedlings after they are exposed to *Rhizopus stolonifer* or other fungi. The activities of enzymes that participate in casbene biosynthesis were measured in cell-free extracts of 67-hour castor bean seedlings (a) that had been exposed to *R. stolonifer* spores 18 hours prior to the preparation of extracts, and (b) that were maintained under aseptic conditions throughout. Activity for the conversion of mevalonate to isopentenyl pyrophosphate does not change significantly after infection. On the other hand, the activities of farnesyl pyrophosphate synthetase (geranyl transferase), geranylgeranyl pyrophosphate synthetase (farnesyl transferase), and casbene synthetase are all substantially greater in infected tissues in comparison with control seedlings maintained under sterile conditions. The subcellular localization of these enzymes of casbene biosynthesis was investigated in preparations of microsomes, mitochondria, glyoxysomes, and proplastids that were resolved by centrifugation in linear and step sucrose density gradients of homogenates of castor bean endosperm tissue from both infected and sterile castor bean seedlings. Isopentenyl pyrophosphate isomerase and geranyl transferase activities are associated with proplastids from both infected and sterile seedlings. Significant levels of farnesyl transferase and casbene synthetase are found only in association with the proplastids of infected tissues and not in the proplastids of sterile tissues. From these results, it appears that at least the last two steps of casbene biosynthesis, geranylgeranyl pyrophosphate synthetase and casbene synthetase, are induced during the process of infection, and that the enzymes responsible for the conversion of isopentenyl pyrophosphate to casbene are localized in proplastids.

The phytoalexin concept was developed to explain the observed resistance of plants to infections by potentially pathogenic microorganisms. Phytoalexins have recently been defined as low mol wt, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms (23). Isoflavonoids and terpenoids have frequently been identified as phytoalexins, although substances of other types have also been found to serve this role.

Cell free extracts of castor bean seedlings are capable of producing at least five identifiable diterpene hydrocarbons from either MVA[4] or GGPP (25, 26), one of which is the macrocyclic diterpene casbene. Sitton and West (28) have proposed that casbene serves the castor bean plant as a phytoalexin based upon (a) the increased level of its production in the seedling after infection by *Rhizopus stolonifer* or other fungi and (b) its antibiotic properties. The radial growth of *Aspergillus niger* on agar plates was significantly inhibited by 10 to 20 μg of casbene per ml. Casbene biosynthesis in castor bean seedlings is elicited by treatments with the *R. stolonifer* extracellular enzyme, endopolygalacturonase (16), or pectic fragments released from the plant cell wall by the action of this enzyme (3).

Increases in the activities of enzymes responsible for phytoalexin biosynthesis in response to fungal or elicitor challenges have been documented in a number of cases. Particularly investigated in this regard have been enzymes catalyzing several of the early steps of isoflavonoid phytoalexin biosynthesis—phenylalanine ammonia lyase, 4-coumarate:CoA ligase, and chalcone synthase. One or more of these enzymes has been shown to increase substantially after elicitation of glyceollin biosynthesis in soybeans (1, 12, 31), phaseolin biosynthesis in bean (15), and pisatin biosynthesis in pea (17). Also, a 4- to 8-fold increase in the overall enzymic activities responsible for the conversion of MVA to IPP was detected in conjunction with the biosynthesis of the sesquiterpenoid phytoalexin, ipomeamarone, that occurs during infection of sweet potato with the fungus, *Ceratocystis fimbriata* (20).

The subcellular localization in organelles of pathway enzymes could be of importance in the regulation of production of a stress metabolite such as casbene. Compartmentation as a means of regulation of isoprenoid pathways was first proposed by Goodwin and his associates as a consequence of their studies of carotenogenesis (9).

Early studies of diterpene biosynthesis in cell-free extracts of castor bean seedlings demonstrated that MVA and GGPP were precursors of casbene (25, 26). The postulated intermediates and enzymes involved in the biosynthesis of casbene from MVA are illustrated in Figure 1. Two geranyl transferase isoenzymes (11), farnesyl transferase (6), and casbene synthetase (7) have been partially purified from castor bean seedling extracts and some of their characteristics have been determined.

This paper describes the changes observed in the activities of

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[4] Abbreviations: MVA, mevalonate; MVAP, mevalonate-5-phosphate; MVAPP, mevalonate-5-pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

the prenyl transferases, casbene synthetase and other enzymes involved in casbene biosynthesis in castor bean seedlings that have been exposed to *R. stolonifer*, and the localization of these enzymes in subcellular compartments.

MATERIALS AND METHODS

Materials. The following were purchased from Sigma Chemical Co.: Tris, piperazine, Tricine, 2-mercaptoethanol, the monobarium salt of DL-glyceraldehyde-3-phosphoric acid diethyl acetal, NADH, cytochrome *c*, ATP, and 2-glycerophosphate dehydrogenase. Thin layer silica gel plates were from E. Merck Laboratories; 2-[^{14}C]mevalonic acid (13.0 and 22.0 mCi/mmol) was from Amersham Searle. QAE Sephadex A-50 and DEAE Sephadex A-25 were purchased from Pharmacia. Geranyl pyrophosphate and dimethylallyl pyrophosphate were gifts from Dr. George Popjak. Farnesyl pyrophosphate and [^{14}C]isopentenyl pyrophosphate were prepared as described elsewhere (6). The castor beans were of an unknown variety obtained from plants grown in the botanical gardens at UCLA.

Analytical Measurements. Sucrose concentrations were measured with a Bausch and Lomb refractometer. Protein concentrations were determined with the Bradford dye-binding assay (2). Samples to be assayed for radioactivity were transferred to 10 ml of toluene-based scintillation fluid for measurement in a Packard 2008 Liquid Scintillation Spectrometer. Counting rates were corrected for background counting rates and converted to disintegrations per minute from measured counting efficiencies in order to calculate the amounts of radioactive product present.

Seed Preparation and Growth. Castor bean seeds were freed of their seed coats, sterilized in 0.02% NaOCl (w/v) for 1.5 min, washed thoroughly in sterile water, and germinated on moist cheesecloth and two layers of filter paper (Whatman No. 1) in sterile crystallizing dishes (19 cm diameter) covered by aluminum foil. No more than 100 seeds were placed in a dish. Growth took place at 30°C in the dark for a total of 67 h.

Intentional infection of the germinating seedlings, when required, was by the addition of a *Rhizopus stolonifer* spore suspension at the desired time prior to the 67th h of germination. The *R. stolonifer* strain employed was maintained on potato-dextrose agar plates (28). A suspension of spores (5×10^5/ml) was prepared as described by Stekoll and West (30). Forty ml of this suspension in combination with 40 ml of a potato-dextrose solution (2% dextrose, 0.4% potato extract; w/v) were added to a sterile crystallizing dish prepared as described above. The germinating seedlings were transferred to the dish containing the spore suspension and the incubation was continued until the 67th h was reached. Infection times were normally 16 to 20 h.

Preparation of Cell-Free Extracts for the Conversion of MVA to IPP and Product Measurement. Cell-free extracts were prepared by mixing the germinated seedlings with one-third of their fresh weight of Polyclar AT and 2.5 ml of homogenization medium per g fresh weight of seedlings (25). The homogenization medium consisted of 50 mM Tris-maleate (pH 7.0), 4 mM $MgCl_2$, 2 mM $MnCl_2$, and 10 mM 2-mercaptoethanol. This suspension was ground at top speed in a Waring Blendor for 30 s at 4°C. The homogenate was then squeezed through eight layers of cheesecloth and the resulting suspension was centrifuged at 27,000*g* for 15 min. The supernatant fraction, minus the floating lipid layer, was centrifuged at 150,000*g* for 1 h. The supernatant fraction from the second centrifugation was utilized as the source of enzyme activity.

The assay mixture for examining the conversion of MVA to IPP contained in a total volume of 0.5 ml: 50 mM Tris-maleate (pH 7.0), 5 mM iodoacetamide, 4 mM $MgCl_2$, 2 mM $MnCl_2$, 4 mM ATP, 11 μM RS-[2-^{14}C]MVA (22 Ci/mol) and 50 to 150 μl of enzyme (200–500 μg protein/ml). Preliminary studies indicated that the reaction rate was linear with this range of enzyme concentrations for 5 min at 30°C. Reactions were stopped after 5 min by the addition of 0.5 ml of acetone.

To determine if 5 mM iodoacetamide was sufficient to inhibit the IPP isomerase and hence the conversion of IPP to DMAPP and higher mol wt products, the following assay was performed. Duplicate reaction sets were prepared as described above and stopped after 5 min of incubation by the addition of 0.5 ml of acetone. One-half ml of 6 N HCl was added to one of the duplicate sets and the incubation was allowed to continue an additional 15 min at 30°C to hydrolyze any acid-labile pyrophosphates present. Any mevalonolactone formed during this acid treatment was hydrolyzed by the addition of 0.5 ml of 10 N KOH. The alcohols resulting from the hydrolysis of allyl pyrophosphates in the acidic solution were then extracted into 3 to 4 ml of petroleum ether, and the extract was transferred to scintillation fluid for the measurement of its radioactivity. These assays indicated that essentially no isoprenoid products beyond IPP were formed under these incubation conditions.

After the reaction was stopped, the acetone was removed from the other set under a stream of N_2 and 30 μl of the reaction mixture was applied to a 3 × 46 cm Whatman No. 2 paper strip. The chromatogram was developed by descending chromatography in a solvent system composed of 1-butanol:HCOOH:water (77:10:33) (v/v/v) (8). The solvent system was freshly prepared each time and the tank was presaturated with the developing solvent. In this system MVA, MVAP, MVAPP, and IPP had R_F values of 0.7 to 0.77, 0.33 to 0.36, 0.01 to 0.06 and 0.17 to 0.2, respectively. After development, the paper strip was air-dried and scanned with a Packard model 7201 radiochromatogram scanner to locate the peaks of radioactivity. A series of 1 cm strips through each peak were assayed for radioactivity by liquid scintillation spectrometry. A unit of activity is defined as the conversion of 1 nmol of MVA to IPP/min.

Estimation of Geranyl Transferase Activity. Geranyl transferase was purified from infected and sterile seedlings by $(NH_4)_2SO_4$ fractionation and QAE Sephadex A-50 chromatography as described by Green and West (11). The geranyl transferase assay was identical to the farnesyl transferase assay described below except that either GPP or DMAPP was employed as the prenyl pyrophosphate substrate, 40 mM Tris maleate (pH 6.8) was employed as the buffer, and no glycerol was present. GPP was routinely employed as the prenyl pyrophosphate substrate, except with assays of lysed organelle preparations, in which case DMAPP was utilized. Quantitation of geranyl transferase activity was performed only after the ion exchange column purification step.

Estimation of Farnesyl Transferase Activity. Whole seedlings were mixed in a Waring Blendor with 150 ml of extraction medium consisting of 50 mM sodium phosphate (pH 6.3), 4 mM $MgCl_2$ and 5 mM 2-mercaptoethanol per 100 seedlings. Homogenization was carried out at half-speed for 5 s and at full speed for 30 s. After pressing the crude slurry through eight layers of cheesecloth, the crude homogenate was centrifuged at 27,000*g* for 10 min. The floating lipid layer was removed by passing the cell-free supernatant fraction through four layers of cheesecloth. The 27,000*g* supernatant was subjected to $(NH_4)_2SO_4$ fractionation. Material precipitating between 40 and 60% saturation with respect to $(NH_4)_2SO_4$ (F_{40}^{60}) was collected for further purification. The F_{40}^{60} pellet was resuspended immediately in 2 ml of the sievorptive buffer consisting of 10 mM piperazine HCl (pH 6.0) 5 mM 2-mercaptoethanol, 4 mM $MgCl_2$, 25% (v/v) glycerol, and 0.02% NaN_3 and subjected to sievorptive chromatography (13) on a 5 × 10-cm column of DEAE A-25 Sephadex equilibrated in this same buffer. An intrinsic gradient was generated with 0.2 M $(NH_4)_2SO_4$, pumped through the column by upward flow at 350 ml/h using a peristaltic pump. The gradient developed on the column was monitored by measuring the conductivity of the

effluent. Six-ml fractions were collected and assayed for transferase activity.

Farnesyl transferase activity was measured with [4-^{14}C]IPP and FPP as substrates. Routine assays were carried out under the following conditions: 0.1 ml of enzyme sample was present in a total volume of 0.5 ml of solution containing 4 mM $MgCl_2$, 25% (v/v) glycerol, 4 μM [4-^{14}C]IPP, 40 μM FPP, and 100 mM Tris chloride (pH 8.5). All components except [^{14}C]IPP were mixed together and equilibrated for 3 min at 30°C. Assays were initiated by the addition of 10 μl of [^{14}C]IPP and stopped 30 min later by the addition of 0.5 ml of absolute ethanol. Prenyl pyrophosphates were hydrolyzed to their corresponding free alcohols by adding 0.5 ml of 6 N HCl and allowing this acidified assay mixture to incubate for an additional 15 min at 30°C. The free alcohols were extracted in a total of 3 to 4 ml of petroleum ether and the extracts were assayed for radioactivity by liquid scintillation spectrometry.

Since the actions of IPP isomerase and phosphatases and background levels of [^{14}C]IPP extracted into the petroleum ether are all sources of error in estimating the true transferase activity, control assays were carried out in the absence of FPP. Transferase activity was taken as the difference between experimental and control assays. One unit of enzyme activity is defined as that amount required to incorporate 1 nmol of [^{14}C]IPP per min into GGPP at 30°C.

Estimation of Casbene Synthetase Activity. Casbene synthetase activity was measured in the crude 27,000*g* or 37,000*g* supernatant fraction with GGPP as the substrate under assay conditions previously described (7).

Preparation of Cellular Organelles from Castor Bean Endosperm. Organelles were isolated from the endosperm of developing castor bean seedlings generally as described by Cooper and Beevers (5). Endosperm tissue was carefully removed from the germinated seedlings, and the rest of the seedling, including cotyledons, was discarded. All subsequent operations were carried out at temperatures below 4°C. Twenty endosperm halves were suspended in 10 ml of grinding medium and chopped with a single edge razor for 7 min or until the final size of the endosperm pieces was 1 to 2 mm. The grinding medium consisted of 20% (w/w) sucrose, 150 mM Tris chloride (pH 7.5), 1 mM EDTA, and 1 mM $MgCl_2$. The homogenate was filtered through Miracloth and centrifuged at 270*g* for 10 min. Four ml of the supernatant suspension, applied directly to the linear gradient, were used as a source of mitochondria, glyoxysomes, and microsomes. The pellet from the low speed centrifugation was resuspended in 4 ml of the grinding medium with the gentle use of a glass rod. This resuspended fraction, which is enriched in proplastids, was applied to the top of the step gradient.

Both the linear and the step sucrose gradients were prepared from a 66% (w/w) sucrose stock solution. The sucrose stock solution, as well as the dilution buffer, contained 100 mM Tris chloride (pH 7.5), 10 mM KCl, 1 mM EDTA, and 1 mM $MgCl_2$. Solutions containing the desired concentrations of sucrose were prepared by mixing the sucrose stock solution and the dilution buffer. All sucrose solutions were prepared on a weight per weight basis. The linear gradient was prepared from 15 ml each of 20 and 60% sucrose with the aid of a gradient maker (60-ml capacity) and layered on a cushion of 2 ml of 60% sucrose. The step gradient was prepared by layering successively in a 40 ml polyallomer centrifuge tube the following solutions: 3 ml of 60%, 6 ml of 57%, 9.5 ml of 50%, 9.5 ml of 44%, and 4 ml of 33% sucrose. This gradient was used immediately after preparation.

Both types of gradients were subjected to centrifugation for 3 h at 20,000 rpm in a SW-27 rotor at 3°C in a Beckman model L5-65 Ultracentrifuge for equilibrium sedimentation. The gradient was fractionated immediately after the conclusion of the centrifugation from the top using an Isco model 640 Density Gradient Fractionator. The fraction size collected was 1.2 ml.

Velocity sedimentation in a linear sucrose gradient as described by Miflin and Beevers (19) was modified in this work to achieve a nearly complete resolution of proplastids and glyoxysomes. Four ml of the resuspended pellet resulting from the 270*g* centrifugation of the homogenate was layered onto a 20 to 60% (w/w) linear sucrose gradient prepared from 14 ml each of 20 and 60% sucrose layered onto a 2 ml cushion of 60% sucrose, followed by a buffer of 2 ml of 20% sucrose layered on top of the gradient. Centrifugation in an SW-27 rotor was as follows: the rotor was accelerated at a low rate to 1,000 rpm, followed by a rapid acceleration over 2 min to a speed of 10,000 rpm. The speed was maintained at 10,000 rpm for 5 min, followed by braking to a stop.

Marker Enzyme Assay Procedures. The marker enzymes for mitochondria, glyoxysomes, microsomes, and proplastids were fumarase, catalase, NADH-Cyt *c* reductase and triose-P isomerase, respectively. Spectrophotometric assays carried out with a Varian Tecktron model 620 Spectrometer were employed in each case. Fumarase was measured from the rate of increase in A_{240} as an indication of the rate of conversion of malate to fumarate. Catalase was assayed from the rate of decrease of A_{240} due to the disappearance of H_2O_2. NADH-Cyt *c* reductase was determined by following the rate of reduction of Cyt *c* at 550 nm. To distinguish between microsomal and mitochondrial NADH-Cyt *c* reductase, the fractions thought to contain mitochondria were reassayed in the presence of 10 μM antimycin A, a specific inhibitor of mitochondrial NADH-Cyt *c* reductase. Triose-P isomerase was measured with a coupled assay system in the presence of added 2-glycerophosphate dehydrogenase. The decrease in A_{340} was followed as a measure of NADH oxidation by the glycerophosphate produced from added glyceraldehyde-3-P through the action of the isomerase.

Hydroxylapatite Chromatography. It was necessary to use hydroxylapatite chromatography with extracts of mitochondrial fractions to separate IPP isomerase activity from the geranyl transferase so the latter could be measured more accurately. Mitochondrial fractions were quick-frozen in liquid N_2, thawed, and diluted 3-fold with the hydroxylapatite equilibration buffer which was 1 mM sodium phosphate (pH 6.0). These lysed suspensions were then applied to a 1.6 × 4 cm column of hydroxylapatite. Elution of the isomerase was with 40 ml of 20 mM sodium phosphate (pH 6) and elution of the transferase was with 40 ml of 100 mM sodium phosphate (pH 6).

RESULTS

Influence of Fungal Infection on the Activities of Enzymes of Casbene Biosynthesis. The activities of enzymes catalyzing reactions of the biosynthetic pathway illustrated in Figure 1 were measured in extracts prepared from seedlings that were maintained under aseptic conditions throughout the germination period and, for comparison, from seedlings that were intentionally exposed to *Rhizopus stolonifer* during the final 18 h of the germination period.

The amounts of MVAP, MVAPP, and IPP produced in the 150,000*g* supernatant fraction from MVA in the presence of sufficient iodoacetamide to inhibit the further metabolism of IPP were determined for extracts of seedlings maintained under aseptic conditions throughout the germination period. The levels of products found at the end of a 5 min incubation period were (in nmol of product per g fresh weight): MVAP, 2.73; MVAPP, 0.36; and IPP, 24.2. The corresponding values from the same experiment performed with the supernatant fraction derived from seedlings that had been infected with *R. stolonifer* spores were not significantly different: MVAP, 3.10; MVAPP, 0.43; and IPP, 23.2. The small pool sizes of MVAP and MVAPP indicate that IPP is being produced rapidly from these intermediates.

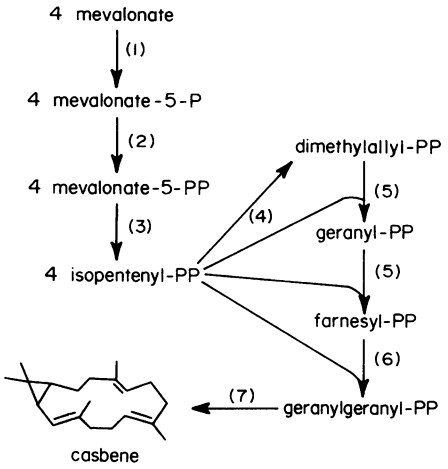

FIG. 1. Biosynthetic pathway from mevalonate to casbene. Enzymes involved are: (1) mevalonate kinase (EC 2.7.1.36); (2) mevalonate-5-P kinase (EC 2.7.4.2); (3) mevalonate-5-pyrophosphate decarboxylase (EC 4.1.1.33); (4) isopentenyl pyrophosphate Δ^3-Δ^2 isomerase (EC 5.3.3.2); (5) farnesyl pyrophosphate synthetase (geranyl transferase or geranyl pyrophosphate: isopentenyl pyrophosphate geranyl transferase) (EC 2.5.1.1); (6) geranylgeranyl pyrophosphate synthetase (farnesyl transferase or farnesyl pyrophosphate: isopentenyl pyrophosphate farnesyl transferase); (7) casbene synthetase.

Table I. *Comparison of Total Enzyme Activities from Infected and Sterile Seedlings*

The preparation of cell-free extracts for assay and the assay procedures are described in "Materials and Methods." The geranyl transferase activity reported is based upon recoveries from the sterile and 18-h infected seedlings after QAE A-50 chromatography. The amount of farnesyl transferase activity represents the yield from the DEAE A-25 sievorptive column chromatography step. The casbene synthetase activities reported were determined by Dueber *et al.* (7) from 37,000*g* supernatant fractions. The unit of activity in each case is defined as the conversion of 1 nmol of substrate to product per min under the assay conditions described in "Materials and Methods."

	Enzyme Activities		
	Sterile (a)	Infected (b)	(b)–(a)
	units/100 seedlings		
Conversion of MVA to IPP	19.2	18.5	
Geranyl transferase	12.3	21.8	9.5
Farnesyl transferase	0.23	5.6	5.37
Casbene synthetase	0	166	166

Therefore, the ability of seedling extracts to utilize MVA can be estimated from the rate of accumulation of IPP alone.

The relative activities for the conversion of MVA to IPP, and for geranyl transferase, farnesyl transferase and casbene synthetase in extracts of sterile and infected seedlings are summarized in Table I. It can be seen from these results that the capacity of seedlings to convert MVA to IPP remains essentially unchanged by the stress of infection. There is significant activity of geranyl transferase present in uninfected seedlings and about a 2-fold increase over this basal level is seen in infected tissues. The increase in the geranyl transferase activity upon infection is sufficient to supply substrate for the levels of farnesyl transferase found in infected tissues. In untreated seedlings there is only a low amount of farnesyl transferase activity, whereas infection causes a 25- to 30-fold increase. As anticipated, casbene synthetase activity appears only upon infection of the castor bean seedlings. The total activity of casbene synthetase in infected seedlings appears from these results to exceed by far the ability of farnesyl transferase to produce GGPP.

It was shown previously that extracts of castor bean seedlings yielded two forms of geranyl transferase activity (geranyl transferase I and II) from a QAE A-50 Sephadex column (11). This observation was confirmed with extracts of 18-h infected seedlings as a source of enzymes (Fig. 2). However, extracts of sterile seedlings prepared under the same conditions gave only one peak of enzyme activity (Fig. 3). Unfortunately, at the present time no properties that distinguish geranyl transferase I from II are known other than their different elution patterns from the ion exchange column. The relationship of the geranyl transferase activity from seedlings maintained under sterile conditions to the two forms in seedlings infected with fungus remains uncertain.

Separation of Organelles in Linear- and Step-Sucrose Gradients. The distributions of marker enzymes observed in successive fractions collected from the top after centrifugation in a linear 20 to 60% sucrose gradient and from fractions isolated

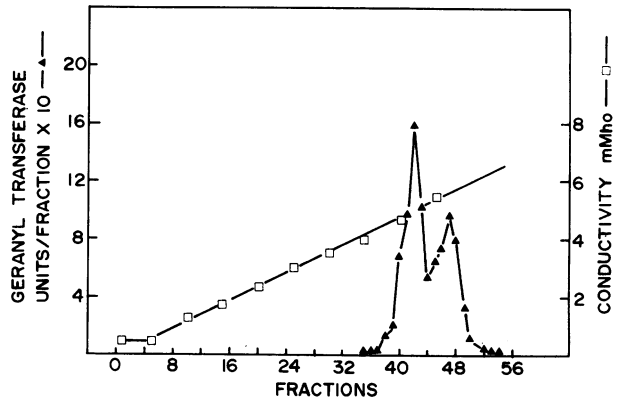

FIG. 2. QAE A-50 Sephadex chromatography of partially purified geranyl transferase from 18-h infected seedlings. The enzyme was prepared as described by Green and West (11). The column was equlibrated in 5 mM citrate (pH 5.0), 1 mM sodium pyrophosphate, and 1 mM 2-mercaptoethanol. The column was developed with 600 ml of a 0 to 0.2 M KCl linear gradient. Approximately 4-ml fractions were collected. Geranyl transferase activity (▲) was determined as described in "Materials and Methods." One unit of enzyme is the amount that catalyzes the formation of 1 nmol of FPP per min under the assay conditions described.

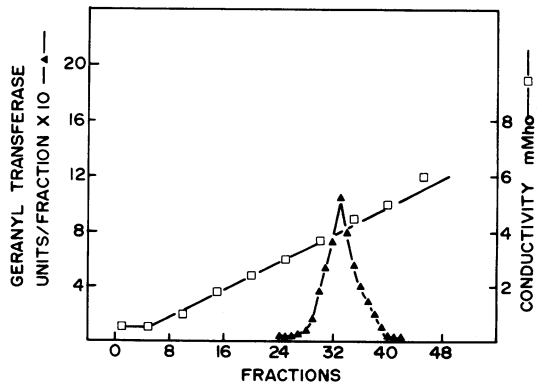

FIG. 3. QAE A-50 Sephadex chromatography of partially purified geranyl transferase from sterile seedlings. The enzyme was prepared as described by Green and West (11). The column was equilibrated in 5 mM citrate (pH 5.0), 1 mM sodium pyrophosphate, and 1 mM 2-mercaptoethanol. The column was developed with 600 ml of a 0 to 0.2 M KCl linear gradient. Approximately 4-ml fractions were collected. Geranyl transferase activity (▲) was determined as described in "Materials and Methods." One unit of enzyme is the amount that catalyzes the formation of 1 nmol of FPP/min under the assay conditions described.

from the step gradient are shown in Figures 4 and 5, respectively. The distribution of organelles throughout both gradients coincides with that seen previously by Beevers *et al.* (5, 21).

Comparison of Enzyme Activities in Organelles from Sterile and Infected Tissues. Preparations of microsomes, mitochondria, glyoxysomes, and proplastids were obtained for analysis by pooling fractions from the sucrose density gradients in accordance with the distributions of marker enzymes. The organelle preparations from both infected and sterile tissues were assayed for geranyl transferase, farnesyl transferase, and casbene synthetase by the procedures outlined in "Materials and Methods." To make valid comparisons of enzyme levels in organelles of sterile and infected tissues it is necessary to correct for differences in organelle yields from the two types of tissues. It is assumed that sterile and infected tissues initially contain the same complement of organelles per unit weight of tissue. If this is so, the relative yields of an organelle from sterile and infected tissues can be estimated from the relative amounts of the marker enzyme associated with that organelle from the two sources. To correct values to the same organelle yield, the enzyme levels from the organelle preparation where the yield was lowest were multiplied by the ratio of the higher to lower values of marker enzyme recovered for that organelle. These corrected levels were still minimal estimates since some enzyme is doubtless lost due to breakage of organelles during the preparation procedures.

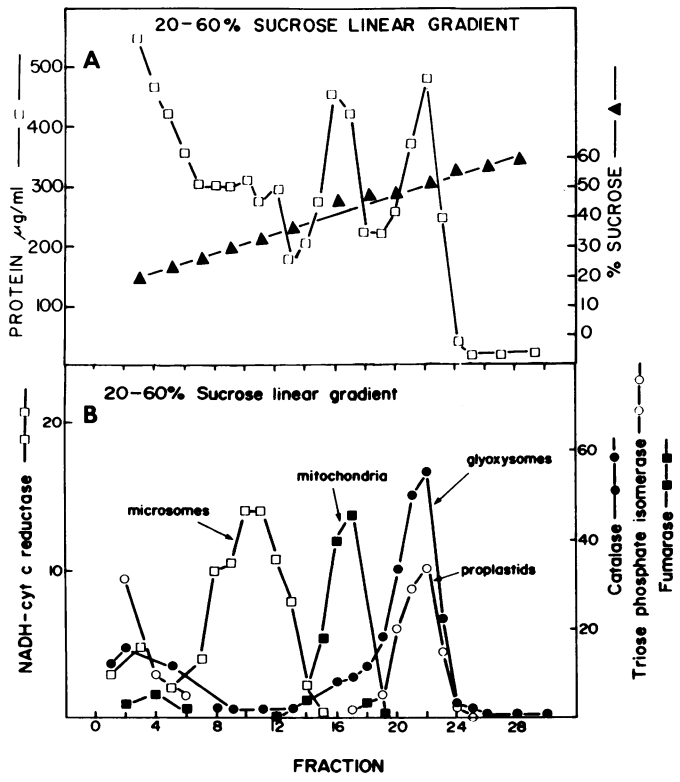

FIG. 4. Equilibrium density gradient separation of organelles from the whole homogenate of endosperm from 3-d-old germinating castor bean seedlings. Endosperm tissue was prepared for analysis in a 20 to 60% linear sucrose gradient as described in "Materials and Methods." A, Protein (□) and sucrose (▲) concentrations and enzyme activities were determined as described in "Materials and Methods." The protein profile was the same for infected (18-h) and sterile seedlings. B, Distribution of the marker enzymes; catalase (●) for glyoxysomes, triose-P isomerase (○) for proplastids, fumarase (■) for mitochondria and NADH:Cyt *c* reductase (□) for microsomes. The gradient is a 20 to 60% linear sucrose gradient. The organelle distribution was the same for infected (18-h) and sterile seedlings.

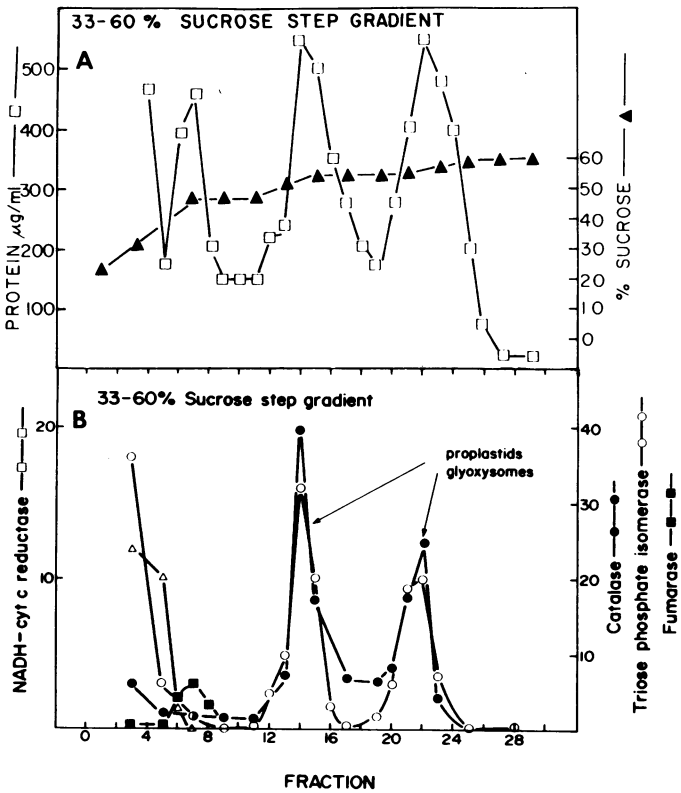

FIG. 5. Equilibrium density gradient separation of organelles from the proplastid-enriched 270*g* pellet of the crude homogenate of endosperm from 3-d-old germinating castor bean seedlings. The 270*g* pellet was prepared for analysis in the step sucrose gradient as described in "Materials and Methods." A, Protein (□) and sucrose (▲) concentrations and enzyme activities were determined as described in "Materials and Methods." The protein profile was the same for infected (18-h) and sterile seedlings. B, Distribution of marker enzymes; catalase (●) for glyoxysomes, triose-P isomerase (○) for proplastids, fumarase (■) for mitochondria and NADH:Cyt *c* reductase (□) for microsomes. The distribution of organelles was the same for 20-h infected and sterile seedlings.

Table II presents a comparison of the relative amounts, corrected as described above, of geranyl transferase, farnesyl transferase, and casbene synthetase observed in mitochondria and proplastids obtained from sterile and infected tissues. None of these enzyme activities were detected in microsomes or glyoxysomes from either sterile or infected tissues, or in mitochondria from sterile tissues. Mitochondria from infected tissues did show geranyl transferase activity, but not farnesyl transferase or casbene synthetase activities. However, the proplastid fraction isolated from infected tissues on a linear gradient appeared to contain all three of these activities. To verify that these activities were actually in the proplastids and not the glyoxysomes, which invariably contaminated the proplastid region of the linear gradient (Fig. 4), the following experiment was performed. The activities of the geranyl transferase and the farnesyl transferase, as well as the organelle marker enzymes for the proplastids and glyoxysomes, triose-P isomerase and catalase, respectively, were determined in (a) the proplastid fraction derived from the linear gradient, and (b) the proplastid fraction derived from the proplastid-enriched 270*g* pellet obtained from a step gradient (Table III). It can be seen that the ratios of activities from the step gradient to those in the linear gradient for geranyl transferase, farnesyl transferase, and triose-P isomerase were about 4 or larger, whereas the catalase activity was at about the same level in both gradients. These results are consistent with the conclusion that the two prenyl transferase activities are localized in the

Table II. *Enzyme Activities from Organelles of Sterile and Infected Endosperm Tissue*

Organelles were isolated from 10 seedlings by sucrose density gradient equilibrium sedimentation as described in "Materials and Methods." Microsomes, mitochondria, and glyoxysomes were isolated from linear gradients, while the proplastids were recovered from step gradients. Infection was by *R. stolonifer* spore suspensions for 18 h as described in "Materials and Methods." Activities were corrected for organelle recoveries as described in the text. One unit of enzyme activity is that amount which catalyzes the formation of 1 nmol of product/min under the conditions of the assay.

Enzyme	Enzyme Activities	
	Mitochondria	Proplastids
	units/10 seedlings × 10^3	
Geranyl transferase		
Sterile	0[a]	11
Infected	13	8
Farnesyl transferase		
Sterile	0	0
Infected	0	83
Casbene synthetase		
Sterile	0	0.07
Infected	0	105
IPP isomerase		
Sterile	0	+[b]
Infected	+	+

[a] 0, Activity not observed. [b] +, Activity observed, but not quantitated.

Table III. *Enzyme Activities from the Proplastid Regions of the Linear and Step Gradients*

The activities of the geranyl and farnesyl transferases and the triose-P isomerase and catalase marker enzymes were determined for (1) the proplastid fraction derived from the whole homogenate from 10 infected seedlings on the linear gradient, and (2) the proplastid fraction derived from the proplastid-enriched 270*g* pellet of the crude homogenate from 10 infected seedlings on the step gradient. Infection was by *R. stolonifer* spore suspensions for 18 h as described in "Materials and Methods." Enzyme assays were as described in "Materials and Methods."

Enzyme	Linear Gradient (1)	Step Gradient (2)	(2)/(1)
			ratio
Geranyl transferase	1.2[a]	5.0[a]	4.2
Farnesyl transferase	2.4[a]	50[a]	21
Triose-P isomerase	12[b]	44[b]	3.7
Catalase	110[b]	100[b]	0.91

[a] Units × 10^3 (nmol of product formed per min). [b] Units (ΔA/min).

proplastids rather than the glyoxysomes of infected tissues. Similar results were obtained in a separate experiment with casbene synthetase (data not shown).

The results of an experiment in which velocity sedimentation in a sucrose gradient was used to separate organelles demonstrated conclusively that casbene synthetase is localized in the proplastids of infected tissues (Fig. 6). Catalase and triose-P isomerase (and hence glyoxysomes and proplastids) are well resolved on this gradient. The profile of casbene synthetase activity coincides closely with that of the proplastid marker enzyme.

Thus it appears that the enzymes necessary for the conversion of IPP to casbene, *i.e.* IPP isomerase, geranyl transferase, farnesyl transferase, and casbene synthetase, are all present in the proplastids of infected tissues. Although the ratio of activities of farnesyl transferase to casbene synthetase is close to one, the amount of geranyl transferase detected was unaccountably low.

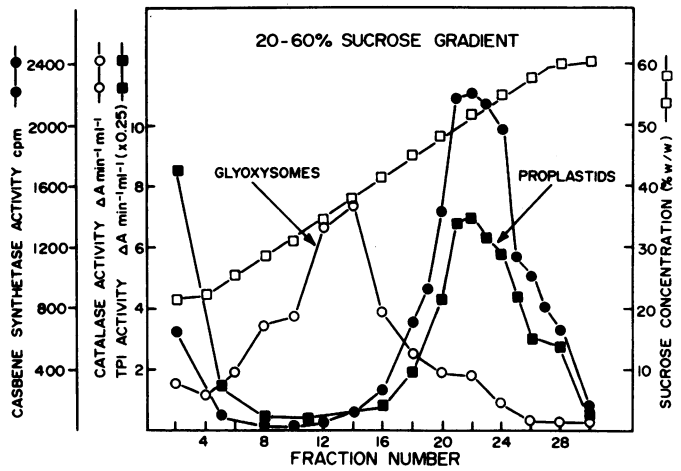

FIG. 6. Velocity sedimentation of casbene synthetase in a linear sucrose density gradient. The resuspended pellet (4.0 ml) from a 270*g* centrifugation of a crude organellar suspension from endosperm tissue of 10 infected seedlings was applied to a linear 20 to 60% sucrose gradient and centrifuged at 10,000 rpm for 5 min as described in "Materials and Methods." Catalase is the marker activity for glyoxysomes and triose-P isomerase (TPI) is the marker activity for proplastids. Sucrose and enzyme activities were assayed as described in "Materials and Methods." Fraction 1 corresponds to the top of the gradient.

Farnesyl transferase activity was not detected in gradients prepared with homogenates from uninfected seedlings (Table II). This result is expected since the results of Table II showed only very low amounts of this enzyme in whole seedling extracts of uninfected tissue. Only a very low level of casbene synthetase was detected in the proplastids from uninfected tissue as well. However, the proplastids of uninfected tissues did contain readily detectable geranyl transferase and IPP isomerase.

DISCUSSION

Extracts of 18-h infected seedlings and control seedlings maintained under aseptic conditions were analyzed for the activities of enzymes of the casbene biosynthetic pathway to determine which had increased after elicitation of casbene synthesis by infection of young seedlings with *Rhizopus stolonifer* spores. When considering the results of these experiments it should be kept in mind that soluble enzymes from various cell compartments are mixed together by the extraction procedure. Also, losses in enzyme activity may occur prior to assay during extraction and processing, and the extent of these losses may differ from one enzyme to another. However, valid comparisons in the activities of a particular enzyme from infected and control tissues should be possible even though meaningful comparisons of the relative activities of different enzymes may be difficult.

Earlier work had demonstrated that casbene synthetase activity increased substantially after elicitation in comparison with the undetectable or very low values found in extracts of uninfected controls (7, 28). The experiments summarized in Table I indicate that elicitation of casbene synthesis in 3-d-old castor bean seedlings results not only in an increase in casbene synthetase activity, but also in the activities of geranyl transferase and farnesyl transferase. Though the overall change in farnesyl transferase activity represents an approximately 25-fold increase and the geranyl transferase activity was only increased about 2-fold, the absolute increase in activity for both enzymes was about the same. The smaller fold-increase in geranyl transferase was due to a higher level of activity in uninfected seedlings that is presumably present to supply FPP for triterpene and sterol synthesis.

The capacity of the castor bean seedling to produce IPP from MVA does not change significantly after infection. This result contrasts with the report by Oba *et al.* (20) that the conversion of MVA to IPP was elevated 4- to 8-fold in sweet potatoes synthesizing the sesquiterpenoid phytoalexin, ipomeamarone, after infection with the fungus *Ceratocystis fimbriata*. The results summarized in Table I suggest that the prenyl transferase activities responsible for the formation of GGPP (steps 5 and 6, Fig. 1) are elicited along with casbene synthetase activity (step 7, Fig. 1) by conditions that stimulate casbene synthesis, whereas the capacity for IPP synthesis (steps 1–3, Fig. 1) is unaffected. IPP isomerase activity (step 4, Fig. 1) was detected in both infected seedlings and sterile controls; however, the level was not quantitated so nothing can be said about changes which may have occurred.

The relative levels of casbene synthetase activity and the prenyl transferase activities reported for infected seedlings in Table I might suggest that the prenyl transferases are the rate limiting enzymes for casbene biosynthesis rather than casbene synthetase. However, it should be borne in mind that the two types of activities were measured at different stages of purification. It was possible to estimate casbene synthetase activity directly in the crude cell free extract, whereas total geranyl and farnesyl transferase activities could not be determined until after both $(NH_4)_2SO_4$ fractionation and an ion exchange chromatography step had been performed. Thus, the low yields of the prenyl transferases may result, at least in part, from losses in enzyme activities brought about during the purification steps. It is not possible to assay reliably the prenyl transferases in crude fractions because of the interferences of phosphatases and, especially, IPP isomerase.

The analysis of enzyme activities associated with organelles isolated from infected castor bean seedlings in a linear sucrose density gradient (Table II) clearly indicates that the terminal steps of casbene biosynthesis catalyzed by farnesyl transferase and casbene synthetase are localized in the proplastid compartment. Although the proplastid fraction was coincident with glyoxysomes in that gradient, the demonstration that an enriched proplastid fraction isolated on a step sucrose gradient was also enriched with respect to the prenyl transferases (Table III) makes it seem likely that these enzymes are associated with the proplastids rather than the contaminating glyoxysomes. This is confirmed by the results of the velocity sedimentation gradient (Fig. 6) that separates glyoxysomes and proplastids and clearly shows casbene synthetase to be associated only with the proplastids. Farnesyl transferase was not detected at all, and casbene synthetase only to a very limited extent, in proplastids isolated from noninfected tissues. On the other hand, geranyl transferase in relatively low amounts was detected in the proplastid fractions from both infected and noninfected tissues. IPP isomerase was likewise present in proplastid fractions from infected and noninfected tissues, although the levels present were not determined. These findings argue for the localization of the enzymes required for the conversion of IPP to casbene in the proplastid compartment. These enzymes were also invariably detected in the fractions at the top of sucrose gradients in which proplastids from infected tissues were sedimented. Presumably these nonsedimentable enzyme activities arose from ruptured proplastids, at least in part, but it is not possible from the evidence at hand to rule out the cytosol as the source of some of these activities.

Although comparison of enzyme activities recovered from organelles must be viewed with some caution, it is interesting that the proplastid fraction from infected tissues contained similar levels of farnesyl transferase and casbene synthetase activities (Table II). This result differs from that seen in extracts of whole seedlings where there was a large disparity in the activities of casbene synthetase and farnesyl transferase recovered (Table I). This supports the suggestion that the higher yields of casbene synthetase activity compared to the prenyl transferase activities in extracts of whole seedlings were due to losses during purification and possible interferences with assays of the prenyl transferases.

The presence of two peaks of geranyl transferase activity from ion exchange columns of extracts of infected seedlings (Fig. 2), while only one was seen in extracts of seedlings maintained under sterile conditions (Fig. 3) led to the initial speculation that the second peak might represent an isoenzyme elicited in the proplastid to provide FPP for casbene biosynthesis. However, the analysis of the enzymes in proplastid fractions has failed to support that speculation. It is evident that more work is required to clarify the relationship of these isoenzymes to casbene biosynthesis.

The appearance of geranyl transferase in mitochondria from infected tissues was not expected, nor is its significance clear. Since neither farnesyl transferase nor casbene synthetase were detected in these mitochondria, it was assumed that the geranyl transferase was not involved with casbene biosynthesis and might instead produce FPP to serve as a precursor of sesquiterpenes and/or triterpenes with a role in disease resistance. However, the results of subsequent investigations by Pargellis (22) do not support this interpretation.

The localization in a plastid compartment of the enzymes involved in the latter stages of casbene biosynthesis is consistent with other reports about the subcellular localizations of isoprenoid biosynthetic pathways in higher plants. Pathways involving the transformation of GGPP to photosynthetic pigments and carotenoids are localized in plastids (4, 29). Also associated with plastids are cyclization enzymes involved in the biosynthesis of *ent*-kaurene and other polycyclic diterpene hydrocarbons (24, 27). An isoprenoid pathway in mitochondria serves to provide the long prenyl side chains required in ubiquinone biosynthesis (18). Some enzymes of sterol synthesis in higher plants are apparently localized in the ER as in other types of organisms (10). Kleinig and his associates (14, 18) have proposed on the basis of their work that IPP synthesized from MVA in the cytosol of plant cells is the precursor for the specialized isoprenoid biosynthetic pathways occurring in the various organelles—GGPP and the various photosynthetic pigments in the chloroplast, ubiquinone in mitochondria, and sterols and glycosyl transfer lipids in the ER. The localization of the enzymes of casbene biosynthesis appears to fit this pattern in that the enzymes for the conversion of IPP to casbene are found in plastids. It seems likely that this compartmentation of the production of such isoprenoid metabolites of specialized function is advantageous from the point of view of regulation of the pathways in response to the need for these products.

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LITERATURE CITED

1. Boerner H, H Grisebach 1982 Enzyme induction in soybean infected by *Phytophthora megasperma* f. sp. *glycinea*. Arch Biochem Biophys 217: 65–71
2. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
3. Bruce RJ, CA West 1982 Elicitation of casbene synthetase activity in castor bean. The role of pectic fragments of the plant cell wall in elicitation by a fungal endopolygalacturonase. Plant Physiol 69: 1181–1188
4. Camara B 1984 Terpenoid metabolism in plastids. Sites of phytoene synthetase activity and synthesis in plant cells. Plant Physiol 74: 112–116
5. Cooper TG, H Beevers 1969 Mitochondria and glyoxysomes from castor bean endosperm. Enzyme constituents and catalytic capacity. J Biol Chem 244: 3507–3513
6. Dudley MW, TR Green, CA West 1986 Biosynthesis of the macrocyclic diterpene casbene in castor bean (*Ricinus communis* L.) seedlings: the

purification and properties of farnesyl transferase from elicited seedlings. Plant Physiol 81: 343–348
7. DUEBER MT, W ADOLF, CA WEST 1978 Biosynthesis of the diterpene phytoalexin casbene: Partial purification and characterization of casbene synthetase from *Ricinus communis*. Plant Physiol 62: 598–603
8. DUGAN RE, E RASSON, JW PORTER 1968 Separation of water-soluble steroid and carotenoid precursors by DEAE-cellulose column chromatography. Anal Biochem 22: 249–259
9. GOODWIN TW 1958 Studies in carotenogenesis. 25. The incorporation of $^{14}CO_2$,[2-^{14}C]acetate and [2-^{14}C]mevalonate into β-carotene by illuminated etiolated maize seedlings. Biochem J 70: 612–617
10. GOODWIN TW 1981 Biosynthesis of plant sterols and other triterpenoids. *In* JW Porter, SL Spurgeon, eds, Biosynthesis of Isoprenoid Compounds, Vol 1. Wiley-Interscience, New York, pp 443–480
11. GREEN TR, CA WEST 1974 Purification and characterization of two forms of geranyl transferase from *Ricinus communis*. Biochemistry 13: 4720–4729
12. HILLE A, C PURWIN, J EBEL 1982 Induction of enzymes of phytoalexin synthesis in cultured soybean cells by an elicitor from *Phytophthora megasperma* f. sp. *glycinea*. Plant Cell Rep 1: 123–127
13. KIRKEGAARD LH 1973 Gradient sievorptive chromatography. A focusing system for the separation of cellular components. Biochemistry 12: 3627–3632
14. KREUZ K, H KLEINIG 1984 Synthesis of prenyl lipids in cells of spinach leaf. Compartmentation of enzymes for formation of isopentenyl diphosphate. Eur J Biochem 141: 531–535
15. LAWTON MA, RA DIXON, K HAHLBROCK, C LAMB 1983 Rapid induction of the synthesis of phenylalanine ammonia-lyase and of chalcone synthase in elicitor-treated plant cells. Eur J Biochem 129: 593–601
16. LEE S-C, CA WEST 1981 Polygalacturonase from *Rhizopus stolonifer*, an elicitor of casbene synthetase activity in castor bean (*Ricinus communis* L.) seedlings. Plant Physiol 67: 633–639
17. LOSCHKE DC, LA HADWIGER, J SCHROEDER, K HAHLBROCK 1981 Effects of light and of *Fusarium solani* on synthesis and activity of phenylalanine ammonialyase in peas. Plant Physiol 68: 680–685
18. LUETKE-BRINKHAUS F, B LIEDVOGEL, H KLEINIG 1984 On the biosynthesis of ubiquinone in plant mitochondria. Eur J Biochem 141: 537–541
19. MIFLIN BJ, H BEEVERS 1974 Isolation of intact plastids from a range of plant tissues. Plant Physiol 53: 870–874
20. OBA K, H TATEMATSU, K YAMASHITO, I URITANI 1976 Induction of furanoterpene production and formation of the enzyme system from mevalonate to isopentenyl pyrophosphate in sweet potato root tissue injured by *Ceratocystis fimbriata* and by toxic chemicals. Plant Physiol 58: 51–56
21. OSMOND CB, T AKAZAWA, H BEEVERS 1975 Localization and properties of ribulose diphosphate carboxylase from castor bean endosperm. Plant Physiol 55: 226–230
22. PARGELLIS CA 1984 Studies of the prenyl transferases from organelles of *Ricinus communis* L. PhD dissertation, University of California, Los Angeles
23. PAXTON JD 1981 Phytoalexins—a working redefinition. Phytopathol Z 101: 106–109
24. RAILTON ID, B FELLOWS, CA WEST 1984 *Ent*-Kaurene synthesis in chloroplasts from higher plants. Phytochemistry 23: 1261–1267
25. ROBINSON DR, CA WEST 1970 Biosynthesis of cyclic diterpenes in extracts from seedlings of *Ricinus communis* L. I. Identification of diterpene hydrocarbons formed from mevalonate. Biochemistry 9: 70–79
26. ROBINSON DR, CA WEST 1970 Biosynthesis of cyclic diterpenes in extracts from seedlings of *Ricinis communis* L. II. Conversion of geranylgeranyl pyrophosphate into diterpene hydrocarbons and partial purification of the cyclization enzymes. Biochemistry 9: 80–89
27. SIMCOX PD, DT DENNIS, CA WEST 1975 Kaurene synthetase from plastids of developing plant tissues. Biochem Biophys Res Commun 66: 166–172
28. SITTON D, CA WEST 1975 Casbene: An antifungal diterpene produced in cell-free extracts of *Ricinis communis* seedlings. Phytochemistry 14: 1921–1925
29. SOLL J, M KEMMERLING, G SCHULTZ 1980 Tocopherol and plastoquinone synthesis in spinach chloroplast subfractions. Arch Biochem Biophys 204: 544–550
30. STEKOLL M, CA WEST 1978 Purification and properties of an elicitor of castor bean phytoalexin from culture filtrates of the fungus *Rhizopus stolonifer*. Plant Physiol 61: 38–45
31. ZAEHRINGER U, J EBEL, H GRISEBACH 1978 Induction of phytoalexin synthesis in soybean. Elicitor-induced increase in enzyme activities of flavonoid biosynthesis and incorporation of mevalonate into glyceollin. Arch Biochem Biophys 188: 450–455