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Evidence from Cell-Free Systems for Differences in the Sterol Biosynthetic Pathway of *Rhizoctonia solani* and *Phytophthora cinnamomi*

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Cell-free preparations of both *Rhizoctonia solani*, a sterol-synthesizing fungus, and *Phytophthora cinnamomi*, a non-sterol-synthesizing fungus, incubated in the presence of [2-^{14}C]mevalonate and iodoacetamide, converted the mevalonate into labelled mevalonate 5-phosphate, mevalonate 5-pyrophosphate and isopentenyl pyrophosphate. In the absence of iodoacetamide, but under anaerobic conditions, the same preparations converted the mevalonate into labelled geraniol, farnesol and squalene, the first two compounds presumably as their pyrophosphates. When cell-free preparations of both organisms were incubated aerobically in the presence of [1-^{14}C]isopentenyl pyrophosphate, only labelled geraniol, farnesol and squalene were recovered from the *P. cinnamomi* reaction mixture, whereas labelled geraniol, farnesol, squalene, squalene epoxide, lanosterol and ergosterol were present in the *R. solani* reaction mixture. When these same preparations were incubated in the presence of ^{14}C-labelled squalene, labelled squalene epoxide, lanosterol and ergosterol were recovered from the *R. solani* reaction mixture. In contrast, the *P. cinnamomi* preparation was unable to convert the squalene into products further along the sterol pathway; instead, a portion of the labelled squalene was converted into water-soluble products, indicating the possible existence of a squalene-degradation process in this organism. It appears that the block in the sterol biosynthetic pathway of *P. cinnamomi* occurs at the level of squalene epoxidation.

The presence or absence of sterols in various groups of fungi has been well documented (Bergmann, 1953; Weete, 1973, 1974). Although this obvious difference in the sterol biosynthetic pathway has been known for a number of years, there have been no detailed studies to determine whether there are differences in the production of earlier components of the pathway in sterol-synthesizing and non-sterol-synthesizing fungi. The results of an investigation to determine the presence or absence of various sterol-pathway compounds in the mycelia of a sterol-synthesizing fungus, *Rhizoctonia solani*, and a non-sterol-synthesizing fungus, *Phytophthora cinnamomi*, were described in the preceding paper (Wood & Gottlieb, 1978). Examination of cell-free preparations of organisms for their ability to enzymically convert precursors into compounds further along the pathway is an important means of determining the extent of a particular pathway in those organisms. The present paper reports evidence of the similarities and differences in various segments of the sterol biosynthetic pathway with cell-free preparations of both *R. solani* and *P. cinnamomi*.

Materials and Methods

General

The materials and methods utilized in both the mycelial and the cell-free phases of the investigation were described in the preceding paper (Wood & Gottlieb, 1978). Only those materials and methods specifically applicable to the cell-free phase of the investigation, and not presented in the preceding paper, are described below.

Chemicals

Enzymes and most co-factors were from Sigma Chemical Co., St. Louis, MO, U.S.A. Nicotinamide and reduced glutathione were from ICN Nutritional Biochemicals Corp., Cleveland, OH, U.S.A. DL-[2-^{14}C]Mevalonic acid lactone (10.9 and 17.5mCi/mmol) and [1-^{14}C]isopentenyl pyrophosphate (ammonium salt; 61.0mCi/mmol) were from Amersham/Searle Corp., Arlington Heights, IL, U.S.A. The mevalonic acid lactone was converted into the acid form by evaporating the benzene solvent, redissolving in water, adjusting to pH11 with 1M-KOH, incubating at room temperature (22.5°C) for about a ½h, and neutralizing with 1M-HCl just before use.

Preparation of cell-free extracts

R. solani and *P. cinnamomi* cultures were grown in glucose/asparagine medium for 2½ days, harvested by suction filtration, and washed with water. Mycelium (5g wet wt.) was suspended in 15ml of 0.1M-potassium

phosphate buffer, pH7.5, containing 30mM-nicotinamide (to inhibit NAD nucleosidase), 5mM-$MgCl_2$ and 2mM-$MnCl_2$. The suspension was homogenized at 4°C in a Sorvall Omni-Mixer homogenizer at high speed for 30s. The homogenized preparation was subjected to further mycelial breakage by passing through a French pressure cell at 138MPa (20000lb/in^2), and then centrifuged at 750*g* for 20min to remove large mycelial fragments. The supernatant was centrifuged at 10000*g* for 30min to remove mitochondria; the 10000*g* supernatant was carefully removed without disturbing the overlying fatty layer or the fluffy surface layer of the pellet. This supernatant, referred to as the S_{10} preparation, contained 5.3mg of protein/ml and all of the sterol-biosynthetic-pathway enzymes that were present in the intact organism.

Conversion of labelled sterol precursors

For the conversion of mevalonic acid into isopentenyl pyrophosphate, the reaction mixtures contained 1.9ml of either *R. solani* or *P. cinnamomi* S_{10} preparation (representing 10mg of protein), 6μmol of ATP, 20μmol of KF (to inhibit adenosine triphosphatase), 10μmol of iodoacetamide (to terminate the reaction at isopentenyl pyrophosphate), and 0.14μmol of [2-^{14}C]mevalonic acid (17.5mCi/mmol) in a total volume of 2.0ml. The reaction tubes were incubated on a Dubnoff metabolic shaker at 37°C for 4h. Reaction mixtures were centrifuged (2000*g* for 5min), and the supernatants analysed directly by scanning thin-layer radiochromatography. The supernatants were then fractionated on Dowex 1 (formate form) columns as described in the Materials and Methods section of the preceding paper (Wood & Gottlieb, 1978); the fractions were monitored for radioactivity described below (under 'Ion-exchange chromatography'), concentrated *in vacuo* on a rotary evaporator, and analysed by scanning thin-layer radiochromatography.

All reaction tubes and flasks described in this section were subjected to the following conditions. The pH of each reaction mixture was adjusted to 7.5 with 1M-KOH if necessary. Water was added, when necessary, to compensate for evaporation during the incubation period. Reactions were terminated by placing the tubes or flasks in a boiling-water bath for 2min. Appropriate boiled (zero-time) control tubes or flasks were run.

For the mevalonic acid-into-squalene and mevalonic acid-into-sterol conversions, the reaction mixtures contained 1.9ml of either *R. solani* or *P. cinnamomi* S_{10} preparation (representing 10mg of protein), 6μmol of ATP, 6μmol of glucose 6-phosphate, 2μmol of NADP, 20μmol of KF and 0.17μmol of [2-^{14}C]mevalonic acid (17.5mCi/mmol) in a total volume of 2.0ml. The reaction tubes were incubated on a Dubnoff metabolic shaker at 37°C for 4h, one set aerobically and another set anaerobically (flushed with N_2 and stoppered with serum-bottle stoppers).

Enzymic hydrolysis of any geranyl pyrophosphate or farnesyl pyrophosphate formed during the reaction was carried out as follows: 0.8ml of 0.1M-glycine/HCl buffer, pH3.0, and a small quantity of crystalline acid phosphatase were added to each reaction tube; the resultant pH was about 4.5; the tubes were incubated in a 37°C-water bath for 1h. The reaction mixtures were extracted with light petroleum (b.p. 30–60°C), and the extracts fractionated on silicic acid columns (40cm×0.5cm; containing 3.5g of silicic acid). Fractions were monitored for radioactivity, and subjected to both g.l.c. and t.l.c. analyses.

To measure the conversion of [1-^{14}C]isopentenyl pyrophosphate into other sterol-pathway compounds, a modification of the procedure described by Kawaguchi *et al.* (1973) was utilized. The reaction mixtures contained 0.1ml of either *R. solani* or *P. cinnamomi* S_{10} preparation (representing 0.5mg of protein), 1μmol of $MgCl_2$, 1μmol of reduced glutathione, 10μmol of glucose 6-phosphate, 0.2μmol of NADP, 1 unit of glucose 6-phosphate dehydrogenase, 10μmol of KF, 50μmol of Tris/HCl buffer, pH7.4, and 3.5nmol of [1-^{14}C]isopentenyl pyrophosphate (61.0mCi/mmol) in a total volume of 1.0ml. The reaction tubes were incubated at 37°C for 2h, one set aerobically and another set anaerobically (flushed with N_2 and stoppered with serum-bottle stoppers). Enzymic hydrolysis of any geranyl pyrophosphate or farnesyl pyrophosphate formed during the incubation was carried out as described above, but using 0.4ml of glycine/HCl buffer per reaction tube.

Before use as a substrate, [^{14}C]squalene was water-solubilized as follows. [^{14}C]Squalene (155nmol; 51.7mCi/mmol) was dissolved in a small volume of acetone, 100μg of Tween 80 was added, and the mixture thoroughly stirred to dissolve the Tween 80; 0.22ml of 0.1M-potassium phosphate buffer, pH7.5, was added, and the mixture again thoroughly stirred to ensure full dissolution of all components; the acetone was evaporated from the solution under a stream of N_2. A 50μl sample of the aqueous solution contained 35nmol of substrate.

The reaction mixtures for the squalene-conversion experiment contained 1.9ml of either *R. solani* or *P. cinnamomi* S_{10} preparation (representing 10mg of protein), 4μmol of NADPH and 35nmol of [^{14}C]-squalene in a total volume of 2.0ml. Erlenmeyer flasks (10ml) were used as reaction vessels to provide the large surface area necessary for adequate aeration during the epoxidation reaction. The flasks were incubated on a Dubnoff metabolic shaker at 37°C for 4h. The reaction mixtures were extracted with light petroleum (b.p. 30–60°C). Both extracts and aqueous phases were monitored for radioactivity. Extracts were analysed by g.l.c. and t.l.c.

T.l.c.

Plastic sheets commercially pre-coated with cellulose (MN-Polygram Cel 300 cellulose, Brinkmann Instruments, Westbury, NY, U.S.A.) were used for t.l.c. separation of mevalonic acid, its phosphorylated derivatives, and isopentenyl pyrophosphate. Chromatograms were developed in Brinkmann Sandwich Chambers using the following solvent systems: butan-1-ol/formic acid/water (77:10:13, by vol.) for the separation of mevalonic acid and 5-phosphomevalonic acid from 5-pyrophosphomevalonic acid and isopentenyl pyrophosphate, but not the latter two from each other; 2-methylpropan-2-ol/formic acid/water (20:5:8, by vol.) for the separation of mevalonic acid and its pyrophosphate from the monophosphate and isopentenyl pyrophosphate, but not the latter two from each other. Samples were generally spotted in lanes on duplicate chromatogram sheets; one sheet was developed in one solvent system and the duplicate sheet in the other solvent system. The chromatograms were then cut into appropriate strips and analysed with the radiochromatogram scanner.

Ion-exchange chromatography

Reaction mixtures containing radioactive mevalonic acid, its phosphorylated derivatives, and/or isopentenyl pyrophosphate were subjected to ion-exchange chromatographic analysis on columns (0.6cm internal diam.) containing Dowex 1 (X10; 200–400 mesh spheres, Cl^- form; J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A.) that had been converted into the formate form by the procedure outlined by Busch (1957). Sufficient Dowex 1 (10ml packed wet vol.) was present in each column to provide at least a 10-fold excess of exchangeable milliequivalents over those in the reaction mixture. The reaction mixture was layered on the surface of the resin, followed by enough water to flush through any components not adhering to the resin. Scintillation counter monitoring of occasional drops during the wash and subsequent elutions was used to determine the completion volume of a given fraction, that is, when radioactivity was no longer being released by the column. Elution was carried out as follows: 1M-formic acid, approx. 80ml; 4M-formic acid, approx. 50ml; 4M-formic acid containing 0.4M-ammonium formate, approx. 50ml; and 4M-formic acid containing 0.8M ammonium formate, approx. 40ml. If the appropriate compounds were present in the reaction mixture, the four eluates contained, respectively, mevalonic acid, mevalonic acid 5-phosphate, mevalonic acid 5-pyrophosphate, and isopentenyl pyrophosphate.

[^{14}C]Squalene synthesis

The synthesis of [^{14}C]squalene was carried out by a hybridized procedure derived from those of several other investigators (Popják, 1969; Popják *et al.*, 1969; Shechter *et al.*, 1970). Fleischmann's 'Active Dry' yeast (4g) was suspended in 40ml of 0.1M-potassium phosphate buffer, pH7.5, containing 30mM-nicotinamide (to inhibit NAD nucleosidase), 5mM-$MgCl_2$ and 2mM-$MnCl_2$. The yeast cells were ruptured by passage through a French pressure cell at 138MPa (20000lb/in^2); the preparation was centrifuged at 750*g* for 20min to remove unbroken cells and cellular fragments. The supernatant was centrifuged at 10000*g* for 30min to remove mitochondria; the 10000*g* supernatant was removed without disturbing the overlying fatty layer or the fluffy surface layer of the pellet. This supernatant, referred to as the S_{10} preparation, contained 18.2mg of protein/ml and all of the squalene-synthesizing enzymes that were present in the intact organism.

The reaction mixture contained 9.5ml of the S_{10} preparation (representing 173mg of protein), 30μmol of ATP, 30μmol of glucose 6-phosphate, 10μmol of NADP, 100μmol of KF (to inhibit adenosine triphosphatase), and 8μmol of [2-^{14}C]mevalonic acid (10.9mCi/mmol) in a total volume of 10ml. After all additions, the pH of the reaction mixture was adjusted to 7.5 with 1M-KOH. The mixture was placed in a 50ml filter flask, which was fitted with a large serum-bottle stopper to facilitate addition of reaction components during incubation. After several alternate evacuations and flushings with N_2, the flask containing the reaction mixture was incubated under N_2 on a Dubnoff metabolic shaker at 34°C. After 2h of incubation and again 1h later, additions of ATP, glucose 6-phosphate and NADP in 0.3ml of water were repeated; incubation was continued 1h longer.

The reaction was stopped by transferring the mixture to a round-bottom flask and adding 20ml of 30% (w/v) KOH in methanol. Saponification was carried out by refluxing under N_2 on a 90°C-water bath for 45min. The saponified mixture was extracted three times with a total of 100ml of light petroleum (b.p. 30–60°C). The extract was washed three times with 0.2vol. of 1% (w/v) $NaHCO_3$, dried over anhydrous Na_2SO_4, and evaporated to dryness in a tared flask *in vacuo*.

Fractionation on a silicic acid column was carried out by the procedure for neutral lipids outlined in the preceding paper (Wood & Gottlieb, 1978). Fractions were monitored for radioactivity. G.l.c. and t.l.c. analyses of the hexane fraction indicated that the [^{14}C]squalene present therein was sufficiently pure to be used without further purification.

Results

Conversion of [2-^{14}C]mevalonic acid into other sterol-pathway compounds

The results of a representative experiment designed to measure the conversion of labelled mevalonic acid

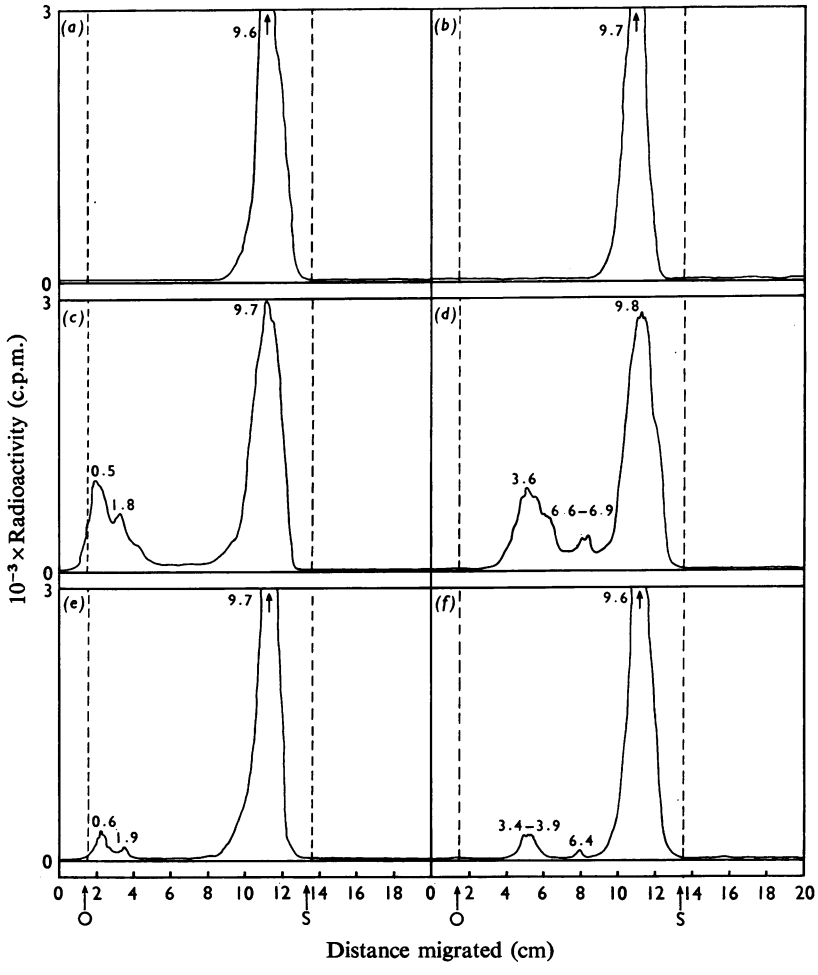

Fig. 1. *Paired thin-layer radiochromatogram scans of R. solani and P. cinnamomi cell-free preparations incubated in the presence of* [2-^{14}*C*]*mevalonic acid and iodoacetamide*

Left-hand chromatograms were developed in butan-1-ol/formic acid/water (77:10:13, by vol.). Right-hand chromatograms were developed in 2-methylpropan-2-ol/formic acid/water (20:5:8, by vol.). (*a*) and (*b*), *R. solani* preparation incubated for 0h; (*c*) and (*d*), *R. solani* preparation incubated for 4h; (*e*) and (*f*), *P. cinnamomi* preparation incubated for 4h. Numbers on peaks are the distance (in cm) from the origin. Solvent front (S) is 12cm from the origin (O).

into isopentenyl pyrophosphate by *R. solani* and *P. cinnamomi* cell-free preparations, carried out in the presence of iodoacetamide to terminate the reaction at isopentenyl pyrophosphate, are presented in Fig. 1 and Table 1. Fig. 1 depicts scans of paired thin-layer radiochromatograms of the *R. solani* zero-time and 4-h reaction-mixture supernatants, and of the *P. cinnamomi* 4-h supernatant. In the scans of the paired zero-time supernatant chromatograms (scans *a* and *b*), only one peak (equivalent to mevalonic acid) is apparent. In each of the scans of the 4-h-supernatant chromatograms (scans *c* and *d* for *R. solani*, and scans *e* and *f* for *P. cinnamomi*), three peaks are discernible. The R_F values of these peaks are listed in Table 1 along with the literature values for mevalonic acid, mevalonic acid 5-phosphate, mevalonic acid 5-pyrophosphate and isopentenyl pyrophosphate in the two solvent systems (Tchen, 1958; Bloch *et al.*, 1959). In all cases, the correlation between the R_F values obtained experimentally and those listed in the literature is good. Thus cell-free preparations of both *R. solani* and *P. cinnamomi* were able to convert mevalonic acid into its two phosphorylated derivatives and further into isopentenyl pyrophosphate.

Table 1. *R_F values of major labelled components in reaction-mixture supernatants of fungal cell-free preparations incubated in the presence of [2-^{14}C]mevalonic acid and iodoacetamide*

Solvent 1, butan-1-ol/formic acid/water (77:10:13, by vol.); solvent 2, 2-methylpropan-2-ol/formic acid/water (20:5:8, by vol.). For details, see the Materials and Methods section.

	R_F values in:					
	Solvent 1			Solvent 2		
Suspected compound	*R. solani*	*P. cinnamomi*	Reference*	*R. solani*	*P. cinnamomi*	Reference†
Mevalonic acid	0.81	0.81	0.75	0.82	0.80	0.80–0.85
Mevalonic acid 5-phosphate	0.15	0.16	0.15	0.55–0.58	0.53	0.53–0.61
Mevalonic acid 5-pyrophosphate	0.04	0.05	<0.1	0.30	0.28–0.32	0.29–0.35
Isopentenyl pyrophosphate	0.04	0.05	<0.1	0.55–0.58	0.53	0.53–0.61

* Reference R_F values are those of Tchen (1958).
† Reference R_F values are those of Bloch *et al.* (1959).

Table 2. *Distribution of radioactivity in Dowex 1 (formate form)-column fractions of fungal cell-free preparations incubated in the presence of [2-^{14}C]mevalonic acid and iodoacetamide*

The amount of radioactivity in the mevalonic acid added to each reaction tube was 5440000d.p.m. For details, see the Materials and Methods section.

		Distribution of radioactivity							
		R. solani				*P. cinnamomi*			
		Boiled control		Experimental		Boiled control		Experimental	
Fraction	Suspected major component	(d.p.m./tube)	(%)	(d.p.m./tube)	(%)	(d.p.m./tube)	(%)	(d.p.m./tube)	(%)
Water	Mevalonic acid lactone	21200	0.39	21700	0.40	19700	0.36	20300	0.37
1 M-Formic acid	Mevalonic acid	5160000	94.85	4060000	74.63	5440000	100.00	5400000	99.26
4 M-Formic acid	Mevalonic acid 5-phosphate	1000	0.02	5430	0.10	1540	0.03	4150	0.08
4 M-Formic acid containing 0.4 M-ammonium formate	Mevalonic acid 5-pyrophosphate	631	0.01	1120000	20.59	750	0.01	11500	0.21
4 M-Formic acid containing 0.8 M-ammonium formate	Isopentenyl pyrophosphate	544	0.01	74000	1.36	338	0.01	8120	0.15

Further confirmation of the mevalonic acid conversion was obtained by subjecting the zero-time (boiled control) and 4-h (experimental) reaction-mixture supernatants of both organisms to ion-exchange chromatography (Table 2). When the supernatants were layered on the Dowex 1 columns and the non-adhering components were washed through with water, about 0.4% of the total label in the supernatants also washed through. Presumably this label represented the small amount of mevalonic acid lactone still present after conversion of the mevalonic acid from the lactone form into the salt form before addition to the reaction mixtures. Except for the 1 M-formic acid fractions, which contained essentially all of the unconverted mevalonic acid, the remaining fractions obtained from the experimental reaction-mixture supernatants of both organisms showed increased amounts of radioactivity when compared with the control fractions. Scanning t.l.c. analysis of the four fractions verified that the four labelled components had been separated into the four individual fractions. In all cases, the major labelled component accounted for more than 90% of the label in that fraction. Further, there is good correlation between

Table 3. *Distribution of radioactivity in silicic acid-column fractions and sterol-pathway compounds of fungal cell-free preparations incubated aerobically and anaerobically in the presence of* [2-^{14}C]*mevalonic acid*

The amount of radioactivity in the mevalonic acid added to each reaction tube was 6600000 d.p.m. N.S., not significant. For details, see the Materials and Methods section.

	Distribution of radioactivity							
	R. solani				*P. cinnamomi*			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
Fraction or compound	(d.p.m./ tube)	(%)	(d.p.m./ tube)	(%)	(d.p.m./ tube)	(%)	(d.p.m./ tube)	(%)
Light petroleum extract	26500	0.40	8820	0.13	5880	0.09	1470	0.02
Hexane	4050	0.06	962	0.01	412	0.01	206	0.003
Squalene	3120	0.05	529	0.01	132	0.002	88	0.001
15% (v/v) Hexane/benzene	3060	0.05	1600	0.02	470	0.01	118	0.002
Squalene epoxide	1440	0.02	N.S.	—	0	—	N.S.	—
5% Diethyl ether/hexane	2970	0.04	868	0.01	824	0.01	200	0.003
15% Diethyl ether/hexane	14000	0.21	3020	0.05	2650	0.04	253	0.004
Lanosterol*	0	—	0	—	0	—	N.S.	—
Farnesol	2570	0.04	1680	0.03	588	0.01	81	0.001
Geraniol	338	0.01	206	0.003	96	0.001	N.S.	—
Ergosterol	N.S.	—	N.S.	—	0	—	0	—
Methanol	2650	0.04	2280	0.03	1480	0.02	741	0.01

* Listed in order of elution from the column.

the relative sizes of the labelled peaks in the scans in Fig. 1 and the relative amounts of radioactivity in the corresponding ion-exchange fractions.

The results of a typical experiment designed to measure the conversion of labelled mevalonic acid into squalene (anaerobic) and into sterol (aerobic) by *R. solani* and *P. cinnamomi* cell-free preparations are presented in Table 3. For the two fungal systems under both aerobic and anaerobic conditions, the light-petroleum extracts of the reaction mixtures and the individual silicic acid-column fractions contained significant quantities of labelled products. However, the amount of label in the fractions was considerably less under anaerobic conditions than when the preparations were incubated aerobically. Further, the radioactivity in these fractions under either aerobic or anaerobic conditions was much less for the *P. cinnamomi* preparation than for the *R. solani* preparation.

The cell-free preparations of both organisms produced significantly labelled farnesol (presumably as the pyrophosphate) and squalene from labelled mevalonic acid under both atmospheric conditions. Geraniol was significantly labelled in all cases except the anaerobically incubated *P. cinnamomi* preparation; the presence of geraniol was detected in all four cases by means of g.l.c.

Only the aerobically incubated *R. solani* preparation contained significantly labelled squalene epoxide (Table 3). Nevertheless, squalene epoxide was detected by g.l.c. in the *R. solani* preparation incubated either aerobically or anaerobically, but not in the *P. cinnamomi* preparation. The squalene epoxide present in the anaerobically incubated *R. solani* preparation probably represented endogenous compound present in the preparation before the incubation period.

Neither the *R. solani* nor the *P. cinnamomi* preparation produced significantly labelled lanosterol or ergosterol under either atmospheric condition. G.l.c. analysis indicated the presence of both compounds in the *R. solani* preparation incubated under either atmospheric condition, but not in the *P. cinnamomi* preparation. The fact that both lanosterol and ergosterol were present in the aerobically incubated *R. solani* preparation, but were unlabelled, suggests that perhaps the incubation period was too short to permit the conversion of labelled mevalonic acid into compounds beyond squalene epoxide, or maybe one or more steps in the conversion were inactive in the reaction mixture.

Conversion of [1-^{14}C]*isopentenyl pyrophosphate into other sterol-pathway compounds*

The data from a typical experiment designed to measure the aerobic and anaerobic conversion of [1-^{14}C]isopentenyl pyrophosphate into other sterol-pathway compounds by cell-free fungal systems are presented in Table 4. Although the reaction mixtures contained identical quantities of labelled substrate and similar amounts of fungal protein, the *P. cinnamomi* preparation converted much more labelled substrate into labelled light-petroleum-extractable products than did the *R. solani* preparation.

Table 4. *Distribution of radioactivity in extracts and sterol-pathway compounds of fungal cell-free preparations incubated aerobically and anaerobically in the presence of* [1-^{14}C]*isopentenyl pyrophosphate*

The amount of radioactivity in the isopentenyl pyrophosphate added to each reaction tube was 47400d.p.m. Compounds were identified by silica-gel t.l.c.; spots were scraped into scintillation vials for determination of radioactive content. N.S., not significant.

	Distribution of radioactivity							
	R. solani				*P. cinnamomi*			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
Extract or compound	(d.p.m./tube)	(%)	(d.p.m./tube)	(%)	(d.p.m./tube)	(%)	(d.p.m./tube)	(%)
Light petroleum extract	49000	10.34	77300	16.31	278000	58.65	216000	45.57
Squalene	138	0.03	301	0.06	800	0.17	975	0.21
Squalene epoxide	120	0.03	N.S.	—	N.S.	—	0	—
Geraniol	1110	0.23	1730	0.36	11700	2.47	16900	3.57
Farnesol	953	0.20	1470	0.31	17100	3.61	24700	5.21
Lanosterol	347	0.07	N.S.	—	N.S.	—	N.S.	—
Ergosterol	347	0.07	N.S.	—	N.S.	—	N.S.	—

Table 5. *Distribution of radioactivity in extracts and sterol-pathway compounds of fungal cell-free preparations incubated in the presence of* [^{14}C]*squalene*

The amount of radioactivity in the squalene added to each reaction tube was 4020000d.p.m. Compounds were identified by silica-gel t.l.c.; spots were scraped into scintillation vials for determination of radioactive content.

	Distribution of radioactivity							
	R. solani				*P. cinnamomi*			
	Boiled control		Experimental		Boiled control		Experimental	
Extract or compound	(d.p.m./tube)	(%)	(d.p.m./tube)	(%)	(d.p.m./tube)	(%)	(d.p.m./tube)	(%)
Aqueous phase	1320000	32.84	574000	14.28	1260000	31.34	2090000	51.99
Light petroleum extract	2770000	68.91	3580000	89.05	2810000	69.90	2140000	53.23
Squalene	2710000	67.41	3160000	78.61	2710000	674.1	1870000	46.52
Squalene epoxide	3430	0.09	7320	0.18	2380	0.06	1990	0.05
Lanosterol	1200	0.03	79000	1.97	2460	0.06	2380	0.06
Ergosterol	940	0.02	20000	0.50	1590	0.04	1260	0.03

All four experimental reaction mixtures, i.e. the enzyme preparations of both organisms under both atmospheric conditions, produced significantly labelled geraniol and farnesol, presumably as their pyrophosphates, and labelled squalene. The aerobically incubated *R. solani* preparation converted the labelled isopentenyl pyrophosphate to labelled squalene epoxide, lanosterol and ergosterol. These three compounds were detected by g.l.c. in the anaerobically incubated *R. solani* preparation, but were probably present in the mycelium before preparation of the cell-free extract. The *P. cinnamomi* preparation, whether incubated aerobically or anaerobically, did not produce any labelled squalene epoxide, lanosterol or ergosterol. Further, none of these three compounds was detected by g.l.c. in the *P. cinnamomi* preparation.

Conversion of [^{14}C]*squalene into other sterol-pathway compounds*

The results of a typical squalene-conversion experiment are outlined in Table 5. Although squalene is generally extracted from aqueous systems quite efficiently by organic solvents, the presence of Tween 80 (to solubilize the squalene) in the reaction mixtures of the present experiment caused a partitioning of the squalene between the aqueous and the light-petroleum phases. Thus approximately one-third of the label (unconverted squalene) in the boiled control

reaction mixture of each fungal preparation remained in the aqueous phase. However, only about 14% of the label remained in the aqueous phase of the *R. solani* experimental reaction mixture, whereas about half of the label remained in the *P. cinnamomi* aqueous phase.

Squalene was the predominant labelled component of each light-petroleum extract. When the light-petroleum extracts of the boiled control reaction mixtures were subjected to thin-layer co-chromatography with authentic squalene epoxide, lanosterol and ergosterol, the spots equivalent to these authentic compounds contained more than just background radioactivity. Unfortunately, when a labelled sample that contains a highly labelled rapidly moving component is subjected to t.l.c., there is always some degree of label 'drag' or 'tailing' below the rapidly migrating component. In the case of the light-petroleum extracts of the boiled control reaction mixtures, the highly labelled rapidly moving squalene left a low-label trail.

When the light-petroleum extract of the *R. solani* experimental reaction mixture was co-chromatographed with authentic squalene epoxide, lanosterol and ergosterol, all three compounds, after elution from the chromatogram, contained considerably higher amounts of radioactivity than did the corresponding three compounds from the control co-chromatogram. Thus *R. solani* was able to synthesize these three compounds from the labelled squalene.

In contrast, the co-chromatogram of the light-petroleum extract of the *P. cinnamomi* experimental reaction mixture showed approximately the same amount of label in the spots corresponding to squalene epoxide, lanosterol and ergosterol as did the control co-chromatogram, indicating no incorporation of label from squalene into any of these compounds by *P. cinnamomi*. In addition, g.l.c. analysis of the light-petroleum extract of the *P. cinnamomi* experimental reaction mixture failed to show any evidence of the three compounds.

Discussion

The presence of the early portion of the sterol biosynthetic pathway, that is, the conversion of mevalonate into isopentenyl pyrophosphate, was demonstrated in both *R. solani* and *P. cinnamomi* by the recovery of significant quantities of label from mevalonate in mevalonate 5-phosphate, mevalonate 5-pyrophosphate and isopentenyl pyrophosphate (Table 2). However, even though the reaction conditions were similar, approx. 22% of the labelled mevalonate was converted into labelled products by the *R. solani* system, whereas less than 1% of the labelled precursor was converted by the *P. cinnamomi* system. Thus although there were no apparent differences in the reactions and components involved in this portion of the pathway in both organisms, there was a considerable quantitative difference in the reactions.

Demonstration of the central portion of the sterol pathway, the conversion of isopentenyl pyrophosphate into squalene, was successful with cell-free preparations of both *R. solani* and *P. cinnamomi*. Under anaerobic conditions, label from isopentenyl pyrophosphate was recovered in geraniol and farnesol, presumably as their pyrophosphates, and in squalene (Table 4). The conversion of labelled isopentenyl pyrophosphate into light-petroleum-extractable labelled products by the *P. cinnamomi* system was several times that of the *R. solani* system. However, when labelled mevalonate was used as the substrate, the conversion of labelled mevalonate into light petroleum-extractable labelled products by the *P. cinnamomi* system was much less than that of the *R. solani* system (Table 3). The especially slow conversion of mevalonate into isopentenyl pyrophosphate by the *P. cinnamomi* system may be the rate-limiting segment of the mevalonate-into-squalene portion of the pathway in this organism. Thus the conclusion arrived at for the earlier portion of the pathway in both organisms also holds for this segment of the pathway: although there were no apparent qualitative differences in the reactions and components involved in this portion of the pathway in both organisms, there was a considerable quantitative difference in the reactions.

The use of cell-free preparations of *R. solani* and *P. cinnamomi* provided evidence for differences at the squalene conversion level of the sterol pathway in these two organisms. Under aerobic conditions, the sterol-producing fungus, *R. solani*, synthesized labelled squalene epoxide when [^{14}C]mevalonate was used as the precursor (Table 3), and labelled squalene epoxide, lanosterol and ergosterol when [^{14}C]isopentenyl pyrophosphate was used as the precursor (Table 4). When preparations of *P. cinnamomi* (the non-sterol-synthesizing fungus) were incubated aerobically in the presence of either labelled substrate, the last labelled compound in the pathway was squalene; no further compounds, squalene epoxide, lanosterol or ergosterol, were detected by any means.

The variation at the level of squalene conversion was further substantiated when [^{14}C]squalene was used as a substrate. After incubation of the *R. solani* cell-free preparation in the presence of the labelled squalene, the major portion of the label was light-petroleum-extractable and consisted of unconverted squalene plus the forward conversion products, squalene epoxide, lanosterol and ergosterol (Table 5). In the *P. cinnamomi* system, however, the only labelled component in the light-petroleum extract was unconverted squalene; squalene epoxide, lanosterol and ergosterol were not detected by either t.l.c. or g.l.c. Moreover, a large percentage of the label in

the *P. cinnamomi* reaction mixture was recovered in the aqueous phase, suggesting that a portion of the squalene may have been degraded to water-soluble products. Identification of these water-soluble products would shed light on the fate of this sterol precursor in an organism that does not make sterols.

The data in this and the preceding paper (Wood & Gottlieb, 1978) indicate that *R. solani* possesses the full sterol biosynthetic pathway from acetate to ergosterol, but that squalene is the terminal compound in the sterol biosynthetic pathway of *P. cinnamomi* (see Fig. 4 of the preceding paper). Apparently *P. cinnamomi* lacks not only the enzyme responsible for the epoxidation of squalene, but also some or all of the enzymes necessary for the reactions involving the cyclization of squalene epoxide and the conversion of lanosterol into ergosterol. The primary reason for *P. cinnamomi*'s inability to synthesize sterols is probably its inability to convert squalene into squalene epoxide.

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