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Metabolism of L-Tyrosine to 4-Hydroxybenzaldehyde and 3-Bromo-4-Hydroxybenzaldehyde by Chloroplast-containing Fractions of *Odonthalia floccosa* (Esp.) Falk[1]

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ABSTRACT

The biosynthesis of 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenzaldehyde from L-[U-^{14}C]tyrosine has been demonstrated in chloroplast-containing fractions obtained by differential and isopycnic centrifugation from the marine red alga *Odonthalia floccosa*. Surfactant and high speed centrifugation studies indicate that the biosynthetic pathway involves a particulate enzyme system, possibly located on the thylakoid membranes. The following scheme, based upon identification of labeled ^{14}C-intermediates, is proposed for the formation of aldehydes: L-tyrosine → 4-hydroxyphenylpyruvic acid → 4-hydroxyphenylacetic acid → 4-hydroxymandelic acid → 4-hydroxybenzaldehyde → 3-bromo-4-hydroxybenzaldehyde.

Odonthalia floccosa is a marine red alga which, along with many others, contains 2,3-dibromo-4,5-dihydroxybenzyl alcohol (lanosol) (10). Cell-free homogenates of *O. floccosa* have been shown to produce 3-bromo-4-hydroxybenzaldehyde from L-[U-^{14}C]tyrosine (20). Based on the identification of the following ^{14}C-labeled compounds from that study, the following pathway was proposed: L-tyrosine → 4-hydroxyphenylpyruvic acid → 4-hydroxyphenylacetic acid → 4-hydroxymandelic acid → 4-hydroxybenzaldehyde → 3-bromo-4-hydroxybenzaldehyde. It was also suggested that lanosol might be produced by further metabolism of this last compound. The marine red algae, *Marginisporum aberrans* (21) and *Dasya pedicellata* (11), have been found to contain 4-hydroxybenzaldehyde. A similar pathway, resulting in the formation of 3-bromo-4-hydroxybenzoic acid, has recently been described (16) for the diatom *Navicula incerta* and the haptophycean alga *Isochrysis galbana*. The formation of 4-hydroxyphenylacetic acid from L-tyrosine, via 4-hydroxyphenylpyruvic acid, has been documented for certain bacteria (5, 28), for the blue-green alga *Anacystis nidulans* (17), and for the higher plant *Astilbe chinensis* (15). The formation of 4-hydroxybenzaldehyde from 4-hydroxymandelic acid has been established for certain bacteria (3), and a fungus (14) grown on the latter compound. The formation of 4-hydroxymandelic acid from 4-hydroxyphenylacetic acid has been postulated to occur in fungi (9, 31) and the studies with *O. floccosa*, *N. incerta*, and *I. galbana* certainly support its occurrence in algae. Despite the apparent widespread occurrence of this pathway, little is known about the site of this pathway within the cell, except in *A. nidulans* where the thylakoid membrane is the site of tyrosine conversion. This study reports results that suggest that the chloroplast is the site of conversion in *O. floccosa*.

MATERIALS AND METHODS

CHEMICALS

All inorganic chemicals were reagent grade unless otherwise stated.

The following compounds were used as standards for TLC and GLC-RC.[3] Chemicals supplied by Aldrich were 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid), 3,4-dihydroxybenzaldehyde (protocatechualdehyde), 4-methoxybenzaldehyde (*p*-anisaldehyde), 3-hydroxy-4-methoxybenzaldehyde (isovanillin), 3,4-dimethoxybenzaldehyde (veratraldehyde), hydroquinone, 4-benzoquinone, 4-hydroxybenzyl alcohol, and 4-hydroxybenzaldehyde. Chemicals supplied by Sigma were L-tyrosine, 4-hydroxycinnamic acid (*p*-coumaric acid), 4-hydroxyphenylpyruvic acid, 2,4-dihydroxyphenylacetic acid (homogentisic acid), 4-hydroxyphenylacetic acid, 4-hydroxyphenyllactic acid, D,L-4-hydroxymandelic acid, D,L-3,4-dihydroxymandelic acid, D,L-3-methoxy-4-hydroxymandelic acid, ubiquinone 50 (bovine), and 4-hydroxybenzoic acid. Pfaltz and Bauer supplied 3,4-dihydroxycinnamic acid (caffeic acid) and 2,3-dibromo-4,5-dihydroxybenzaldehyde. K & K-ICN supplied 3,4-dihydroxybenzoic acid (protocatechuic acid) and M.C.B. supplied 4-hydroxy-3-methoxybenzaldehyde (vanillin). Drs. J. S. Craigie and W. Fenical kindly provided samples of 2,3-dibromo-4,5-dihydroxybenzyl alcohol (lanosol) and 3,5-dibromo-4-hydroxybenzyl alcohol, respectively. Incubation reagents were supplied by Sigma. Triton X-100 was obtained from Sigma, digitonin (grade A) from Calbiochem and sodium desoxycholate was obtained from K & K-ICN. The silylation reagents BSTFA and TMCS were obtained from Supelco and K & K-ICN, respectively.

CHEMICAL SYNTHESES

The following compounds were synthesized as described below.

4-Hydroxyphenylacetaldehyde. This was synthesized from D,L-synephrine (Sigma) and purity was determined as described by Robbins (26) using his solvent system B. MS (70 ev) gave M^+ 136 m/e; $(M\text{-}CO)^+$, 108 m/e; $(M\text{-}CHO)^+$ 107 m/e.

The synthesis of the 3-bromo derivatives of various phenolic

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[3] Abbreviations: GLC-RC: gas-liquid chromatography-radiocounting; BSTFA: *N,O*-bis-trimethylsilyl trifluoracetamide; TMCS: trimethylchlorosilane; FAD: flavin adenine dinucleotide (oxidized); FID: flame ionization detector; RAM; radioactive monitor; P-5-P: pyridoxal-5-phosphate; R_t: retention time.

compounds was attempted using the method of Paal (23) for 3-bromo-4-hydroxybenzaldehyde. The reactant was dissolved in 15 times its weight of an appropriate solvent and an equimolar amount of Br_2, in a small amount of solvent, was added dropwise for 20 h. The solution was bubbled with N_2 until crystals formed. The products were recrystallized from boiling water except for 3-bromo-4-hydroxyphenylacetic acid which was recrystallized from 50% ethanol.

3-Bromo-4-hydroxybenzaldehyde. This was synthesized from 4-hydroxybenzaldehyde (6 g) in warm chloroform. M.P. (corrected) 123 to 125 C. Lit. 124 C. Yield 98%. MS (70 ev), gave M^+ 202,200 m/e; $(M-1)^+$ 201, 199 m/e; $(M-CHO)^+$ 171, 173 m/e.

3-Bromo-4-hydroxybenzoic acid. This was synthesized from 4-hydroxybenzoic acid (6 g) in ethyl ether. M.P. (corrected) 174 to 176 C. Lit. 177 C. Yield 97%.

3-Bromo-4-hydroxyphenylacetic acid. This was synthesized from 4-hydroxyphenylacetic acid (2 g) in ethyl ether. M.P. (corrected) 102 to 104 C. Lit. 107 C. Yield 97%. MS (70 ev) gave M^+ 230,232 m/e; $(M-CO_2H)^+$ 185, 187 m/e; $C_7H_7O^+$, 107 m/e.

3-Bromo-4,5-dihydroxybenzaldehyde. This was synthesized from 3,4-dihydroxybenzaldehyde (6 g) in ethanol. M.P. (corrected) 227 to 229 C. Lit. 230 C. Yield 81%. MS (70 ev) gave M^+ 216,218 m/e; $(M-1)^+$ 215,217 m/e; $(M-CHO)^+$ 187,189 m/e.

3-Bromo-4,5-dihydroxybenzoic acid. This was synthesized from 3,4-dihydroxybenzoic acid in ethyl ether. M.P. (corrected) 224 to 225 C. Lit. 227 to 229 C. Yield 68%. Contained some unreacted 3,4-dihydroxybenzoic acid.

3,5-Dibromo-4-hydroxybenzaldehyde. This was synthesized from 4-hydroxybenzaldehyde (6 g) in warm chloroform with twice the molar amount of Br_2. M.P. (corrected) 178 to 180 C. Lit. 179 to 180 C. Yield 97%. MS (70 ev) gave M^+ 277,279,281 m/e; $(M-CHO)^+$ 249,251,253 m/e; $(M-C_2O_2H)^+$ 221,223,225 m/e.

Radioactive chemicals. Radioactive L-[U-^{14}C]tyrosine obtained from Schwarz/Mann (Orangeburg, N.Y.) had a specific radioactivity of 460 mCi/mmol and a concentration of 50 μCi/ml in 0.01 N HCl. Solid L-[carboxyl-^{14}C]tyrosine (Amersham Corp., Arlington Heights, Ill.) had a specific radioactivity of 54 mCi/mmol and was prepared as a 100 μCi/ml solution by the addition of 0.01 N HCl. All of the L-[^{14}C]tyrosine was checked for impurities via two dimensional TLC-autoradiography using solvent systems 2 and 3 as described later. A 20-μl aliquot of each solution was spotted, chromatographed, and exposed to film for 5 days. A 10-μl aliquot of L-[U-^{14}C]tyrosine, silylated with BSTFA and TMCS, was checked for purity by GLC-RC as described later. No labeled 4-[U-^{14}C]hydroxybenzaldehyde or any other identifiable compounds were detected. Radiochemical purity was determined to be greater than 99%.

Separation Techniques. TLC-autoradiography was performed on Silica Gel G, type 60 (Brinkmann) 0.5 mm thick, activated for 40 min at 100 C. Solvent front was allowed to rise 16 cm unless otherwise noted. The solvent systems employed were (all ratios by volume): system 1: toluene-ethyl formate-formic acid (97%), 5:4:1 (32); system 2: chloroform-ethanol-acetic acid (glacial), 18:1:1; system 3: benzene-acetic acid (glacial)-water, 10:7:3 (16) using the organic phase after thorough mixing and separation of the phases. Spots were visualized by spraying the plate with Fast Blue B(*o*-dianisidine diazotate, K & K-ICN) in a 0.05% aqueous solution (24). Spots were also visualized by their absorption or fluorescence under UV light (254 nm). Those TLC plates utilized for autoradiography were dried overnight and were exposed to Kodak No Screen Medical x-ray film for 2 weeks and processed manually. Identification was made by the alignment of the visualized chromatograph with the autoradiograph.

Gas-liquid radiochromatography was performed with a Selectra System 5000, GLC and radiocounter (Nuclear-Chicago-Analytical Biochemistry Labs., Inc., Columbia, Mo.) consisting of a 6-foot silanized glass column packed with 3% (w/w) SE-30 on 80/100 mesh, acid-washed Chromosorb W (Supelco). Retention times were determined over a temperature program of 100 to 300 C at 5 C/min. Injection port and detector temperatures were 270 and 300 C, respectively. Argon was used as the carrier gas with a 8.5:1 split for the radiocounter. The flow rate at the detector was 67 ml/min.

Preparation of Algal Material. *O. floccosa* (Esp.) Falkenberg was collected at Shell Beach, Sonoma County, Calif., through the kindness of Dennis Shevlin, thoroughly cleaned of invertebrates and epiphytes, and rinsed with agitation in distilled H_2O and ice (10 C) for 30 min to remove surface microorganisms, especially diatoms. Microscopic examination of the thallus revealed no algal or fungal contaminants. Cleaned *O. floccosa* (160 g) was cut into small pieces, homogenized in a VirTis-45 homogenizer at maximum speed for 3 min with either 240 ml of 25% (w/w) or 10% (w/w) of sucrose in 0.02 M glycylglycine buffer (pH 7.5) at 4 C. The homogenate was filtered through four layers of cheesecloth (with or without one layer of Miracloth) and subjected to a sequential series of 30-min centrifugations at 100*g*, 4,000*g* and 39,000*g*. The pellets from each step were recovered and resuspended in the buffered sucrose. Care was taken to preserve the integrity of the organelles during resuspension with a TenBroeck tissue homogenizer, although it was considered marginally successful.

FACTORS INFLUENCING THE *IN VITRO* FORMATION OF 4-HYDROXYBENZALDEHYDE

The incubations were performed at 30 C for 1 h (surfactant study), 20 min (cofactor study), and 30 min (pH study) with shaking. The incubation mixtures contained 10 μl L-[U-^{14}C]tyrosine (0.5 μCi, 1.0 nmol) or 10 μl L-[carboxyl-^{14}C]tyrosine (1 μCi, 18.5 nmol), 20 μl of surfactant (0.5% w/v), 100 μl of the 4,000*g* pellet, cofactors (if present) see below, K-phosphate buffer (0.02 M, pH 7) in a total volume of 1 ml. A control containing no surfactants was included. The incubations were stopped by the addition of 100 μl of 99% trichloroacetic acid. The mixtures were then centrifuged at 8,000*g* for 20 min. The supernatant (50 μl) was then spotted with a mixture of 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenzaldehyde for two-dimensional TLC (solvent systems 1 and 2). Spots were visualized with UV, recovered and counted for radioactivity in 5 ml of scintillation cocktail 3a70B (Research Products, Inc.) in a Beckman liquid scintillation system LS-150.

The formation of 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenzaldehyde was followed with time in an incubation mixture containing Triton X-100, and double the amount of material and volume. Aliquots of 0.2 ml were taken at given times and added to 5 μl of the trichloracetic acid solution.

Cellular Localization of Activity. Chl *a* was determined according to the method of Arnon (1). Protein was determined according to Lowry *et al.* (19). The possibility of phenolic interference with the protein assay is considered insignificant since no significant difference was seen between values obtained from 39,000*g* supernatant that had been dialyzed and that which had not been dialyzed. Also the only phenolic compounds identified were three bromophenols present in less than 0.06% (unpublished data).

Catalase activity was determined by measuring the disappearance of H_2O_2 at 240 nm (7). Glycolate oxidase/dehydrogenase activity was measured using the phenylhydrazine method (2); no attempt was made to determine which enzyme was present.

The formation of 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenzaldehyde from L-tyrosine was determined using the incubation mixture containing Triton X-100 and with and without the presence of FAD, NADP, α-ketoglutarate and $MnBr_2$ together for 30 min.

A control incubation mixture containing the 100*g* pellet was boiled for 3 min prior to the addition of the labeled tyrosine.

The 39,000*g* supernatant was then centrifuged at 95,000*g* for 3 h and the pellet resuspended; both supernatants and the resus-

pended pellet were assayed for protein and their ability to form 4-hydroxybenzaldehyde.

The 100*g* pellet (homogenate from the 25% sucrose) was carefully resuspended in 2 parts of buffered sucrose and layered (1 ml) on a discontinuous sucrose density gradient consisting of 5 ml of 70% (w/w) sucrose, 4 ml of 67%, 5 ml of 60%, 5 ml of 50%, 7.5 ml of 45%, and 7.5 ml of 24%. The gradient was then centrifuged at 77,000*g* for 3 h (SW 27 rotor, Beckman). One-ml fractions were collected as drops from the bottom of the punctured tube. All steps were conducted at 4 C. Chl was determined by adding 0.5 ml pyridine to solubilize the sucrose and extract the Chl from 0.2 ml of the sample, and reading the *A* at 670 nm. A pellet that formed at the bottom of the gradient tube was resuspended and also assayed.

Identification of Labeled Compounds. The remaining incubation mixtures containing the 100*g* resuspended pellets were lyophilized, desiccated for 24 h over P_2O_5, and silylated by the addition of 50 μl of acetonitrile and 50 μl of BSTFA with 1% TMCS. This mixture was heated for 20 min at 60 C and 5 μl of this mixture added to 2 μl of silylated standards and subjected to GLC-RC. Fifty-μl aliquots of all of the incubation mixtures were separately spotted with standards and subjected to two-dimensional TLC with solvent systems 1 and 2. An additional 50 μl of the 100*g* incubation mixture was spotted with suitable standards and subjected to two-dimensional TLC with solvent systems 2 and 3. These two solvent systems successfully separated *p*-coumaric acid, 2,3-dibromoprotocatechualdehyde, 4-hydroxybenzoic acid, 4-hydroxyphenylacetaldehyde, 3-bromo-4-hydroxyphenylacetic acid, 3-bromo-4-hydroxybenzoic acid from 4-hydroxybenzaldehyde, and protocatechualdehyde, 3-bromoprotocatechualdehyde from 4-hydroxyphenylacetic acid.

RESULTS

Incubation Conditions. The presence of Triton X-100 in the incubation mixture more than doubled the production of 4-hydroxybenzaldehyde and was more effective than digitonin or desoxycholate.

The formation of 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenzaldehyde was linear with time to about 30 min (Fig. 1, a and b), while the pH optimum for the formation of these two compounds is pH 7 (Fig. 1, c and d).

The following compounds caused an insignificant increase in the formation of 4-hydroxybenzaldehyde (less than 15%): FAD, NADP, ATP, α-ketoglutarate and pyruvate. Pyridoxal phosphate, NAD, CoASH, and thiamine caused an insignificant decrease. Activity showed a significant increase in response to $MnBr_2$ (1.24 × 10^4 dpm of 4-hydroxybenzaldehyde in 50 μl of incubation mixture), while oxaloacetic acid (5.4 × 10^3 dpm) and $MgSO_4$ (6.6 × 10^3 dpm) caused a significant decrease in activity (control: 9.8 × 10^3 dpm). When NADP, FAD, $MnBr_2$, and α-ketoglutarate were present in the same incubation mixture no significant increase (less than 15%) in *p*-hydroxybenzaldehyde resulted.

Cellular Localization of Activity. Microscopic examination of the resuspended pellets revealed the following.

100g fraction. Both samples contained slight amounts of cell debris, most of which was unidentifiable except for a few cell wall fragments. The fraction contained chloroplasts (3- to 7-μm diameter), what appeared to be chloroplast fragments and aggregations of organelles, but apparently lacked whole cells.

4,000g and 5,000g. Both contained chloroplasts (3- to 4-μm diameter), microbodies (1.3-μm diameter), and chloroplast fragments.

39,000g. This contained (<1-μm diameter) microbodies and chloroplast fragments and presumptive mitochondria (1-μm diameter).

Supernatant. The supernatant was free of visible particles and faintly blue in color. In all cases the pellets were very sticky and difficult to resuspend. The results of the various assays are shown in Table I. Almost half of the Chl was found in the 100*g* fraction confirming the presence of chloroplasts. In fractions from homogenates made in either 10 or 25% sucrose the greatest activities of catalase and of glycolate oxidase/dehydrogenase were in the 5,000*g* fraction with considerable activity in the 39,000*g* fraction presumably reflecting the presence of microbodies.

The ability to synthesize 4-hydroxybenzaldehyde from L-[U-^{14}C]tyrosine corresponded with the distribution of Chl in each fraction, with the greatest activity being associated with the 100*g* pellet. No 4-hydroxybenzaldehyde was detected in the boiled control. Assuming a specific radioactivity of 357 mCi/mmol for C_6-C_1-U-^{14}C-compounds (7/9 of 460 mCi/mmol), calculations reveal that approximately 10 to 30% of L-[U-^{14}C]tyrosine was converted to 4-hydroxybenzaldehyde by the 100*g* fraction depending on the batch of *O. floccosa* used. The ability to produce

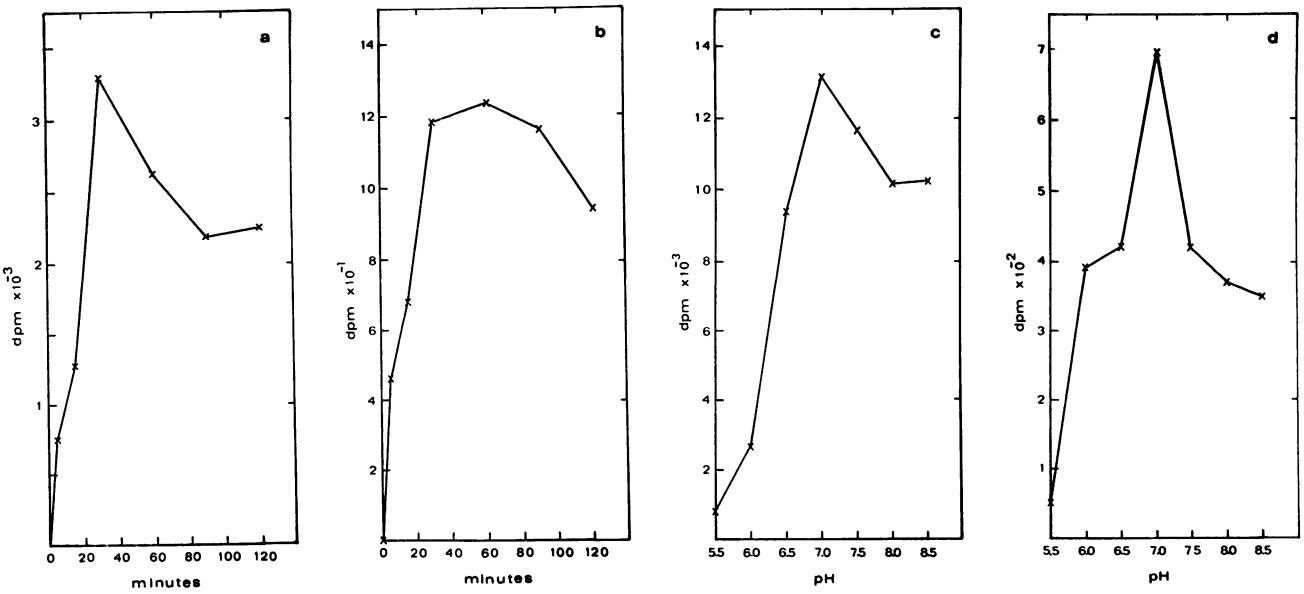

FIG. 1. Time dependence of the formation of 4-hydroxybenzaldehyde (a) and 3-bromo-4-hydroxybenzaldehyde (b) and the effect of pH on the formation of 4-hydroxybenzaldehyde (c) and 3-bromo-4-hydroxybenzaldehyde (d). The effect of pH was determined by preparing K-phosphate buffers at various pH values and incubating as described in the text with the addition of Triton X-100, FAD, NADP, and $MnBr_2$.

Table I. *Analyses of Fractions Obtained by Differential Centrifugation*

"Per cent of total activity in homogenate" signifies the amount of the measured quantity that was formed for that fraction, expressed as a percentage of the total for the total homogenate. "Relative specific activity" signifies the specific radioactivity (as dpm/mg protein) relative to the highest value which is designated as 100. One unit of enzymic activity equals 1 μmol H_2O_2 consumed or glyoxalate formed per min.

	Sample			
	100*g*	4,000*g*	39,000*g*	Supernatant
Chl				
Total Chl (mg)	7.56	3.76	3.73	1.81
% of Total homogenate	46.2	23.0	19.7	11.1
Protein				
Total protein (mg)	320	231	251	1170
% of Total homogenate	16.2	11.7	12.7	59.4
Catalase activity				
Total activity (units)	245	396	376	0
% of Total activity in homogenate	24.2	38.7	37.1	0
Relative specific activity (×100)	49.8	100	97.2	0
Glycolate oxidase/dehydrogenase activity				
Total activity (units)	10.8	20.0	18.2	0
% of Total activity in homogenate	21.9	40.8	37.1	0
Relative specific activity (×100)	41.2	100	90.5	0
4-Hydroxybenzaldehyde formation from L-[^{14}C]tyrosine				
Without cofactors				
Total dpm × 10^{-6}	7.34	2.57	2.10	1.91
% of Total dpm of homogenate	52.6	18.4	15.1	13.8
With cofactors				
Total dpm × 10^{-6}	8.36	3.92	2.46	2.90
% of Total dpm of homogenate	46.6	21.9	15.4	16.2
Relative dpm/mg protein (×100)	100	64.9	41.9	9.6
3-Bromo-4-hydroxybenzaldehyde formation from L-[^{14}C]tyrosine with cofactors				
Total dpm × 10^{-5}	8.06	3.35	2.68	0
% of Total dpm of homogenate	57.2	23.8	19.0	0
Relative dpm/mg protein (×100)	100	54.8	42.0	0

Table II. *Analyses of Fractions Obtained from High Speed Centrifugation of 39,000g Supernatant*

	Sample		
	39,000*g* supernatant	95,000*g* supernatant	95,000*g* pellet
4-Hydroxybenzaldehyde formation from L-tyrosine			
Total dpm in fraction	3.4×10^5	0	3.0×10^5
Protein (dpm/mg)	1.63×10^3	0	3.33×10^6
Total protein (mg)	214.8	173.7	0.09

4-hydroxybenzaldehyde from L-tyrosine in the 39,000*g* supernatant appears to be associated with very small particles as it was localized in the pellet formed by high speed centrifugation (Table II).

The analysis of the density gradient (Fig. 2) showed that 4-hydroxybenzaldehyde synthesis corresponded with the Chl-containing bands. The top of the tube contained some phycocyanin, which indicated that some chloroplasts had ruptured. Band 1 contained presumptive proplastids 2 μm in diameter; band 2 contained chloroplasts, the majority being 5.3 μm in diameter and some damaged chloroplasts. Bands 3 and 4 and the pellet contained chloroplasts 4 to 7 μm in diameter. Bands 2, 3, and 4 and the pellet sedimenting through the 70% sucrose contained a very slight amount of unidentifiable debris.

Identification of Intermediates. The R_F values and retention times from GLC-RC are given in Table III, while the FID and RAM traces from GLC-RC are shown in Figure 3. The autoradiograph of the incubation mixture containing L[U-^{14}C]tyrosine and the 100*g* fraction is shown in Figure 4 and is identical to the autoradiograph for the incubation mixtures containing the other fractions. The autoradiograph of the TLC plate chromatographed in solvents 2 and 3 did not reveal any additional compounds. Three unknown spots appear on the TLC autoradiograph of the L-[U-^{14}C]tyrosine incubation mixture, two of which correspond with unknown spots from the L-[carboxyl-^{14}C]tyrosine TLC-autoradiograph.

The RAM trace shows a possible peak (shoulder of peak that aligns itself with 4-hydroxymandelic acid) that corresponds with the FID trace of 3,5-dibromo-4-hydroxybenzaldehyde (peak 5). This is, however, not verified by TLC-autoradiography.

DISCUSSION

Microscopic examination of the chloroplast fractions (100*g*) indicated the presence of chloroplasts with little apparent contamination. The added use of Miracloth removed slightly more contaminants than the four layers of cheesecloth alone. The exact nature of the contaminants was difficult to determine microscopically, although some aggregated organelles (chloroplasts, presumptive peroxisomes) were present.

The labeled compounds from all incubation mixtures, identified by TLC-autoradiography and GLC-RC, were the same as those identified from the whole homogenate (20), and did not show any addition or reduction in the number of labeled products.

The ability to metabolize L-tyrosine to 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenzaldehyde appears to reside in the chloroplast-containing fraction. Analysis of the cell fractions revealed that the per cent total dpm (4-hydroxybenzaldehyde) of the homogenate, and dpm of 4-hydroxybenzaldehyde per mg protein are highest in the 100*g* fraction, which in turn corresponds with the fraction containing the most Chl. Per cent total dpm (4-hydroxybenzaldehyde) from the homogenate corresponds closely to the per cent total Chl of the homogenate and not with the marker enzymes indicative of peroxisomes (Table I). The disap-

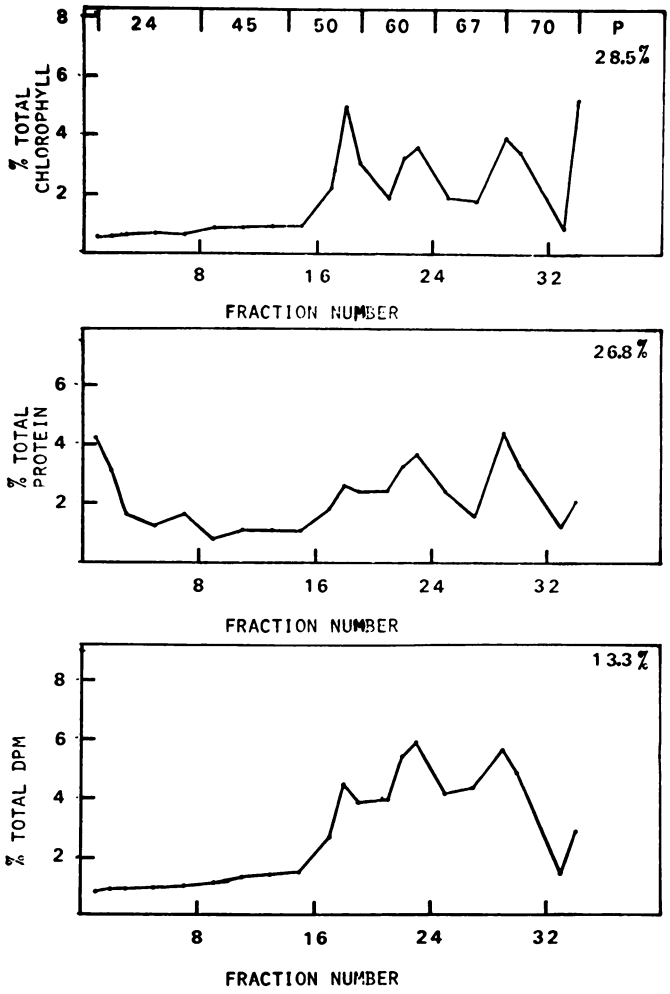

FIG. 2. Analysis of the sucrose density gradient. Upper numerals represent the per cent of sucrose, while the percentage values below P indicate the per cent of total in the pellet.

pearance of H_2O_2 may be due in part to the presence of a peroxidase in the 39,000*g* fraction and supernatant.

It is very difficult to obtain organelle-enriched fractions from *O. floccosa* in which membrane integrity is maintained after resuspension due to the adhesiveness of the pelleted material. The existence of a faint blue color at the top of the gradient tube after centrifugation indicates the presence of phycocyanin and hence the rupturing of some chloroplasts.

The data presented suggest that the chloroplast is the site of 4-hydroxybenzaldehyde biosynthesis from L-tyrosine. However, chloroplast fractions completely free of contaminants were not obtained, and the presence of microsomes (ER) likely to contain aromatic-oxidizing systems and mitochondria were not monitored. It is possible that they adhered to the chloroplasts.

The radiochemical assay was performed using very low substrate concentrations and therefore the rate of reaction was not maximal. Thus, the interference of endogenous substrates, inhibitors, and competing pathways, if present, could have been pronounced (22). Evidence for the existence of another metabolic sequence for L-tyrosine was not observed. The above conditions can, of course, affect localization experiments. Thus, a more rigorous proof of location is needed. Yet, a similar pathway resulting in 4-hydroxyphenylpyruvic acid from L-tyrosine was shown to occur on thylakoid isolates of *A. nidulans* (17).

The possibility of bacterial contamination being responsible for the partial or complete metabolism of L-tyrosine is considered remote, since the formation of 4-hydroxymandelic acid from 4-hydroxylphenylacetic acid has not yet been demonstrated in those bacteria studied and no common bacterial products of L-tyrosine metabolism, homogentisic acid, homoprotocatechuic acid, and protocatechuic acid were detected.

The absolute necessity of the cofactors for the formation of 4-hydroxybenzaldehyde by the crude fractions used was not shown. Based on the postulated pathway, FAD, NADP, and/or NAD may be cofactors for various oxidations, thiamine for oxidative decarboxylation, and α-ketoglutarate and P-5-P for the transamination reaction. Manganese bromide was added to the incubation mixture as a source of bromide for bromination and for the possible requirement of Mn^{2+} by an enzyme. The stimulation of the production of 4-hydroxybenzaldehyde is likely due to the Mn^{2+}. Mg^{2+} may not replace Mn^{2+} in certain reactions, or may be required for the metabolism of 4-hydroxybenzaldehyde resulting, in either case, in the detection of less 4-hydroxybenzaldehyde in those mixtures containing Mg^{2+}.

The optimal pH for the entire pathway of formation of 4-hydroxybenzaldehyde from L-tyrosine in the incubation mixture is 7 (Fig. 1c). The formation of 4-hydroxybenzaldehyde with time was linear for approximately 30 min with a significant reduction thereafter, not accounted for in the increase of 3-bromo-4-hydrox-

Table III. *Separation of Standard Compounds by Thin Layer and Gas-Liquid Chromatography*

Compound	$R_F \times 100$			GLC Retention Times in Min. with Program 100 C at 5 C/min of Silylated Derivatives
	TLC Solvent system 1	Solvent system 2	Solvent system 3	
L-Tyrosine	4	0		[a]
p-Coumaric acid	58	42	25	19.7
Caffeic acid	42	31		
4-Hydroxyphenylpyruvic acid	46	21[b]		22.0
4-Hydroxyphenyllactic acid	29	10		19.4
2,5-Dihydroxyphenylacetic acid	35	11		18.2
4-Hydroxyphenylacetaldehyde	48	54	28	
4-Hydroxyphenylacetic acid	46	39	19	13.5
3,4-Dihydroxyphenylacetic acid	38	22		18.2
4-Hydroxymandelic acid	26	8		16.1
3,4-Dihydroxymandelic acid	19	5		
3-Methoxy-4-hydroxymandelic acid	28	24		
4-Hydroxybenzaldehyde	51	58	21	7.9
4-Hydroxybenzoic acid	50	46	20	13.5
3,4-Dihydroxybenzoic acid	42	29		18.2
4-Hydroxybenzylalcohol	43	27		10.6
3,4-Dihydroxybenzaldehyde	42	37	7	13.5
3-Bromo-4-hydroxyphenylacetic acid	52	59	31	18.2
3-Bromo-4-hydroxybenzaldehyde	55	69		12.2
3-Bromo-4-hydroxybenzoic acid	53	58	32	18.2
3-Bromo-4-hydroxybenzylalcohol	48	43		15.5
3-Bromo-4,5-dihydroxybenzaldehyde	50	41	13	17.3
3,5-Dibromo-4-hydroxybenzaldehyde	68	80		16.5
3,5-Dibromo-4-hydroxybenzylalcohol	54	60		
2,3-Dibromo-4,5-dihydroxybenzaldehyde	52	48	21	21.3
Lanosol	49	14		22.9
p-Anisaldehyde	65	83		
Vanillin	54	72		
Isovanillin	54	71		
Veratraldehyde	54	86		
Hydroquinone	48	84		
4-Benzoquinone	60	68		
Ubiquinone 10	86	98		

[a] Does not come off column after 20 min upon reaching 300 C.

[b] Estimated because of extreme tailing when preceded by solvent system 1 in first dimension.

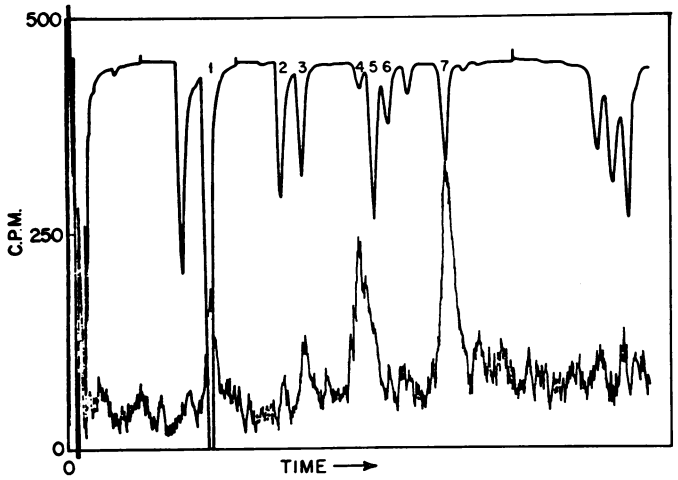

FIG. 3. FID (upper) and RAM (lower) traces from GLC-RC of the 100*g* incubation mixture and standard compounds (silylated). 1: 4-hydroxybenzaldehyde; 2: 3-bromo-4-hydroxybenzaldehyde; 3: 4-hydroxyphenylacetic acid; 4: 4-hydroxymandelic acid; 5: 3,5-dibromo-4-hydroxybenzaldehyde; 6: 3-bromo-4,5-dihydroxybenzaldehyde; 7: 4-hydroxyphenylpyruvic acid.

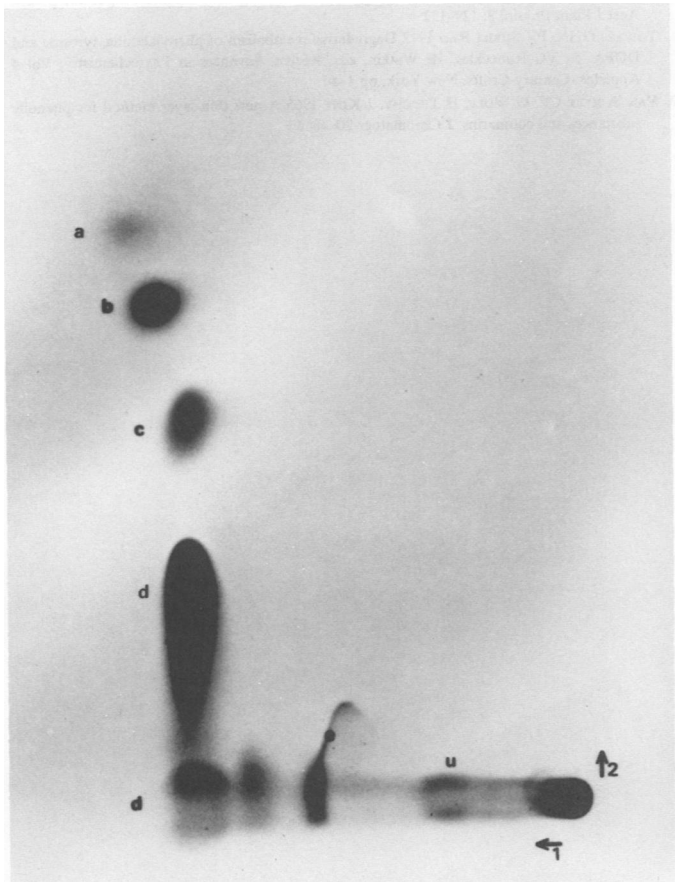

FIG. 4. TLC-autoradiograph of the L-[U-^{14}C]tyrosine incubation mixture with solvent systems 1 and 2. a: 3-bromo-4-hydroxybenzaldehyde; b: 4-hydroxybenzaldehyde; c: 4-hydroxyphenylacetic acid; d: 4-hydroxyphenylpyruvic acid (smears in solvent 2); e: 4-hydroxymandelic acid; u: unknown; tyrosine at origin.

ybenzaldehyde (Fig. 1, a and b). A branch point for a variety of phenolic compounds including 4-hydroxybenzyl alcohol (via aromatic alcohol dehydrogenases) and ubiquinone (neither of which were detected) may occur at 4-hydroxybenzaldehyde.

Increased activity resulting from the addition of surface active agents supports the contention that the enzyme system responsible for 4-hydroxybenzaldehyde formation is in an organelle (possibly chloroplast) and the association of this activity with the 95,000*g* pellet from the 39,000*g* supernatant indicates that it is associated with a membrane.

3-Bromo-4-hydroxybenzaldehyde was identified in the incubation mixtures which contained bromide. The absence of 3-bromo-4-hydroxyphenylacetic acid and 3-bromo-L-tyrosine indicates that bromination does not occur prior to the formation of 4-hydroxybenzaldehyde. This is in contrast to the apparent situation in the red alga *Halopitys incurvus* which contains 3,5-dibromo-4-hydroxyphenylacetic acid and 3,5-dibromo-4-hydroxyphenylpyruvic acid (8).

No other brominated compounds were positively identified from the L-[U-^{14}C]tyrosine incubations. This may be due to specificity of this enzyme for C_6-C_1 compounds; chloroperoxidase, however, has the ability to utilize L-tyrosine as a substrate (12). The presence of a multienzyme complex or membrane-bound enzyme system containing bromoperoxidase could conceivably prevent bromination of certain potential substrates. Those intermediates which diffuse from the complex or system would not be as likely to become bound once again to an enzyme, including bromoperoxidase, of the system. It has been demonstrated that the bromoperoxidase from the red alga *Cystoclonium purpureum* is a particulate enzyme (25). H_2O_2, required for the activity of bromoperoxidase, could be readily available in the chloroplast via the Mehler reaction. Bromide ion, the source of bromine, could conceivably find its way into the chloroplast via a mechanism similar to that for the chloride ion.

Although the RAM trace from GLC-RC suggests the presence of 3,5-dibromo-4-hydroxybenzaldehyde, this large RAM peak could possibly be due to 4-hydroxyphenylglyoxylic acid. This could not be determined since pure 4-hydroxyphenylglyoxylic acid could not be obtained, and attempts to synthesize it from 4-hydroxyacetophenone (6) repeatedly resulted in the formation of 4-hydroxybenzaldehyde.

It has been proposed (20) that the bromophenols of marine red algae may be produced from L-tyrosine via 4-hydroxybenzaldehyde. Although bromophenols were not detected under the conditions of these experiments, it may still be that they are formed in the chloroplasts. Floridorubin, a polyphenolic compound containing bromophenols, found in certain Rhodomelacean algae but not *O. floccosa*, has been tentatively located in the chloroplasts of *Lenormandia prolifera* based on X-ray emission microanalysis (13). Since the pathway to 3-bromo-4-hydroxybenzaldehyde, and presumably then to the bromophenols, may exist in the chloroplast, L-tyrosine must be readily available within this organelle. The biosynthesis of tyrosine has been demonstrated in isolated chloroplasts of *Acetabularia* (27) and the aromatic amino acids have been synthesized from [1,6-^{14}C]shikimate in isolated spinach chloroplasts (4).

The isolation and purification of U-^{14}C-intermediates (4-hydroxyphenylacetic acid, 4-hydroxybenzaldehyde, and 3-bromo-4-hydroxybenzaldehyde) and studies on their metabolism are currently under investigation.

A finding incidental to the main thrust of this study is that glycolate oxidase or glycolate dehydrogenase activity was detected in a marine red alga. The method used does not distinguish between the two possible enzymes since activity with and without O_2 was not determined. Previous attempts have failed to detect the existence of such enzymes in a number of marine algal species (30). "Catalase activity" (H_2O_2 disappearance) corresponded to the glycolate-oxidizing activity implying that a peroxisome-like organelle is present. The existence of catalase does not preclude the existence of glycolate dehydrogenase in favor of the oxidase since unicellular green algae which contain the former still contain low levels of catalase activity (29).

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