ABSTRACT

PATHWAY OF PHLOROGLUCINOL DEGRADATION BY A *PSEUDOMONAS* SP. MAC 451

Ph.D. Yong Deng Hang Microbiology

Phloroglucinol reductase was successfully isolated and purified approximately 23-fold. The enzyme is able to catalyse a rapid oxidation of NADPH with phloroglucinol as the acceptor. Phloroglucinolcarboxylic acid, various quinones and potassium ferricyanide can replace phloroglucinol as the acceptor. The enzyme is NADPH-specific. It is most stable at pH 7.0 and has an optimum pH at 7.4 in phosphate buffer. The Km for phloroglucinol is 6.25×10^{-6}M. No stimulation was obtained with FAD,FMN, cytochrome c or various cations; the enzyme was inhibited by mercuric chloride, iodoacetate and p-chloromercuribenzoate. The reaction catalysed by phloroglucinol reductase is postulated as

$$\text{Phloroglucinol} + \text{NADPH} + H^{+} \rightleftharpoons \text{Dihydrophloroglucinol} + \text{NADP}^{+}$$

The detection of some of the intermediates involved in phloroglucinol degradation was successful. Dihydrophloroglucinol and resorcinol were isolated successfully with Sephadex G-10 and were identified as two intermediates from a growing cell fermentation liquor.

The pathway of phloroglucinol degradation by *Pseudomonas* sp. Mac 451 is postulated. This pathway involves the reduction of phloroglucinol to dihydrophloroglucinol which is dehydrated then to resorcinol. The dihydroxy phenol is further metabolized.

PATHWAY OF PHLOROGLUCINOL DEGRADATION BY A PSEUDOMONAD

by

Yong Deng Hang

PATHWAY OF PHLOROGLUCINOL DEGRADATION BY A PSEUDOMONAS SP. MAC 451

by

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A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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CLAIM OF CONTRIBUTIONS TO KNOWLEDGE

The following information, as revealed in this work, is claimed to be a contribution to knowledge.

1. Phloroglucinol reductase, the first enzyme involved in the pathway of phloroglucinol degradation by *Pseudomonas* sp. Mac 451 was successfully isolated and purified approximately 23-fold. The enzyme is able to catalyse a rapid oxidation of NADPH with phloroglucinol as the acceptor. It is most stable at pH 7.0 and has an optimum pH at 7.4 in phosphate buffer. The Km for phloroglucinol is 6.25×10^{-6}M. The substrate specificity and kinetic properties of the enzyme were studied in detail. The product of enzymatic reduction of phloroglucinol is tentatively identified as dihydrophloroglucinol. The reaction catalysed by phloroglucinol reductase is postulated as

$$\text{Phloroglucinol} + \text{NADPH} + H^{+} \rightleftharpoons \text{Dihydrophloroglucinol} + NADP^{+}$$

2. Dihydrophloroglucinol, the first intermediate of phloroglucinol degradation, was successfully isolated and tentatively identified from a growing cell fermentation liquor. The product of the chemical reduction of phloroglucinol was also tentatively identified as dihydrophloroglucinol.

3. Resorcinol, the second intermediate of phloroglucinol degradation, was successfully isolated and definitely identified from a growing cell fermentation liquor.

4. A new pathway of phloroglucinol degradation by Pseudomonas sp. Mac 451 is postulated. This pathway involves the reduction of phloroglucinol to dihydrophloroglucinol which is dehydrated then to resorcinol. The dihydroxy phenol is further metabolized.

INTRODUCTION

Aromatic compounds accumulate in plants and are deposited in soils when plants die. A number of soil microorganisms, notably species of Pseudomonas, have been found to metabolize these compounds as carbon and energy sources. This process is very important in maintaining the balance of chemical substances available to living things and thus plants incorporate carbon atoms into benzene rings in compounds like lignins which cannot be metabolized further by plants or animals and soil microorganisms are necessary to free the carbon atoms for other uses.

Phloroglucinol (sym. 1,3,5-trihydroxybenzene) occurs in nature frequently as a glucoside in plants. One well known compound is phloridzin (from Greek phloos "bark" and riza "root") from which the phenol gets its name. Phloroglucinol is a constituent of several naturally occurring substances, e.g., quercetin,hesperidin, flavones, anthocyanins, filicic acid and the catechins and is obtained by acid hydrolysis of these compounds. The usual route of chemical synthesis involves the reduction of trinitrobenzene or trinitrobenzoic acid with tin or iron and concentrated HCl to triaminobenzene followed by neutralization and hydrolysis to phloroglucinol (Clark and Hartman, 1929). Biosynthesis of phloroglucinol has been

shown to involve the head-to-tail linkage of acetate units (Birch and Donovan, 1953).

A review of the literature on the occurrence and importance of phloroglucinol was made recently by Robern (1965). Pascoe (1955) reviewed the chemistry and industrial application of phloroglucinol.

The aims of this work are to isolate and characterize the intermediates involved in the pathway of phloroglucinol degradation by Pseudomonas sp. Mac 451 using growing culture and resting cells and to isolate, purify and study the properties of phloroglucinol reductase.

This thesis is divided into three parts. The first describes the detection of the intermediates of phloroglucinol degradation by growing culture and resting cells. The second part deals with the isolation and characterization of the intermediates of phloroglucinol degradation by growing culture. The third part describes the purification and properties of phloroglucinol reductase and the role this enzyme plays in the pathway of phloroglucinol degradation.

LITERATURE REVIEW

Robern (1965) has made a survey of the literature on the metabolism of phloroglucinol and other phenolic compounds by microorganisms and animals. This literature review concerns only the degradation of phloroglucinol by microorganisms.

Wagner (1914) was the first person to isolate bacteria from soil and faeces able to metabolize phloroglucinol, catechol, phenol and benzene. Organisms, capable of growing in medium containing phloroglucinol as sole carbon source, were not able to assimilate resorcinol, catechol, benzene, pyrogallol or hydroquinone. Subsequently, Gray and Thornton (1928) isolated from soil, bacteria capable of growing in pure culture on phloroglucinol, cresol, resorcinol, resorcylic acid and other related compounds as the sole carbon source. Bennetti and Schlesser (1950) isolated five species of Flavobacterium able to utilize phloroglucinol, resorcinol, benzoic acid and pyrogallol as the sole source of carbon. In a recent study Mullakhanhai and Bhat (1966) found that a basal mineral medium containing 0.001% yeast extract and phloroglucinol, orcinol, resorcinol, catechol, phenol, gallic acid and benzene could support the growth of Arthrobacter spp.

Higuchi (1953) isolated and partially purified an enzyme from the mycelial mat of Coriolus hirustus capable of carrying out the oxidation of phloroglucinol,

resorcinol, catechol and pyrogallol. Bernheim (1956) reported that an adaptive enzyme was formed in a Mycobacterium sp. if the organism was preincubated with phloroglucinol. If one of the three hydroxyl groups meta to each other on the ring was substituted by a methyl group as in orcinol, the enzyme was formed much more slowly, but the enzyme once formed was able to oxidize orcinol rapidly. The substitution of a carboxyl group as in 3,5-dihydroxybenzoic acid failed to induce the formation of the enzyme. The oxygen uptake varied between four and six atoms per mole of phloroglucinol metabolized and carbon dioxide was produced. The conclusion was that the ring was split and that some assimilation of the end products occurred and accounted for the variability in oxygen uptake. Undoubtedly, more than one enzyme was involved in the over-all reactions. Subsequently, Nakagawa and Takeda (1962) described the partial purification and some properties of phenol hydroxylase from Brevibacterium fuscum and found that the enzyme catalyzed also the oxidation of various phenol derivatives including orcinol, phloroglucinol, resorcinol and cresol.

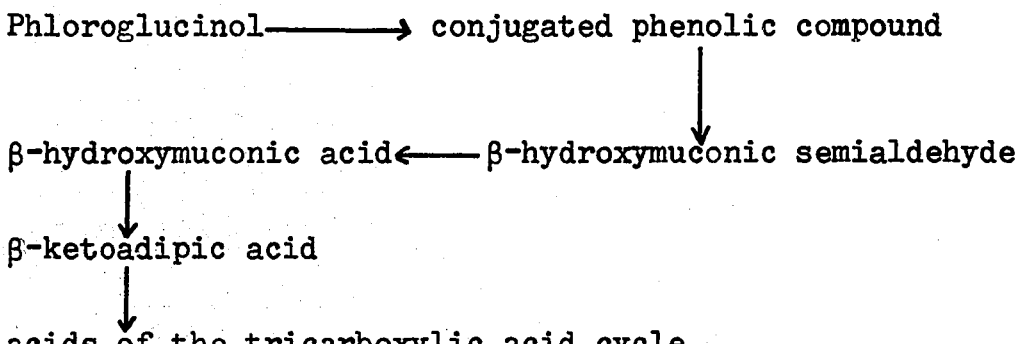
Craigie et al. (1965) observed the release of $C^{14}O_2$ from uniformly labelled C^{14}-phloroglucinol incubated aseptically with several marine algae. The destruction of the aromatic ring was enhanced several-fold by pre-

incubating the algae with non-radioactive phloroglucinol and this probably represented an adaptive mechanism similar to that found in other organisms.

Robern (1965) working in this Department isolated a Penicillium sp. and a Pseudomonas sp. from soil both capable of utilizing phloroglucinol as the sole carbon source in a medium containing various inorganic salts. Utilization of phloroglucinol by the Penicillium sp. was shown by paper chromatographic techniques and no intermediates could be detected either from a growing cell culture or from a resting cell suspension. However, spectrophotometric analyses of the resting cell fermentation liquor showed the accumulation of an unidentified compound which was not further metabolized by the fungus.

The effects of various factors such as temperature, pH and nutrition on the metabolism of phloroglucinol by the Pseudomonas sp. were also reported by Robern (1965). Under optimum conditions, 0.1% phloroglucinol was utilized in 12 hours by the bacterium. The cell-free extracts prepared from the bacterium required NADH or NADPH in the decomposition of phloroglucinol. The optimal pH of the fractionated crude enzyme ranged from 7.2 to 7.6 and the Km for phloroglucinol was 6.08×10^{-7}M. Manometric experiments showed that the fractionated crude enzyme was not active oxidatively, but was NADPH dependent (NADH was less active) producing a conjugated phenolic compound

when incubated in the presence of phloroglucinol. Based on these findings, Robern concluded that the first enzymatic process was probably NADPH dependent and reduction of phloroglucinol took place by removal of a hydroxyl group from the benzene ring. The pathway of phloroglucinol degradation by the Pseudomonas sp. was then proposed as follows:



PART I

DETECTION OF INTERMEDIATES OF PHLOROGLUCINOL DEGRADATION BY GROWING CULTURES AND BY RESTING CELLS

INTRODUCTION

Robern (1965) first studied the pathway of phloroglucinol degradation by growing culture and by resting cells. Using paper chromatographic techniques, he showed the presence of an intermediate, (giving a green colored spot with vanillin toluene p-sulfonic acid) in a growing cell fermentation medium and two other intermediates (giving green and pink colorations respectively) in a resting cell fermentation medium. The compound producing the latter green color was identified as β-ketoadipic acid and the one producing the pink color was postulated as a conjugated phenolic compound which gave rise to resorcinol after acid hydrolysis of the fermentation liquor.

Randerath (1963) has summarized the R_f values and color reactions of a number of simple phenols and some related compounds on silica gel G layers but no reports have appeared on the detection of the intermediates involved in the pathway of phloroglucinol degradation by thin-layer chromatographic techniques. The aim of the present investigation is the detection by thin layer chromatography of the intermediates of phloroglucinol degradation by Pseudomonas sp. Mac 451, and a comparison of the results with those reported by Robern (1965).

MATERIALS AND METHODS

Organism-Pseudomonas sp. Mac 451 used in this work was isolated and described by Robern (1965).

Preparation of Media- The medium used for growing the bacterium was prepared according to Robern (1965) and the basal medium contained 0.1% $(NH_4)_2SO_4$, 0.05% $MgSO_4.7H_2O$, 10% of a 1 M potassium phosphate buffer (pH 7.2) and 0.1% phloroglucinol. The salts and substrate were sterilized separately and then combined. Slants and plating media were prepared by adding 1.5% agar to the basal medium.

Growth and Maintenance of Culture- The bacterium was grown on a 0.1% phloroglucinol basal medium at 30° C for 18 hours. The culture was stored on agar slants at 4° C and rejuvenated periodically.

Preparation of Cells- From a phloroglucinol agar slant a 50-ml Erlenmeyer flask containing 25 ml of phloroglucinol basal medium was inoculated and incubated at 30° C on a rotary shaker. After 18 hours incubation, the growth medium was transferred to a 2000-ml Erlenmeyer flask containing 500 ml of medium of the same composition and incubated at 30° C for 18 hours on a rotary shaker

having one inch eccentricity at a rate of 250 rpm.

Resting cell suspensions were prepared by harvesting cells grown for 18 hours in a phloroglucinol basal medium at 30° C by centrifuging in a Servall Superspeed centrifuge at 14,600 x g for 15 minutes. The cells (about 7 g) were then washed in 0.01 M phosphate buffer (pH 7.2) three times and resuspended in a 2000-ml Erlenmeyer flask containing 500 ml of the same medium except that $(NH_4)_2SO_4$ was deleted. The medium was then incubated at 30° C on a rotary shaker.

Technique of Thin-Layer Chromatography- The thin-layer plates were prepared by vigorous shaking 40 g of silica gel G with 80 ml of distilled water in a 250-ml stoppered conical flask for 45 seconds. The suspension was then immediately transferred to an open spreader (Desaga Co., Heilderberg,Germany) having set previously the desired layer thickness (500μ). The coated plates (4 plates 20 x 20 cm or 8 plates 10 x 20 cm or 16 plates 5 x 20 cm) were air-dried overnight and stored in a desiccator. They were activated at 105° C for 30 minutes before use.

The chromatography tanks were rectangular glass vessels. The atmosphere in the tanks was saturated by covering the walls with filter paper which was soaked with solvent by swirling the vessel before putting in the plates.

Solvent systems used in this work were benzene-methanol-acetic (45:8:4) (Randerath,1963); 95% ethanol-concentrated ammonium hydroxide (100:1) (Kennedy and Barker, 1951) and benzene-dioxane-acetic acid (90:25:4) (Randerath,1963).

The chromatograms were prepared by applying samples on the starting line of the plates and allowing them to dry. The plates were then placed in the chromatography tank which had been filled to a depth of 0.5 cm with the solvent. The plates were removed from the tank as soon as the solvent front travelled a distance of 10 cm. The chromatograms were dried at room temperature.

The color developing reagents used for the detection of the chromatograms were described as follows:

1. Tetrazotized benzidine (Randerath,1963):

Solution I: 5 g benzidine was dissolved in 14 ml of concentrated HCl and diluted with distilled water to 1000 ml.

Solution II: Aqueous 10% sodium nitrite solution.

Equal volumes of the two solutions were mixed just before use. This mixture can be kept for 2-3 hours. After spraying, the plates were placed in the drying cabinet at 105° C for a few minutes until the spots were clearly visible.

2. Vanillin toluene p-sulfonic acid (Roux and Maihs, 1960): 2 g vanillin and 1 g toluene p-sulfonic acid were dissolved in 100 ml of absolute ethanol. The reagent was stable for several weeks if kept in the cold. After spraying, the plates were placed in the drying cabinet at 105° C for a few minutes until the spots were clearly visible.
3. Bromocresol purple (Reid and Lederer,1951): 40 mg bromocresol purple were dissolved in 100 ml of 1:5 dilution on formalin in ethanol and the pH was adjusted to about 5 with 0.1 N NaOH. The sprayed chromatograms were held for 2-3 minutes in atmosphere saturated with 3% aqueous NH_3, and the acids appeared as bright yellow spots on a purple background.
4. Sucrose reagent (Roux, 1951): 2 g of sucrose were mixed with 10 ml of concentrated HCl and 90 ml of absolute ethanol. Complete solution was not achieved, but the suspension was sprayed on the chromatograms. After spraying, the plates were placed in the drying cabinet at 105° C until the spots were clearly visible. Fructose could be used to replace sucrose.

Preparation of Samples for Thin-Layer Chromatography- 1000 ml of medium containing 0.1% phloroglucinol in basal medium was inoculated and then incubated at 30° C on a

rotary shaker for 18 hours. After removal of the cells by centrifugation at 14,600 x g for 15 minutes, the supernatant solution was acidified to pH 2.0 with concentrated HCl and extracted with ethyl ether in a continuous ether extractor overnight. The ethereal extract was then evaporated to dryness under reduced pressure at 35° C. The residue was dissolved in 2 ml of 95% ethanol and 50 lambda were spotted on silica gel G layer.

Samples of a resting cell fermentation were taken at intervals to study both the rate of utilization of substrate and the production of intermediates. Samples (20 ml) of fermentation liquor were withdrawn at specified times and after removal of the cells by centrifigation, 0.5 ml of each sample was used for ultraviolet absorption spectrophotometric analysis and the remaining fermentation liquor was acidified to pH 2.0 with concentrated HCl and extracted with 10 ml of ethyl ether five times. The ethereal extracts were then pooled and evaporated to dryness under reduced pressure at 35° C. The residue was dissolved in 1 ml of 95% ethanol and 50 lambda aliquot were spotted on silica gel G layer.

Ultraviolet Absorption Spectrophotometry- To a cuvette having a capacity of 3 ml with 10 mm light path 0.5 ml of sample and 2.5 ml of distilled water were added. In a control cuvette, 0.5 ml of distilled water was added in place of the sample. A recording UNICAM SP. 800 ultraviolet spectrophotometer was used for all analyses.

RESULTS

Chromatography of Fermentation Liquor from Growing Culture- The R_f values and color reactions of the intermediates involved in the pathway of phloroglucinol degradation by growing culture are summarized in Table I. Five intermediates were successfully detected when suitable solvent systems and detectors were used. The intermediates I and II gave blue fluorescence and yellow fluorescence respectively when examined under UV light. They gave negative results when sprayed with color developing reagents. The intermediates III and IV, however, gave positive results when sprayed with known phenolic detecting reagents such as vanillin toluene p-sulfonic acid, sucrose reagent and tetrazotized benzidine and suggests strongly that these two intermediates are phenolic compounds. Intermediate V is probably an aliphatic acid since it appeared as a bright yellow spot on a purple background when sprayed with bromocresol purple.

Chromatography of Fermentation Liquor from Resting Cells- Both the rate of utilization of substrate and production of intermediates by resting cells were studied chromatographically. Fig. 1 shows the tracings of thin-layer chromatograms of samples of a resting cell fermentation. Development was with benzene-methanol-acetic acid

Table I R_f values and color reactions of intermediates of phloroglucinol degradation

Intermediate	R_f in Solvent System[A] 1	2	3	Ultraviolet light	Vanillin toluene p-sulfonic acid	Sucrose reagent	Tetrazotized benzidine	Bromocresol purple
I	0.79	0.82	0.88	Blue fluorescence	-	-	-	-
II	0.49	0.60	0.43	Yellow fluorescence	-	-	-	-
III	0.40	0.86	0.56	-	Pink	Pink	Reddish brown	-
IV	0.22	0.75	0.07	-	Yellow	Pink	Brown	-
V	-	0.81	-	-	-	-	-	Yellow

[A] Solvent System 1: benzene-methanol-acetic acid (45:8:4)
2: 95% ethanol-concentrated ammonium hydroxide (100:1)
3: benzene-dioxane-acetic acid (90:25:4)

and vanillin toluene p-sulfonic acid was used as the detecting spray. Note the gradual decrease in the concentration of phloroglucinol with a simultaneous increase in two products, one yellow and the other pink, with R_f values of 0.22 and 0.40 respectively. The concentration of the products increased up to the second hour and after longer incubation gradually disappeared.

Ultraviolet Absorption Spectrophotometry of a Resting Cell Fermentation Liquor- Both the rate of utilization of substrate and the production of intermediates by resting cells were studied spectrophotometrically. Note a shift in the absorption peak from 268 mμ to 278 mμ after two hours incubation (Fig.2). The presence of the new peak at 278 mμ indicates the formation of a new compound which reached its maximum concentration after two hours incubation, after five hours incubation the new peak disappeared.

Fig.1 Tracings of thin-layer chromatograms of a resting cell fermentation liquor

1. Pink spot with a R_f value of 0.40 (intermediate III)
2. Pink spot with a R_f value of 0.28 (phloroglucinol)
3. Yellow spot with a R_f value of 0.22 (intermediate IV)

Solvent system: benzene-methanol-acetic acid (45:8:4)

Color developing reagent: vanillin toluene p-sulfonic acid

Fig.1 Tracings of thin-layer chromatograms of a resting cell fermentation liquor

1. Pink spot with a R_f value of 0.40 (intermediate III)
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Solvent system: benzene-methanol-acetic acid (45:8:4)

Color developing reagent: vanillin toluene p-sulfonic acid

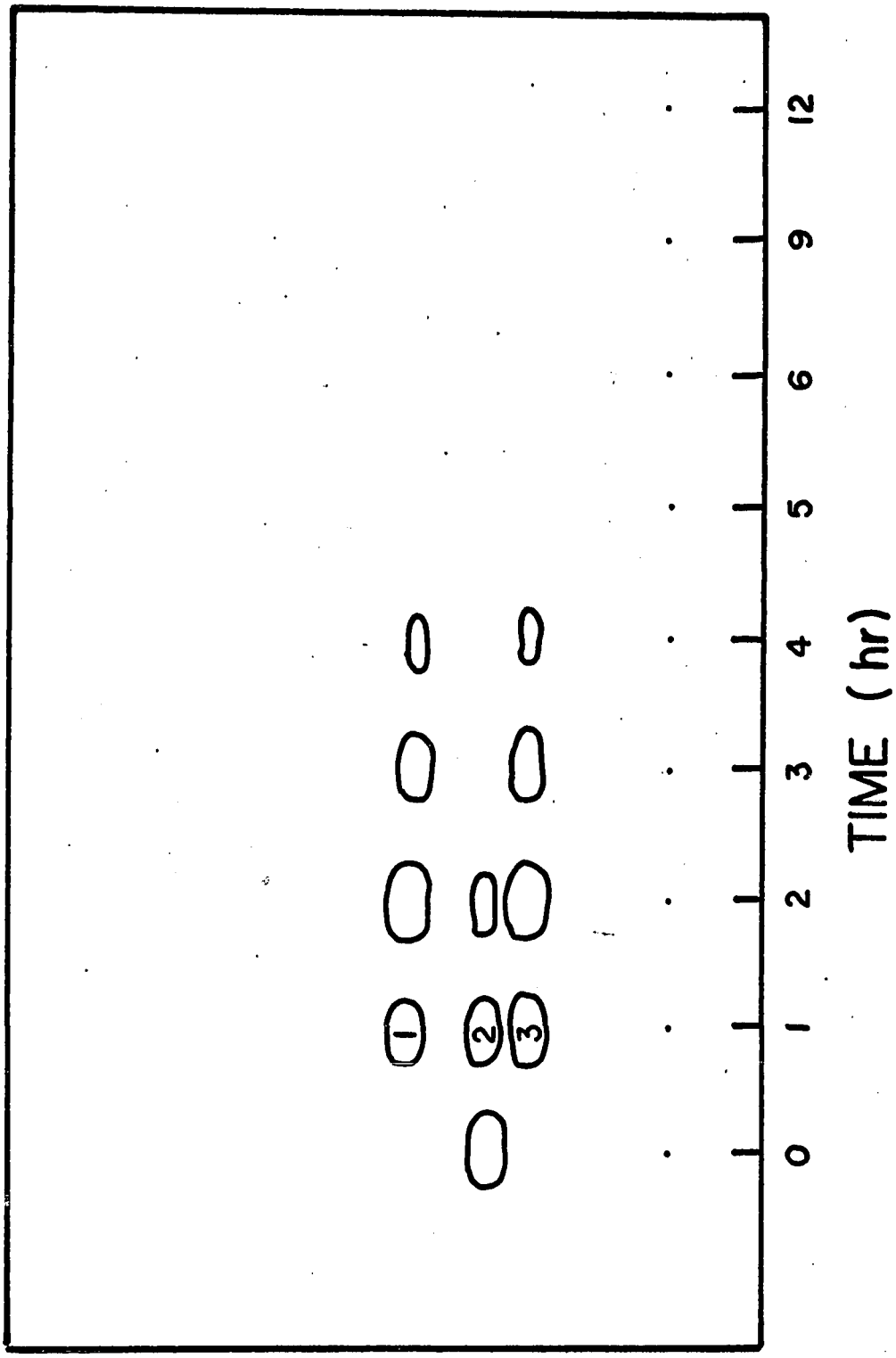
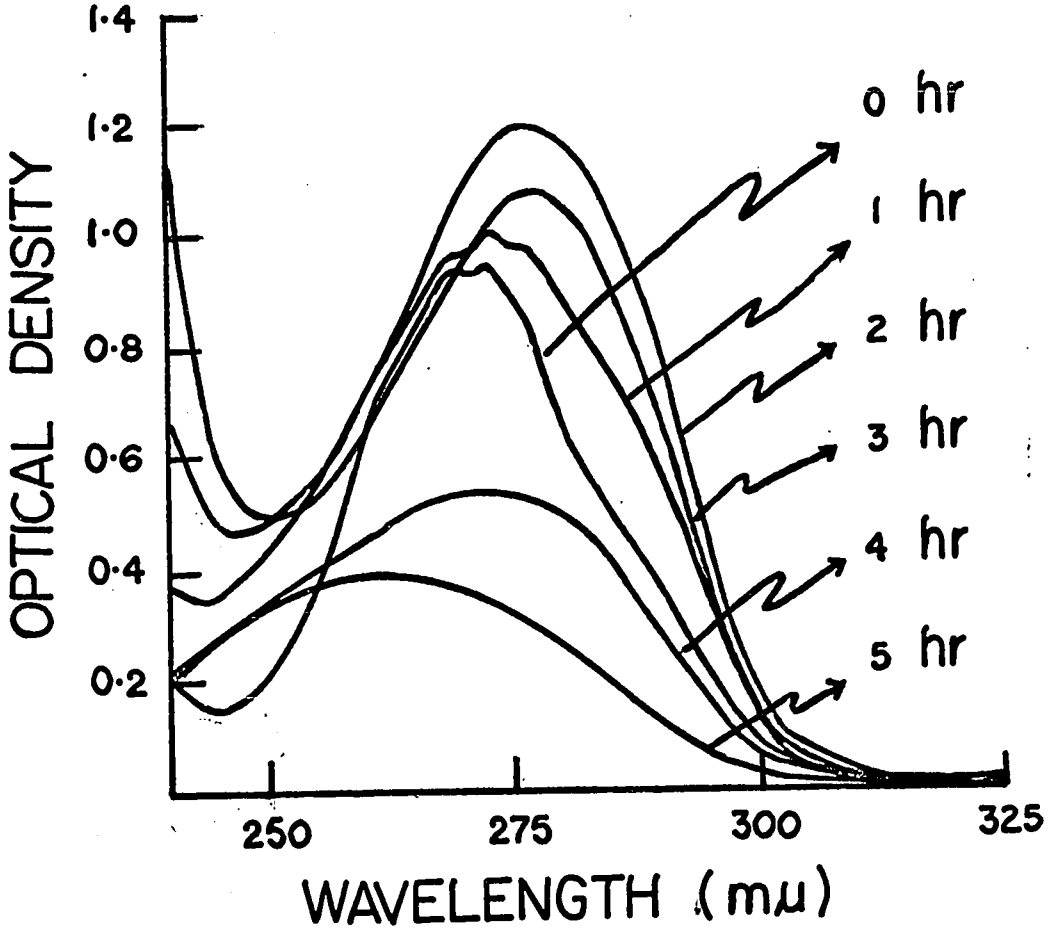

0
1
2
3
4
5
6
9
12
TIME (hr)

Fig.2 Ultraviolet absorption spectra of the fermentation liquor at different time intervals. The spectra were recorded in a UNICAM SP. 800 ultraviolet absorption spectrophotometer.


1·4
1·2
1·0
0·8
0·6
0·4
0·2
OPTICAL DENSITY
0 hr
1 hr
2 hr
3 hr
4 hr
5 hr
250
275
300
325
WAVELENGTH (mμ)

DISCUSSION

With paper chromatographic techniques, Robern (1965) reported that one green spot was observed on the chromatograms of samples obtained from a growing cell fermentation when sprayed with vanillin toluene p-sulfonic acid. In the present investigation, detection of the intermediates involved in the pathway of phloroglucinol degradation by Pseudomonas sp. Mac 451 was attempted with thin-layer chromatographic techniques. Two intermediates, tentatively identified as phenolic compounds, were successfully detected on the chromatograms of samples obtained from a growing cell fermentation. Three additional intermediates, two fluorescent compounds and an aliphatic acid could also be detected on the chromatograms developed in suitable solvent systems when examined under UV light and sprayed with bromocresol purple respectively. The intermediate giving a green color which Robern (1965) observed on his paper chromatograms, was not detected on the thin-layer chromatograms even when sprayed with the same color developing reagent (vanillin toluene p-sulfonic acid).

Robern (1965) also showed the presence of two other intermediates with green and pink colorations when sprayed with vanillin toluene p-sulfonic acid on the paper chromatograms of samples of a resting cell fermentation. The intermediate

giving a green spot was identified as β-ketoadipic acid and the pink intermediate was postulated as a conjugated phenolic compound which was shown to give rise to resorcinol after acid hydrolysis of the fermentation liquor. In the present investigation, two products, one yellow and the other pink, as detected with vanillin toluene p-sulfonic acid were observed on the thin-layer chromatograms of samples of a resting cell fermentation. The green spot and the pink spot which were identified as β-ketoadipic acid and a conjugated phenolic compound respectively by Robern (1965), however, were not observed on the chromatograms even when sprayed with the same color developing reagent (vanillin toluene p-sulfonic acid). This discrepancy is probably due to different methods used in the investigations.

In his investigation during a fermentation trial using resting cell of a Penicillium sp., Robern (1965) reported that a compound accumulated in the culture medium which absorbed at 278 mμ. This compound was not utilized by the fungus and could not be detected by paper chromatographic techniques. In the present work, both the rate of utilization of phloroglucinol and the production of intermediates were also followed spectrophotometrically. A shift in the absorption peak from 268 mμ to 278 mμ was noted after two hours incubation. The presence of the new peak at 278 mμ indicates the

formation of a new compound which reached its maximum concentration after two hours incubation; after five hours incubation the new peak was not observed. These data indicate that both intermediates are possibly identical since they have similar absorption peak at 278 mμ.

PART II

ISOLATION AND CHARACTERIZATION OF INTERMEDIATES OF PHLOROGLUCINOL DEGRADATION BY GROWING CULTURE

INTRODUCTION

In his studies on the metabolism of phloroglucinol ~~degradation~~ by a Pseudomonas sp., Robern (1965) observed with paper chromatographic techniques that a conjugated phenolic compound and β-ketoadipic acid accumulated transiently in the resting cell fermentation liquors. The conjugated phenolic compound was shown to give rise to resorcinol after acid hydrolysis of the fermentation liquor. Robern (1965) was subsequently able to confirm his observations by the direct isolation of these intermediates from the fermentation liquors. Resorcinol was isolated from a hydrolyzed fermentation medium and characterized by its melting point, UV spectrum and the formation of the derivative resorcinol dibenzoate. β-ketoadipic acid was isolated as levulinic acid 2,4-dinitrophenylhydrazone. The formation of levulinic acid was shown to be the result of decarboxylation of β-ketoadipic acid. Attempts to isolate β-ketoadipic acid 2,4-dinitrophenylhydrazone were unsuccessful.

Studies reported in Part I of this thesis concerning the detection of the intermediates involved in the pathway of phloroglucinol degradation by growing culture and

by resting cells as detected on the thin-layer chromatograms revealed the presence of two phenolic compounds, two fluorescent compounds and an aliphatic acid in a growing cell fermentation, and only two phenolic compounds in a resting cell fermentation. The aim of the present study involves the isolation and characterization of these intermediates from a growing cell fermentation liquor.

MATERIALS AND METHODS

Preparation of Sample for Sephadex Column Chromatography- Ten liters of medium containing 0.1% phloroglucinol in basal medium was inoculated and incubated at 30° C on a rotary shaker for 18 hours. After removal of the cells by centrifugation at 14,600 x g for 15 minutes, the supernatant solution was concentrated to 2 liters under reduced pressure at 35° C. The concentrated solution was then acidified with HCl to pH 2.0 and extracted with ethyl ether in a continuous extractor for 24 hours. The ethereal extract was then flash evaporated to dryness at 35° C. The residue was dissolved in 2.5 ml of distilled water, then passed through a column of Sephadex G-10.

Preparation of Sephadex G-10 Column- A 1.5 x 64 cm column was prepared from 45 g of Sephadex G-10 suspended in distilled water. After equilibrating the column with distilled water overnight, the sample was added, and then eluted with distilled water, collecting 5 ml fractions at room temperature in an automatic fraction collector (Gilson Medical Electronics, Middleton,Wis., U.S.A.). To detect the intermediates of phloroglucinol degradation, 50 lambda of each fraction were spotted on silica gel G thin-layer plate which was prepared and developed as described previously.

Preparation of Purified Silica Gel H Plates- 60 g of silica gel H were washed twice with 120 ml of methanol. After the second filtration through a sintered glass funnel, the silica gel was suspended in 60 ml of methanol. This suspension was then poured on two 20 x 20 cm plates and evenly distributed by tapping and shaking. The plates were air-dried overnight and stored in a desiccator. They were activated at 105° C for 30 minutes before use.

Chemical Reduction of Phloroglucinol- Fieser (1955) demonstrated that reduction of benzil was accomplished rapidly with sodium borohydride in ethanol. In the present investigation, attempts to reduce phloroglucinol with $NaBH_4$ were made. Phloroglucinol (100 mg) were dissolved in a 250-ml Erlenmeyer flask containing 100 ml of distilled water. Then, 20 mg of $NaBH_4$ (large excess) were added and 0.5 ml aliquots of the reaction mixture were withdrawn at specified times. Fifty lambda of each sample were spotted on silica gel G thin-layer plate and the remaining solution was used for ultraviolet absorption spectrophotometric analysis.

The isolation of the product of chemical reduction of phloroglucinol was attempted as follows: 500 mg of phloroglucinol were dissolved in a 250-ml Erlenmeyer flask containing 60 ml of distilled water and 100 mg of $NaBH_4$ (large excess); after 10 minutes, the reaction mixture was flash evaporated to remove water. The viscous residue was

dissolved in 1 ml of distilled water and passed through a column of Sephadex G-10 column with distilled water as the eluant. Five milliliter fractions were collected and those containing the chemically reduced product were detected with vanillin toluene p-sulfonic acid; after pooling, water was removed by flash evaporation and the viscous residue was stored at 4° C.

RESULTS

Isolation of the Intermediates Involved in the Pathway of Phloroglucinol Degradation on Sephadex G-10 Column

Three intermediates arising from phloroglucinol degradation were successfully separated from one another through Sephadex G-10. The elution pattern observed on Fig.3 indicates that intermediate II (fractions 11-13) appeared first and intermediate IV (fractions 17-19) appeared before intermediate III (fractions 106-136) in the eluate.

Isolation of Intermediate II- Fractions 11-13 obtained from Sephadex G-10 column were pooled and evaporated to dryness under reduced pressure at 35° C. The residue was dissolved in 1 ml of methanol and the resulting solution was then applied to a 20 x 20 cm plate of purified silica gel H. The chromatogram was developed with benzene-methanol-acetic acid (45:8:4). When the development of the chromatogram had been completed, the position of the spots were established under UV light, then, the layer was removed with a scalpel and transferred to a centrifuge tube. The layer was extracted with 10 ml of methanol, then centrifuged. The clear solution was decanted and evaporated to dryness in a small vial at room temperature. The sample obtained was used for mass spectroscopic analysis.

Isolation of Intermediate III- Fractions 106-136 obtained from Sephadex G-10 column were pooled and evaporated

Fig.3 Chromatography of intermediates of phloroglucinol degradation on Sephadex G-10
Solvent System: benzene-methanol-acetic acid (45:8:4)

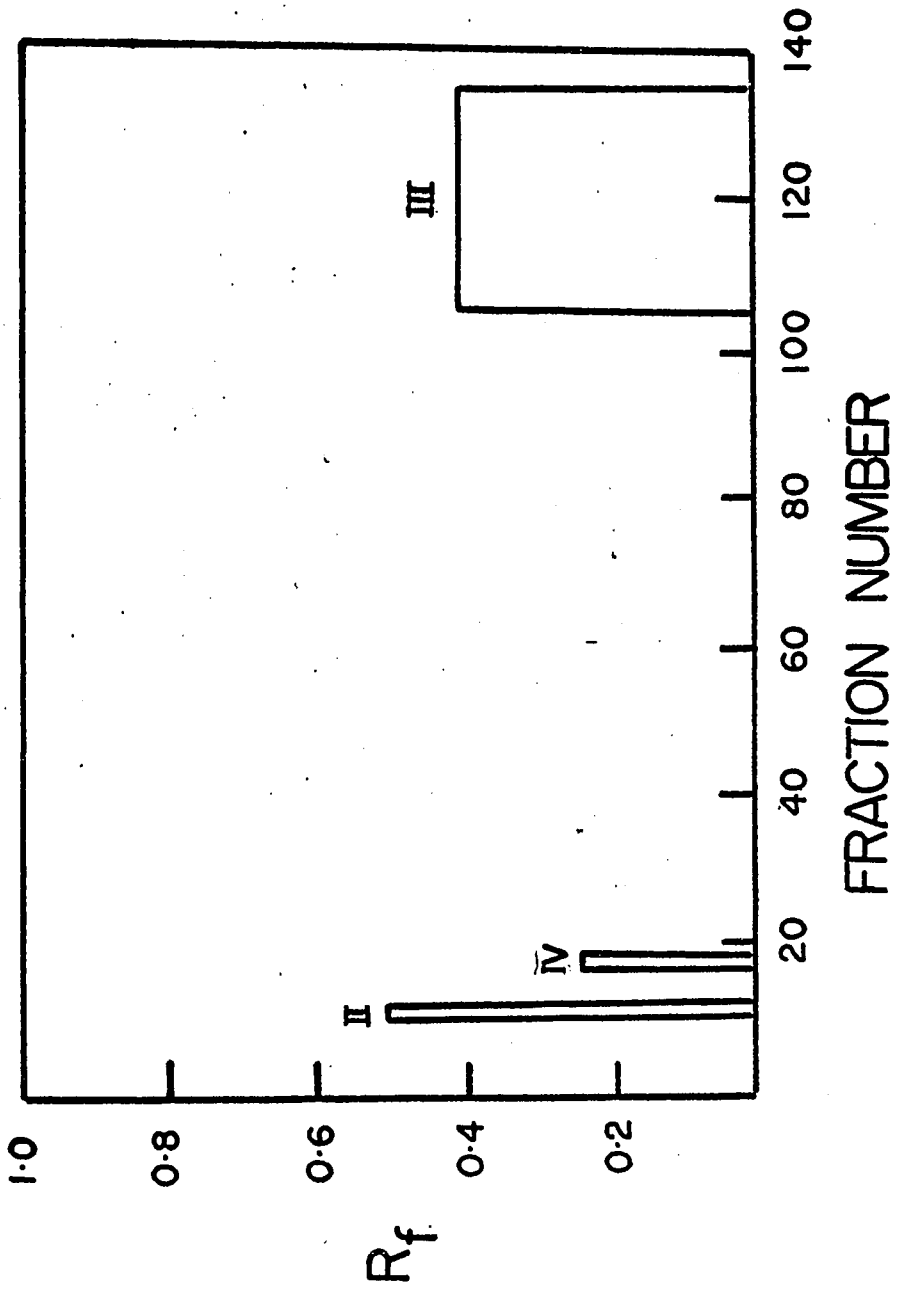

1·0
0·8
0·6
0·4
0·2
R_f
II
IV
III
20
40
60
80
100
120
140
FRACTION NUMBER

Table II R_f values and color reactions of Sephadex G-10 column eluates and of authentic samples

Intermediate	Fraction number	R_f Solvent System* 1	2	3	Ultra-violet light	Vanillin toluene p-sulfonic acid	Sucrose reagent	Tetrazotized benzidine
II	11-13	0.49	0.60	0.43	Yellow fluores-cence	-	-	-
III	106-136	0.40	0.86	0.56	-	Pink	Pink	Reddish brown
IV	17-19	0.22	0.75	0.07	-	Yellow	Pink	Brown
Resorcinol		0.40	0.86	0.56	-	Pink	Pink	Reddish brown
Chemically reduced product		0.22	0.75	0.07	-	Yellow	Pink	Brown

* Solvent System. 1: benzene-methanol-acetic acid (45:8:4)
2: 95% ethanol-concentrated ammonium hydroxide (100:1)
3: benzene-dioxane-acetic acid (90:25:4)

to dryness under reduced pressure at 35° C. The residue was dissolved in boiling benzene and the needle-like crystals formed at 4° C were collected by filtration and dried in vacuo at room temperature.

Isolation of Intermediate IV- Fractions 17-19 obtained from Sephadex G-10 column were pooled and evaporated to dryness under reduced pressure at 35° C. The residue was dissolved in 1 ml of methanol and the resulting solution was applied to a 20 x 20 cm plate of purified silica gel H. The chromatogram was developed with benzene-methanol-acetic acid (45:8:4). When the development of the chromatogram had been completed, the larger part of the layer was covered with a sheet of glass and the uncovered part of the chromatogram was sprayed with tetrazotized benzidine solution. After the position of the spots were established, the layer was removed carefully with a scalpel and transferred to a centrifuge tube. The sample was extracted with 10 ml of methanol. The clear solution was decanted and evaporated to dryness in a small vial at room temperature. The sample obtained was used for mass spectroscopic analysis.

Chemical Reduction of Phloroglucinol

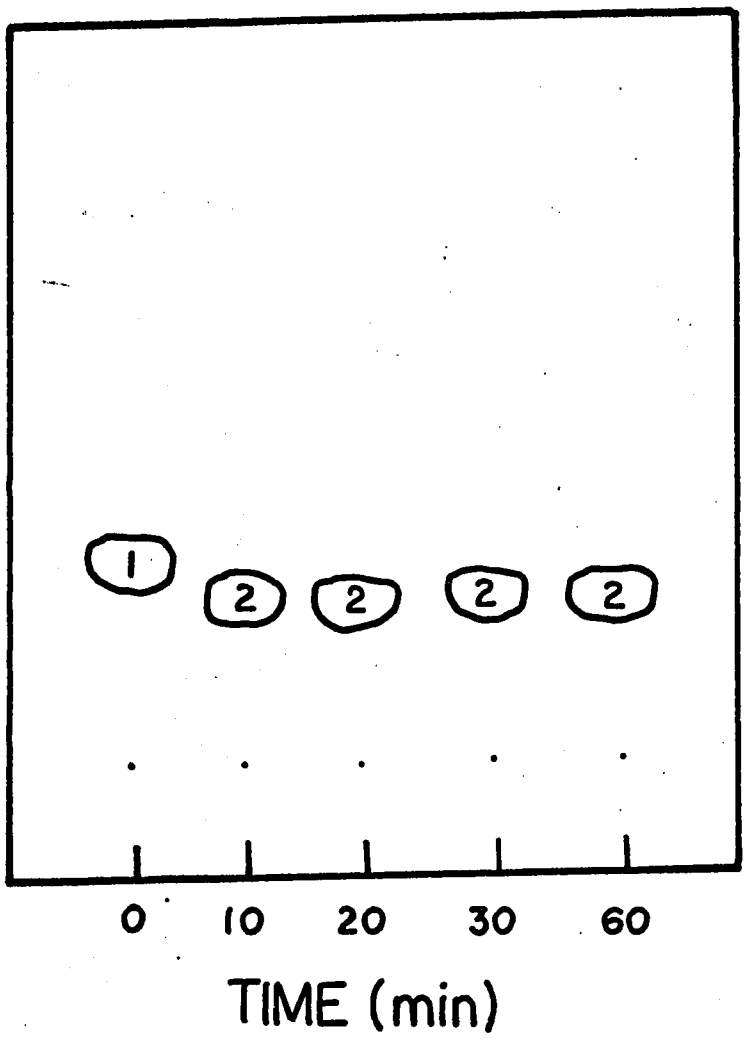
The rate of chemical reduction of phloroglucinol was followed chromatographically. Fig.4 shows the tracings of thin-layer chromatogram of the rate of chemical reduction of phloroglucinol. Note the complete reduction of phloroglucinol in 10 minutes. The product formed remains unchanged even on prolonged incubation of the reaction

Fig.4 Tracings of thin-layer chromatogram of the time study of the reduction of phloroglucinol with sodium borohydride

1. A pink spot with a R_f value of 0.28 (phloroglucinol
2. A yellow spot with a R_f value of 0.22 (chemically reduced product)

Solvent System: benzene-methanol-acetic acid (45:8:4)

Color reagent: vanillin toluene p-sulfonic acid


1
2
2
2
2
0
10
20
30
60
TIME (min)

mixture. Its R_f value and color reaction are shown in Table II. The rate of chemical reduction of phloroglucinol was also followed spectrophotometrically. A shift in the absorption peak from 268 mμ to 278 mμ was found after ten minutes incubation and this new peak at 278 mμ did not disappear when the reaction mixture was incubated for 1 hour.

Characterization of the Intermediates Involved in the Pathway of Phloroglucinol Degradation

The isolated intermediates were characterized by melting point determination, thin-layer chromatography, ultraviolet absorption spectrophotometry and mass spectroscopy.

Characterization of Intermediate II- The R_f values and color reactions of intermediate II are shown in Table II. Note that a yellow fluorescence was found when examined under UV light. No maximum absorption was observed from 190 mμ to 700 mμ when the sample was examined in a recording UNICAM SP.800 ultraviolet spectrophotometer. The mass spectrum of the sample and the interpretation were provided by Dr.G.P.Arsenault, Atlantic Regional Laboratory, National Research Council. The mass spectrum of the sample did not give any information except that it contained some dioctylphthalate.

Characterization of Intermediate III- The crystallized material (yield 8.2 mg) and authentic resorcinol both melted at 110° C (uncorrected) and gave no melting point depression on admixture. The ultraviolet spectra were also comparable (Fig.5). The R_f values and color reactions of the two compounds were identical in the thin-layer chromatograms (Table II). When mixed together, both moved as a single spot. The results obtained from mass spectrometry showed the presence of peaks at m/e 110 in the spectra of the sample and authentic resorcinol.

On the basis of the data given above, the intermediate III is identified definitely as resorcinol.

Characterization of Intermediate IV- The mass spectrum of the sample showed the presence of the spectrum of resorcinol (m/e 110). An explanation for this result is that the intermediate IV (molecular weight 128) which loses elements of water (molecular weight 18) on electron impact gives rise to ions at m/e 110 which have identical structure to the molecular ion of resorcinol.

The R_f values and color reactions of intermediate IV are shown in Table II. Its ultraviolet absorption peak was found to be 278 mμ (Fig.6).

The results obtained from enzymatic studies described in Part III of this thesis indicate that intermediate IV is identical to the product of enzymatic reduction of phloroglucinol (see Table IV). Stoichiometry of phloroglucinol

Fig.5 Ultraviolet absorption spectra of authentic and biologically obtained resorcinol. The solid line indicates the authentic compound and the broken line indicates the biologically obtained specimen. The spectra were recorded in a UNICAM SP.800 ultraviolet absorption spectrophotometer

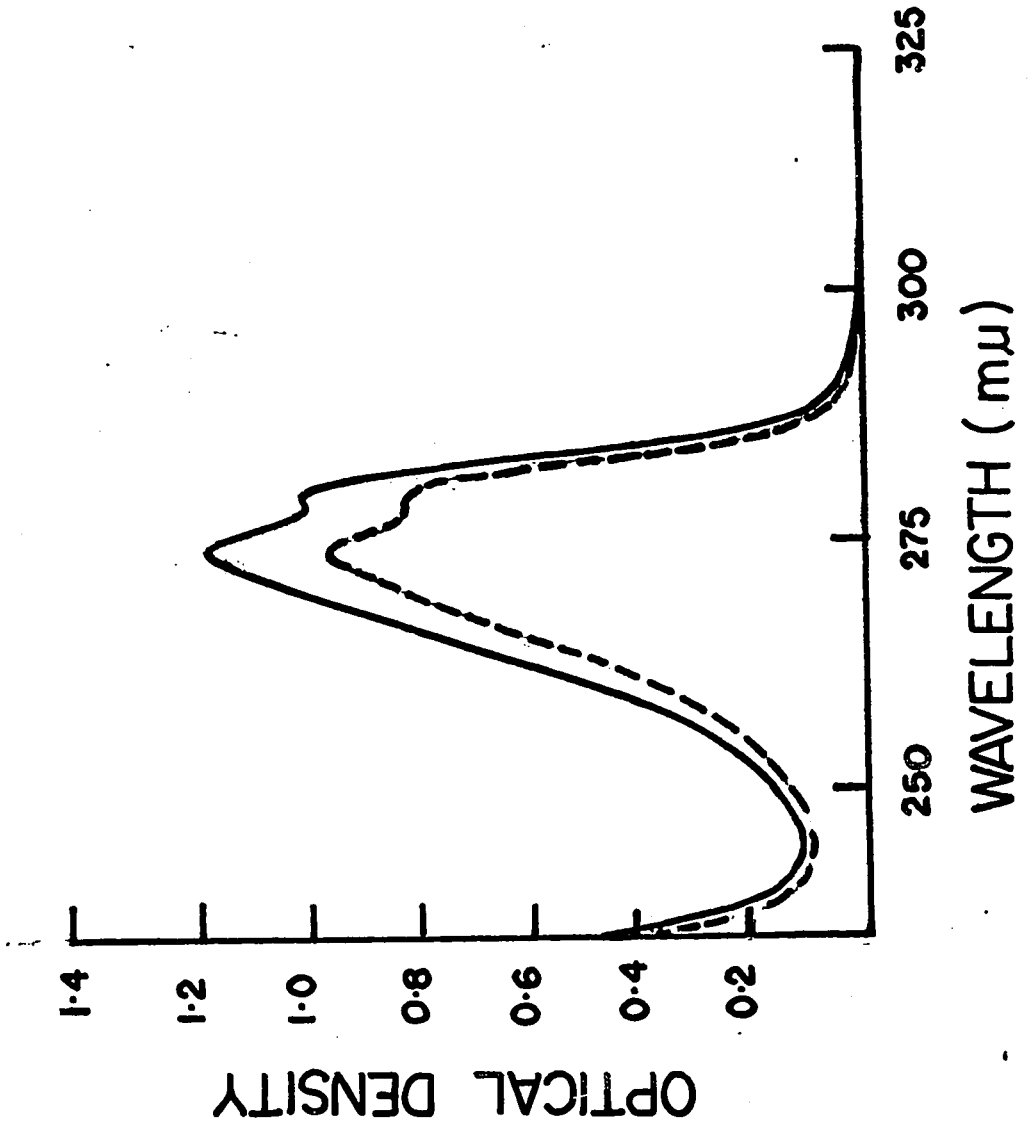
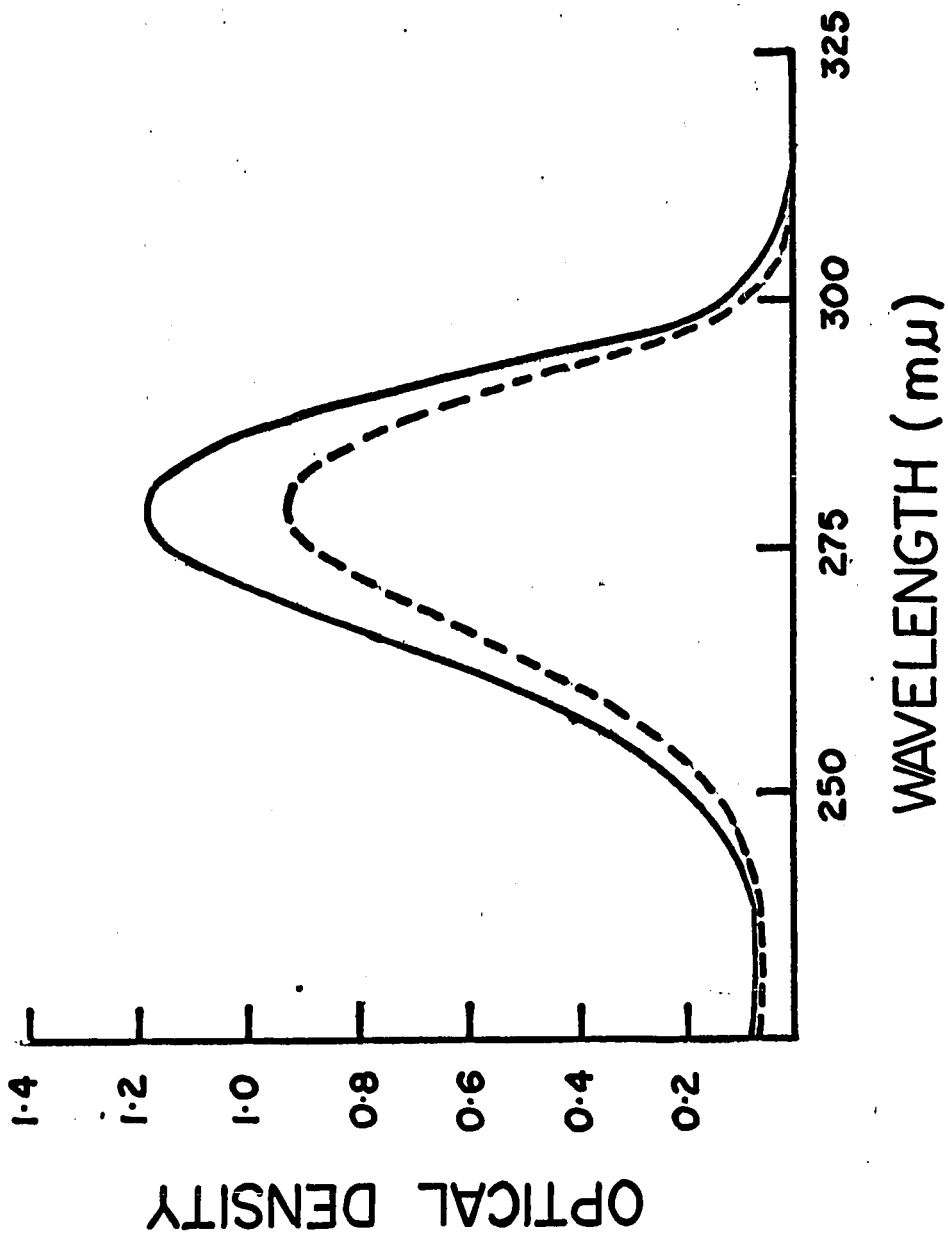

OPTICAL DENSITY
1·4
1·2
1·0
0·8
0·6
0·4
0·2
250
275
300
325
WAVELENGTH (mμ)

Fig.6 Ultraviolet absorption spectra of chemically reduced and biologically obtained dihydrophloroglucinol. The solid line indicates the chemically reduced compound and the broken line indicates the biologically obtained specimen. The spectra were recorded in a UNICAM SP.800 ultraviolet absorption spectrophotometer


OPTICAL DENSITY
1·4
1·2
1·0
0·8
0·6
0·4
0·2
250
275
300
325
WAVELENGTH (mμ)

reductase reaction indicates a mole for mole relationship between the oxidation of NADPH and the reduction of phloroglucinol (see Table V). The data obtained thus give the evidence that the enzymatic product (molecular weight 128) is the result of the addition of two atoms of hydrogen (molecular weight 2) to phloroglucinol (molecular weight 126) with NADPH as hydrogen donor.

On the basis of all the data, intermediate IV is tentatively identified as dihydrophloroglucinol.

Characterization of the Product of the Chemical Reduction of Phloroglucinol

The crystalline material (yield 106 mg) melted at 55-57° C (uncorrected). The R_f values and color reactions were identical to intermediate IV (Table II). Both compounds had their maximum absorption at 278 mμ (Fig.6) and similar mobility through Sephadex G-10 (fractions 17-19).

On the basis of the data given above, the chemically reduced product is identified also as dihydrophloroglucinol.

DISCUSSION

Studies on the detection of the intermediates involved in the pathway of phloroglucinol degradation by growing culture, as reported in Part I, revealed the presence of two phenolic compounds, two fluorescent compounds and an aliphatic acid in the fermentation liquor. In the present investigation, three intermediates of phloroglucinol degradation by growing culture were successfully separated from one another through Sephadex G-10. Intermediate III was identified definitely as resorcinol and intermediate IV was tentatively identified as dihydrophloroglucinol. The data obtained thus confirm the earlier observations that the intermediates III and IV were phenolic compounds. The intermediate II, a fluorescent compound, was also isolated but attempts to identify it were unsuccessful.

The data on enzymatic reduction of phloroglucinol are reported in Part III of this thesis and indicate that dihydrophloroglucinol, a product of enzymatic reduction of phloroglucinol, is the result of the addition of two atoms of hydrogen to phloroglucinol with NADPH as the donor. Experiments were then undertaken to reduce ~~dihydro~~phloroglucinol chemically. The results obtained in the present investigation showed that the product of chemically reduced phloroglucinol had the same R_f value, color reaction,

ultraviolet absorption spectrum and mobility through Sephadex G-10 as the product obtained from fermentation liquor. This indicates that the product of chemically reduced phloroglucinol was also dihydrophloroglucinol which was the result of the addition of two atoms of hydrogen to phloroglucinol with sodium borohydride as donor.

Naming intermediate IV as dihydrophloroglucinol is based on the rules which are taken from the "Definitive Report of the Commission on the Reform of the Nomenclature of Organic Chemistry" (Patterson,1933). According to rule 12, the prefix hydro, proceded by di-, tetra-, etc., will be used to name partially saturated polycyclic aromatic compounds. Dihydroanthracene is one of the examples given. Dihydrophloroglucinol isolated and tentatively identified as one of the intermediates involved in the pathway of phloroglucinol degradation by Pseudomonas sp. Mac 451 is a new compound which neither Beilstein's "Handbuch der Organischen Chemie" (1940) nor The Merck Index (1960) have listed.

The elution pattern observed in the present work indicates that dihydrophloroglucinol and the intermediate II appeared only in three fractions respectively while resorcinol was present in a great number of fractions. This was apparently a result of the greater affinity of resorcinol to Sephadex gels while dihydrophloroglucinol

and the intermediate II had little or no affinity to the gels, due to the different structures of their molecules. The data presented in the present investigation thus confirm the earlier report (Pharmacia,1966) that aromatic compounds have greater affinity to Sephadex gels than non-aromatic compounds of similar size and are thus retarded to varying extents.

In his studies on the metabolism of phloroglucinol by a Pseudomonas sp., Robern (1965) successfully isolated and characterized a conjugated phenolic compound and β-ketoadipic acid from the resting cell fermentation liquors. In the present investigation these intermediates were not detected on the thin-layer chromatograms of the fermentation liquor even when sprayed with vanillin toluene p-sulfonic acid and these products were not isolated.

PART III

PURIFICATION AND PROPERTIES OF PHLOROGLUCINOL REDUCTASE

INTRODUCTION

Wosilait and Nason (1954a) demonstrated that quinones could serve as electron acceptors for the enzymatic oxidation of reduced pyridine nucleotides, and their survey of plant, animal and microorganism extracts (Wosilait et al., 1954) revealed the general occurrence of reduced pyridine nucleotide-quinone reductase activity. Previously, Dam (1942) reported that vitamin K which was a common constituent of plants and microorganisms might be the physiological acceptor for quinone reductase and Wosilait and Nason (1954b) demonstrated that cell-free extracts of Escherichia coli could catalyse an oxidation of reduced pyridine nucleotide with menadione as the electron acceptor. Menadione reductase activity was subsequently demonstrated in extracts of Achromobacter fischeri (Cormier and Totter, 1954), Streptococcus faecalis (Dolin,1954), Azotobacter vinelandii (Schils et al., 1960) and Hydrogenomonas eutropha (Repaske and Lizotte,1965).

Robern (1965) reported that cell-free extracts prepared from Pseudomonas sp. Mac 451 were able to catalyse the oxidation of NADPH or NADH with phloroglucinol as the substrate. This reductase has now been successfully isolated and partially purified and this report describes the properties of the enzyme.

MATERIALS AND METHODS

Special Precautions- All solutions were prepared in glass distilled water and all glassware was washed with cleaning solution.

Source of Chemicals- Reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), cytochrome c and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Company (St.Louis,Mo., U.S.A.). Phloroglucinol, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone (menadione) and benzoquinone were purchased from Matheson, Coleman and Bell (East Rutherford,N.J., U.S.A.). Sodium iodoacetate, toluquinone and p-xyloquinone were purchased from Eastman Organic Chemicals Company (Rochester,N.Y., U.S.A.). Resorcinol, pyrogallol, orcinol, phenol and potassium ferricyanide were obtained from Fisher Scientific Company (Montreal, P.Q., CANADA). Phloroglucinolcarboxylic acid was purchased from Morton Chemical Company (Ringwood, Ill., U.S.A.). Phloroglucinaldehyde was obtained from Aldrich Chemical Company (Milwaukee,Wis., U.S.A.). Diethylaminoethyl (DEAE)-cellulose, Bio-Gel P-150, Bio-Gel P-300 and p-chloromercuribenzoate were purchased from CALBIOCHEM (Los Angeles, Calif., U.S.A.). Mercuric chloride was obtained from Anachemia Chemical Company (Montreal,P.Q.,

CANADA). All types of Sephadex gels were purchased from Pharmacia (CANADA)Ltd., Montreal,P.Q., CANADA.

Methods of Analysis- Protein was estimated by the method of Lowry et al. (1951) with crystalline egg albumin as the standard (Fig.7). Phloroglucinol was determined by the method of Jayasankar and Bhat (1966) and standard curve for this trihydroxy phenol is shown in Fig.8.

Thin-layer chromatographic analyses were performed as described previously.

Throughout purification, assays for phloroglucinol reductase were at room temperature in 1.8 ml of reaction mixture containing 100 micromoles of potassium phosphate buffer (pH 7.2); 0.2 micromoles of NADPH, 5 micromoles of phloroglucinol and 0.1 ml of various enzyme fractions diluted appropriately with 0.01 M phosphate buffer (pH 7.2). The reaction, timed from the addition of NADPH, was followed at 340 mμ in a Carl Zeiss Spectrophotometer, Model PMQ. A blank without NADPH was used as the reference. Controls without enzyme were periodically included and no nonenzymatic oxidation of NADPH by phloroglucinol was found. One unit of enzyme activity is defined as the amount of enzyme producing an optical density change at 340 mμ of 0.010 per minute under assay conditions. Specific activity was calculated as enzyme units per mg of protein.

Fig.7 Standard curve for egg albumin

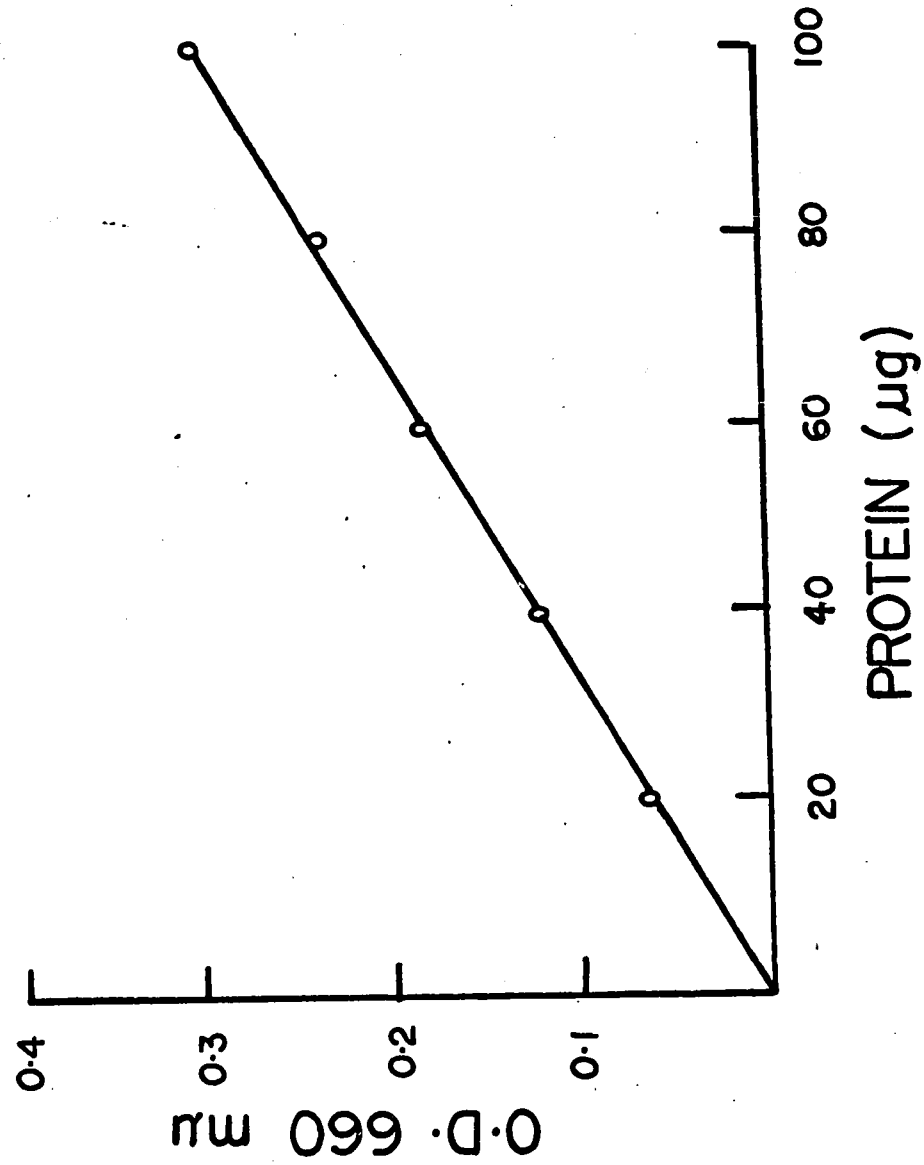
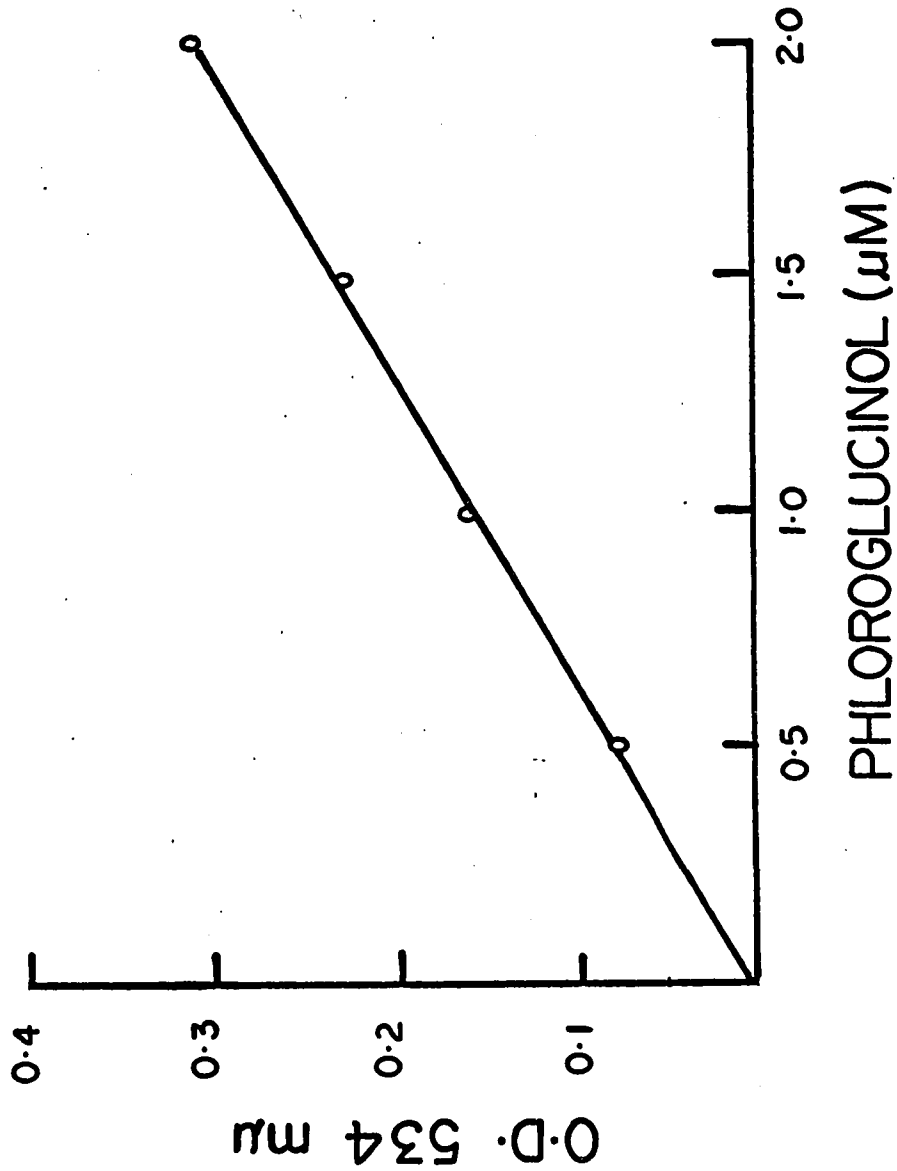

0·D· 660 mμ
0·4
0·3
0·2
0·1
20
40
60
80
100
PROTEIN (μg)

Fig.8 Standard curve for phloroglucinol


O.D. 534 mμ
0·4
0·3
0·2
0·1
0·5
1·0
1·5
2·0
PHLOROGLUCINOL (μM)

Production and Purification of Enzyme- Pseudomonas sp. Mac 451 was grown on a phloroglucinol basal medium as described previously. Wet cells (about 10 g) harvested from 4 liters of growth medium were washed three times with 0.01 M potassium phosphate buffer containing 1 mM EDTA and 1 mM cysteine, pH 7.2. The washed cells were suspended in 20 ml of the same buffer and then treated in a Raytheon ultrasonic distintegrator for 20 minutes. The suspension was centrifuged at 44,110 x g for 1 hour and the pellet was discarded.

Precipitation of nucleic acid from the crude extract was by the method of Burchall and Hitchings (1965). A 10% solution of streptomycin sulfate was added to the crude extract in an amount equal to one-tenth of the volume of the crude extract. After stirring for 10 minutes, the inactive precipitate was removed by centrifugation and discarded.

The streptomycin-treated supernatant solution was then passed through a column of DEAE-cellulose (1.5 x 22 cm) prepared by the method of Peterson and Sober (1962) and equilibrated with 0.01 M phosphate buffer containing 1 mM EDTA and 1 mM cysteine (pH 7.2) for 4 hours. The column was successively eluted with 30 ml of the same buffer, 40 ml of 0.1 M KCl, and 80 ml of 0.2 M KCl, collecting 5 ml fractions throughout.

The active pooled fractions from DEAE-cellulose chromatography were then concentrated by adding solid ammonium sulfate to 65% of saturation. The active precipitate was col-

lected by centrifugation.

The active precipitate from ammonium sulfate fractionation was dissolved in 0.5 ml of 0.01 M phosphate buffer containing 1 mM EDTA and 1 mM cysteine (pH 7.2) and then passed through a column of Bio-Gel P-300 (1.5 x 25 cm) which had been equilibrated with the same buffer for 12 hours with the soft gel formed was stabilized by applying a 3 cm layer of Sephadex G-75 at the top of the column bed. The elution was performed with the same buffer and 4 ml fractions were collected.

The enzyme purification steps were completed over a 24 hour period.

RESULTS

Nomenclature of the Enzyme- According to the Reports of the Commission on Enzymes of the International Union of Biochemistry (1961), all enzymes catalysing oxidoreductions will be named "oxidoreductases" in the systematic nomenclature, and the name is formed from the pattern "donor: acceptor oxidoreductase". This scheme of classification and nomenclature is used in the present report. The enzyme which has been isolated and partially purified is named "NADPH: phloroglucinol oxidoreductase" based on the fact that the enzyme is able to catalyse the oxidation of NADPH with phloroglucinol as the acceptor. The trivial name of the enzyme is phloroglucinol reductase which will be used throughout the present report.

Production of Enzyme- The phloroglucinol reductase of Pseudomonas sp. Mac 451 is an inducible enzyme and is produced only when phloroglucinol is added to the growth medium. Cells grown in a basal mineral medium containing glucose, acetate or succinate had no enzymatic activity.

Preliminary Purification of Enzyme- Robern (1965) obtained a two-fold purified enzyme after protamine sulfate treatment and ammonium sulfate fractionation of the cell-free preparation. Dixon and Webb (1964) reported that more rapid purification could be achieved by alternating the different fractionation methods than by repetition of fractions of one type and thus experiments were undertaken

to fractionate the enzyme by other methods.

Ammonium sulfate fractionation of the crude extracts showed that the enzyme activity distributed in the fractions precipitated from 25% to 65% of saturation.

Fractional precipitation of the crude extracts with cold acetone resulted in the inactivation of the enzyme.

Chromatography of the crude extracts on DEAE-cellulose afforded about five-fold purified enzyme. The enzyme activity was completely eluted from the column with 0.2 M KCl.

The results obtained by gel filtration of the crude extracts showed that the enzyme activity was excluded from the gels of Sephadex G-75, Sephadex G-100 and Bio-Gel P-150, indicating that the molecular weight of the enzyme is above the upper limit of the fractionation ranges of the gels. When chromatographied on Bio-Gel P-300, the enzyme was not excluded from the upper limit of the fractionation ranges of the gels and about four-fold purified enzyme was obtained.

The results obtained above suggest strongly that ammonium sulfate fractionation, column chromatography on DEAE-cellulose and gel filtration on Bio-Gel P-300 are valid methods for the purification of the enzyme.

Purification of Enzyme- The data obtained from various steps of a typical preparation are summarized in Table III. The specific activity of the crude extracts was 15. After streptomycin precipitation, the specific activity rose only

Table III Purification of phloroglucinol reductase

Purification step	Total units	Total protein mg	Specific* activity	Recovery %
Crude extracts	4,400	288.0	15.0	100
1% Streptomycin	3,800	239.4	15.8	84
DEAE-cellulose	2,920	28.4	103.0	66
65% Ammonium sulfate fraction	2,860	18.8	152.0	65
Bio-Gel P-300	712	2.1	339.0	16

* Specific activity was calculated as enzyme units per mg of protein.

to 15.8 but this treatment was useful in removing nucleic acid from the crude extracts which was necessary to enhance the efficiency of the fractionation methods used in the subsequent steps. Chromatography of the streptomycin-treated crude extracts afforded about seven-fold purified enzyme with a recovery of 66%. Fig.9 shows a typical experiment in the next step where the enzyme was chromatographed on DEAE-cellulose column. The enzyme activity was completely eluted from the column with 0.2 M KCl. The active pooled fractions (26-33) from DEAE-cellulose column were concentrated by the addition of solid ammonium sulfate to 65% of saturation. The specific activity of the enzyme at this point was increased to 152.0 with a recovery of 65%. Chromatography of the 65% ammonium sulfate fraction on Bio-Gel P-300 gave about twenty-three-fold purified enzyme with a recovery of 16% and Fig. 10 shows a typical elution pattern of the enzyme on a column of Bio-Gel P-300. The fractions (6-7) containing the highest enzyme activity were pooled, stored at -20° C and used for the studies on the properties of the enzyme.

Characterization of the Product of Enzymatic Reduction of Phloroglucinol- Having successfully isolated and identified dihydrophloroglucinol and resorcinol as the intermediates involved in the pathway of phloroglucinol degradation by growing culture, attempts were then made to confirm this finding using partially purified enzyme. The reaction mixture

Fig.9 Chromatography of phloroglucinol reductase on DEAE-cellulose by KCl in 0.01 M phosphate buffer - 1 mM EDTA - 1 mM cysteine, pH 7.2. The solid bar on the abscissa indicates fractions pooled

Fig.9 Chromatography of phloroglucinol reductase on DEAE-cellulose by KCl in 0.01 M phosphate buffer - 1 mM EDTA - 1 mM cysteine, pH 7.2. The solid bar on the abscissa indicates fractions pooled

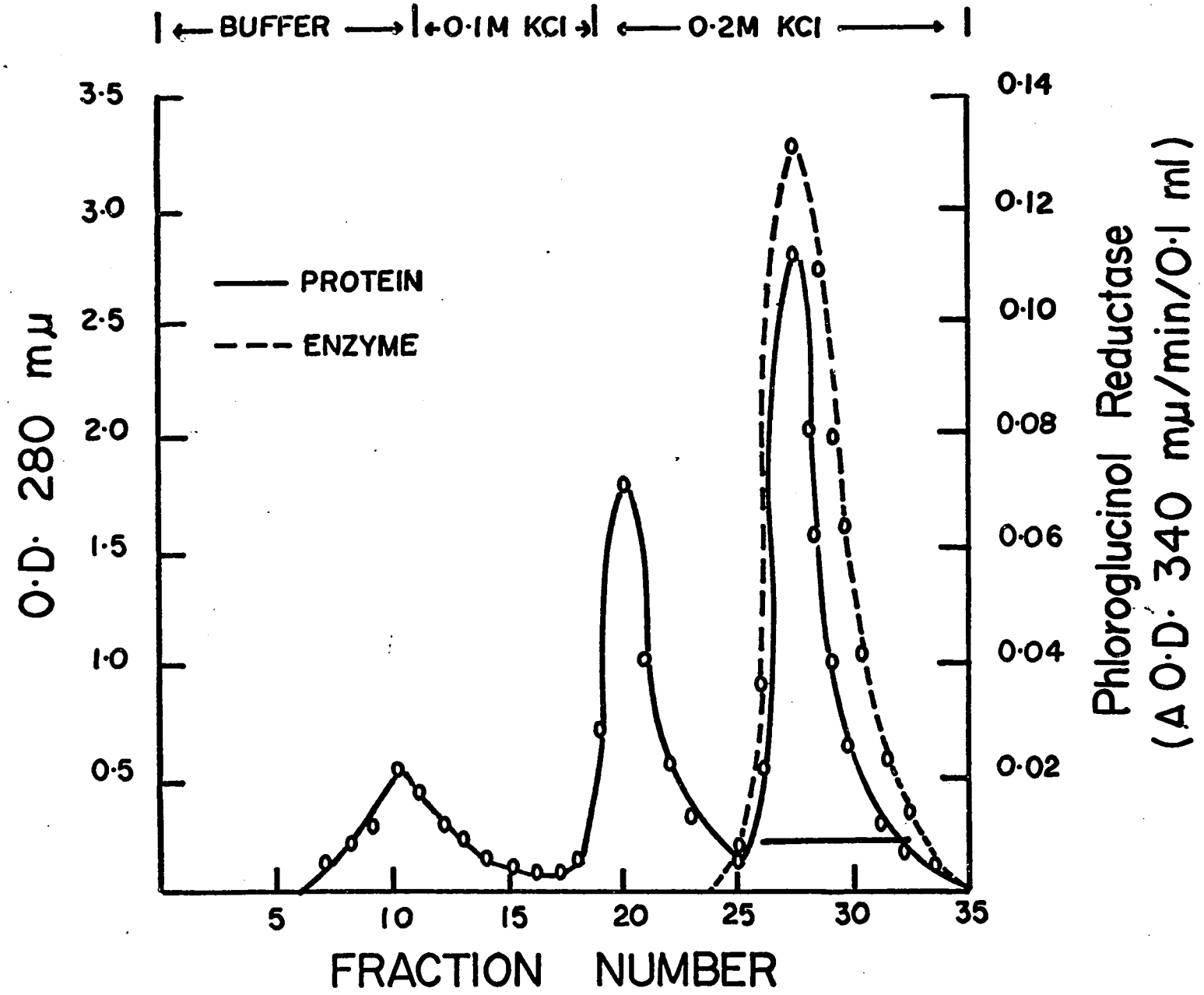
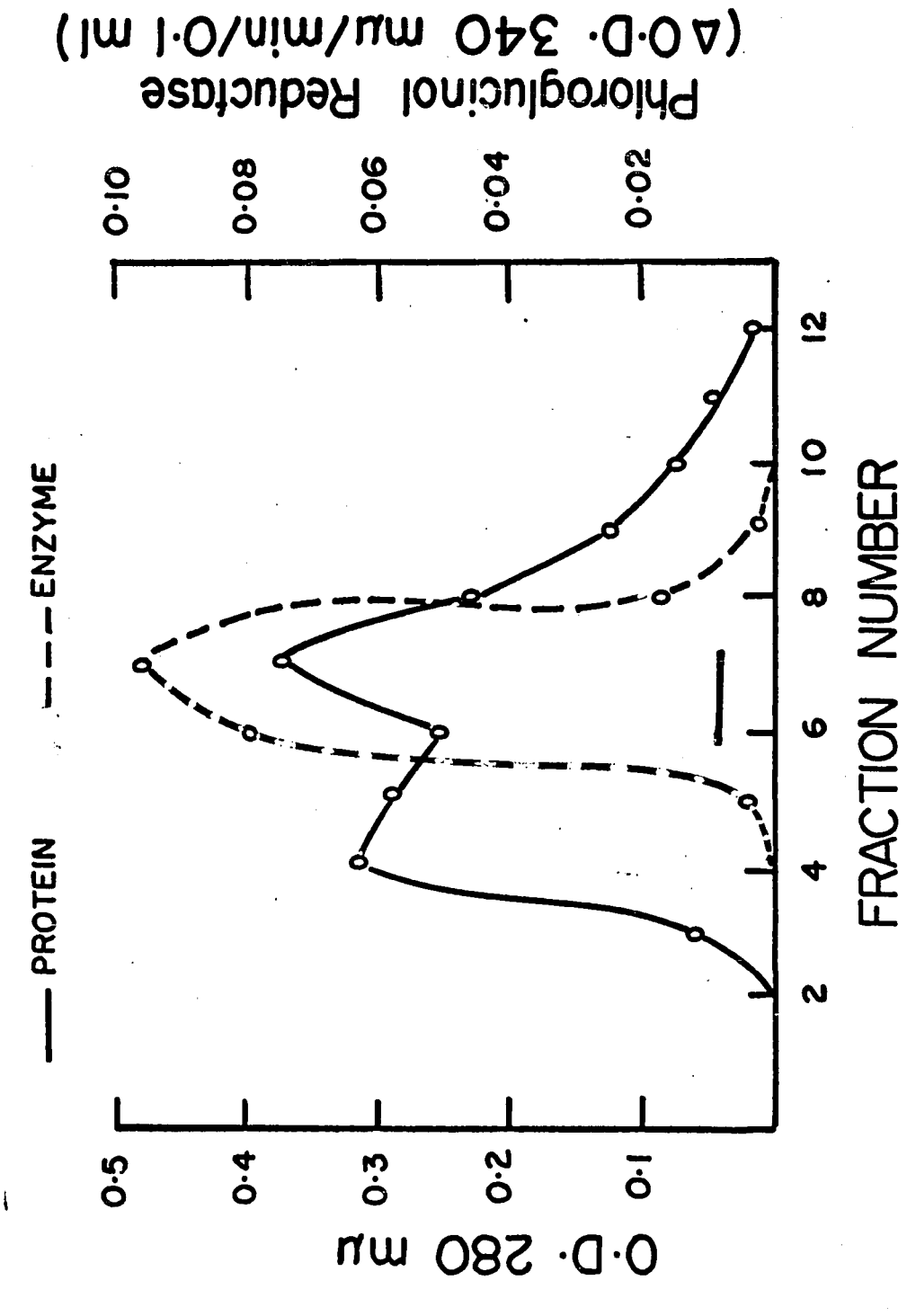

BUFFER
0·1M KCl
0·2M KCl
PROTEIN
ENZYME
O·D· 280 mμ
Phloroglucinol Reductase
(Δ O·D· 340 mμ/min/0·1 ml)
FRACTION NUMBER
3·5
3·0
2·5
2·0
1·5
1·0
0·5
0·14
0·12
0·10
0·08
0·06
0·04
0·02
5
10
15
20
25
30
35

Fig.10 Chromatography of phloroglucinol reductase on Bio-Gel P-300
The eluting solution contained 0.01 M phosphate buffer, 1 mM EDTA and 1 mM cysteine at pH 7.2. The solid bar on the abscissa indicates fractions pooled


O·D· 280 mμ
0·5
0·4
0·3
0·2
0·1
PROTEIN
ENZYME
2
4
6
8
10
12
FRACTION NUMBER
0·10
0·08
0·06
0·04
0·02
Phlorogluicinol Reductase
(Δ O·D· 340 mμ/min/O·1 ml)

(1.8 ml) contained, in micromoles: NADPH, 20; potassium phosphate buffer (pH 7.2), 300; phloroglucinol, 30; and enzyme, 35.6 units. A control was run without NADPH. Oxidation of NADPH was followed spectrophotometrically. When no further change in absorbance at 340 mμ was found, the reaction was terminated by the addition of 0.2 ml of 25% trichloroacetic acid and the sample was centrifuged. The clear supernatant was tested for the presence of the product of enzymatic reduction of phloroglucinol by thin-layer chromatographic techniques. Fig.11 shows tracings of the results and indicates that the product of the enzymatic reduction of phloroglucinol appears as a yellow spot with a R_f value of 0.22 and phloroglucinol gives a pink spot with a R_f value of 0.28. The product of enzymatic reduction of phloroglucinol is not formed when NADPH is not added to the enzymatic reaction mixture. The results obtained above thus give$ the evidence that the formation of the product of enzymatic reduction of phloroglucinol is a result of reduction of this trihydroxy phenol with NADPH as hydrogen donor. The product of enzymatic reduction of phloroglucinol has been successfully separated from the substrate by passing the reaction mixture through a column of Sephadex G-10 (1.5 x 64 cm). It was found to have the same R_f value, color reaction and ultraviolet absorption peak as dihydrophloroglucinol (Table IV). The product of enzymatic reduction of phloro-

Fig.11 Tracings of thin-layer chromatogram of the product of enzymatic reduction of phloroglucinol

A. NADPH was omitted from the reaction mixture

B. Complete system

1. A pink spot with a R_f value of 0.28 (phloroglucinol)
2. A yellow spot with a R_f value of 0.22 (dihydrophloroglucinol)

Solvent System: benzene-methanol-acetic acid

Color developing reagent: vanillin toluene p-sulfonic acid

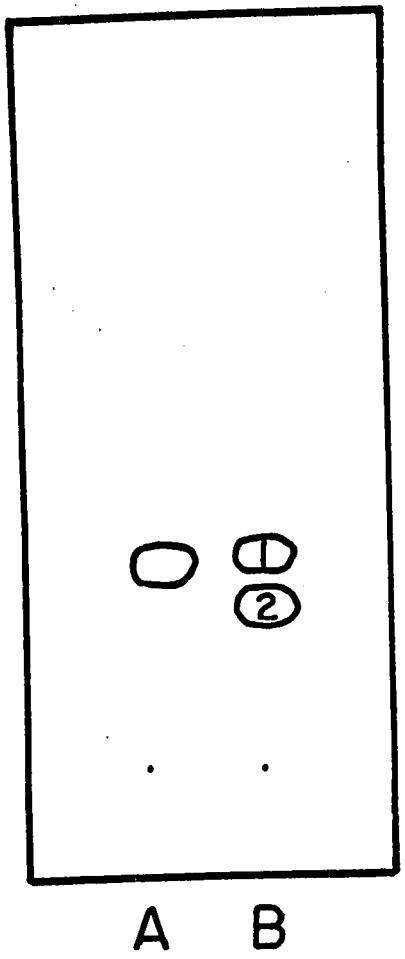

1
2
A
B

Table IV Comparison of the product of enzymatic reduction of phloroglucinol and dihydrophloroglucinol

	R_f in Solvent System*			Color developing reagent	Absorption peak
	1	2	3	Vanillin toluene p-sulfonic acid	mμ
Enzymatic product	0.22	0.75	0.07	Yellow	278
dihydrophorogluci-nol	0.22	0.75	0.07	Yellow	278

* Solvent System 1: benzene-methanol-acetic acid (45:8:4)
2: 95% ethanol-concentrated ammonium hydroxide (100:1)
3: benzene-dioxane-acetic acid (90:25:4)

glucinol is thus tentatively identified as dihydrophloroglucinol, confirming the earlier postulate that dihydrophloroglucinol is one of the intermediates involved in the pathway of phloroglucinol degradation by growing culture.

Having successfully identified the product of enzymatic reduction of phloroglucinol as dihydrophloroglucinol, attempts were then made to demonstrate the oxidation of dihydrophloroglucinol by $NADP^+$ in the reverse direction. When dihydrophloroglucinol is incubated with $NADP^+$ and phloroglucinol reductase, an increase in absorbance at 340 mμ is observed, indicating the reduction of $NADP^+$ to NADPH. The presence of phloroglucinol was confirmed by its R_f value and color reaction on a thin-layer chromatogram. The results show that the product of enzymatic reduction of phloroglucinol, dihydrophloroglucinol, can be enzymatically oxidized to phloroglucinol by $NADP^+$ in the reverse direction.

Properties of the Enzyme

Stoichiometry of Enzymatic Reduction of Phloroglucinol- The following three examples illustrate experiments showing the relation of the amounts of NADPH oxidized to the amounts of phloroglucinol reduced by the enzyme (Table V). Note the mole for mole relationship between NADPH oxidized and phloroglucinol reduced enzymatically. These

Table V Stoichiometry of phloroglucinol reductase reaction

Experiment number	NADPH oxidized μM	Phloroglucinol reduced μM	Ratio
1	0.10	0.08	1.25
2	0.20	0.17	1.18
3	0.30	0.25	1.20

Reaction mixture (1.8 ml) contained, in micromoles: Potassium phosphate buffer (pH 7.2), 100; phloroglucinol, 4; enzyme, 26.7 units and NADPH as indicated. NADPH was omitted from the control. The rate of NADPH oxidation was followed spectrophotometrically. When the complete oxidation of NADPH was noted, the reaction was terminated by the addition of 0.2 ml of 25% trichloroacetic acid and the sample was centrifuged. The clear supernatant was then assayed colorimetrically for residual phloroglucinol.

results further confirm the previous conclusion that the product of enzymatic reduction of phloroglucinol is dihydrophloroglucinol, a result of the addition of two atoms of hydrogen to the substrate with NADPH as hydrogen donor.

Nucleotide Specificity- Phloroglucinol reductase from Pseudomonas sp. Mac 451 is NADPH-specific; no activity occurs when NADH is supplied.

Specificity of Electron Acceptors- The data in Table VI compare the relative rates of enzymatic reaction of the various acceptors with NADPH as the donor. Besides phloroglucinol, phloroglucinolcarboxylic acid, various quinones and potassium ferricyanide act as acceptors, but the rate of NADPH oxidation with phloroglucinol is much faster. Phenol, catechol, resorcinol, pyrogallol, orcinol and phloroglucinaldehyde did not act as acceptors for phloroglucinol reductase.

Effect of Enzyme Concentration- The effect of enzyme concentration on the rate of NADPH oxidation is shown in Fig.12. The initial rate of NADPH oxidation is proportional to the amount of enzyme added but this relationship eventually decreases with increasing concentrations of enzyme.

Effect of Substrate Concentration- The effect of phloroglucinol concentrations on the rate of NADPH oxidation is shown in Fig.13. The Lineweaver-Burk plot (1934) shows a linear relation between $1/v$ and $1/(s)$ (Fig.14). The Km for phloroglucinol at pH 7.2 in phosphate buffer is cal-

Table VI Comparative rates with various acceptors for phloroglucinol reductase with NADPH as donor

Acceptor	Relative rate
Phloroglucinol	100
Phloroglucinolcarboxylic acid	65
1,4-Naphthoquinone	43
2-Methyl-1,4-naphthoquinone (menadione)	12
p-Benzoquinone	53
Toluquinone	60
p-Xyloquinone	39
Potassium ferricyanide	28
Phenol	0
Resorcinol	0
Catechol	0
Pyrogallol	0
Orcinol	0
Phloroglucinaldehyde	0

Reaction mixture (1.8 ml) contained, in micromoles: NADPH,0.5; potassium phosphate buffer (pH 7.2), 100; acceptor, 5; and enzyme, 5.4 units.

Fig.12 Effect of enzyme concentration on the oxidation of NADPH

Reaction mixture (1.8 ml) contained, in micromoles: NADPH, 0.5; phloroglucinol, 5; potassium phosphate buffer (pH 7.2), 100; and enzyme as indicated

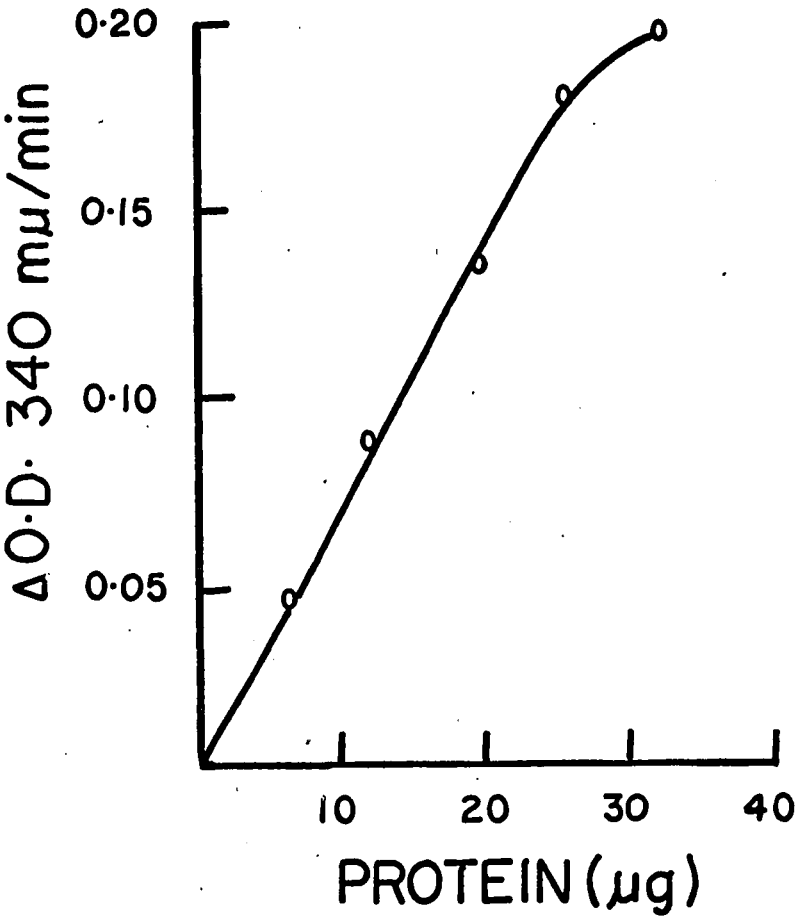

0·20
0·15
0·10
0·05
ΔO·D· 340 mμ/min
10
20
30
40
PROTEIN (μg)

Fig.13 Effect of substrate concentration on the oxidation of NADPH

Reaction mixture (1.8 ml) contained, in micromoles: NADPH,0.5; potassium phosphate buffer (pH 7.2), 100; enzyme, 5.4 units and phloroglucinol as indicated

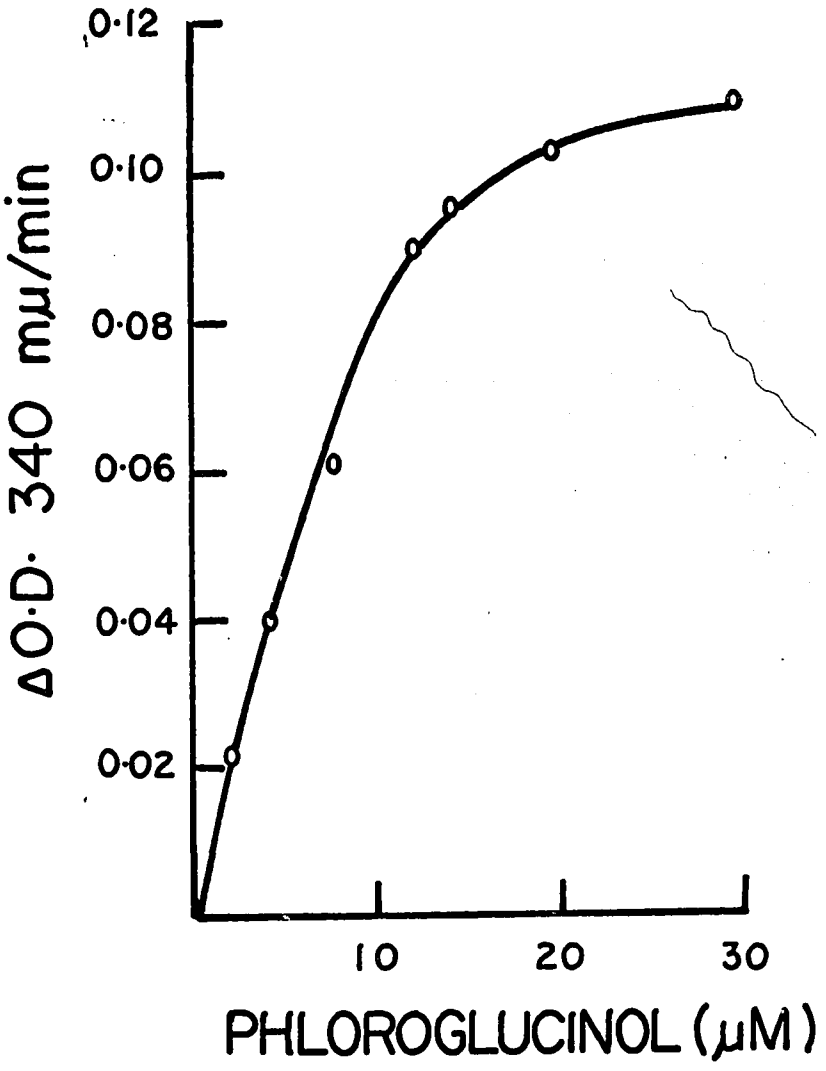
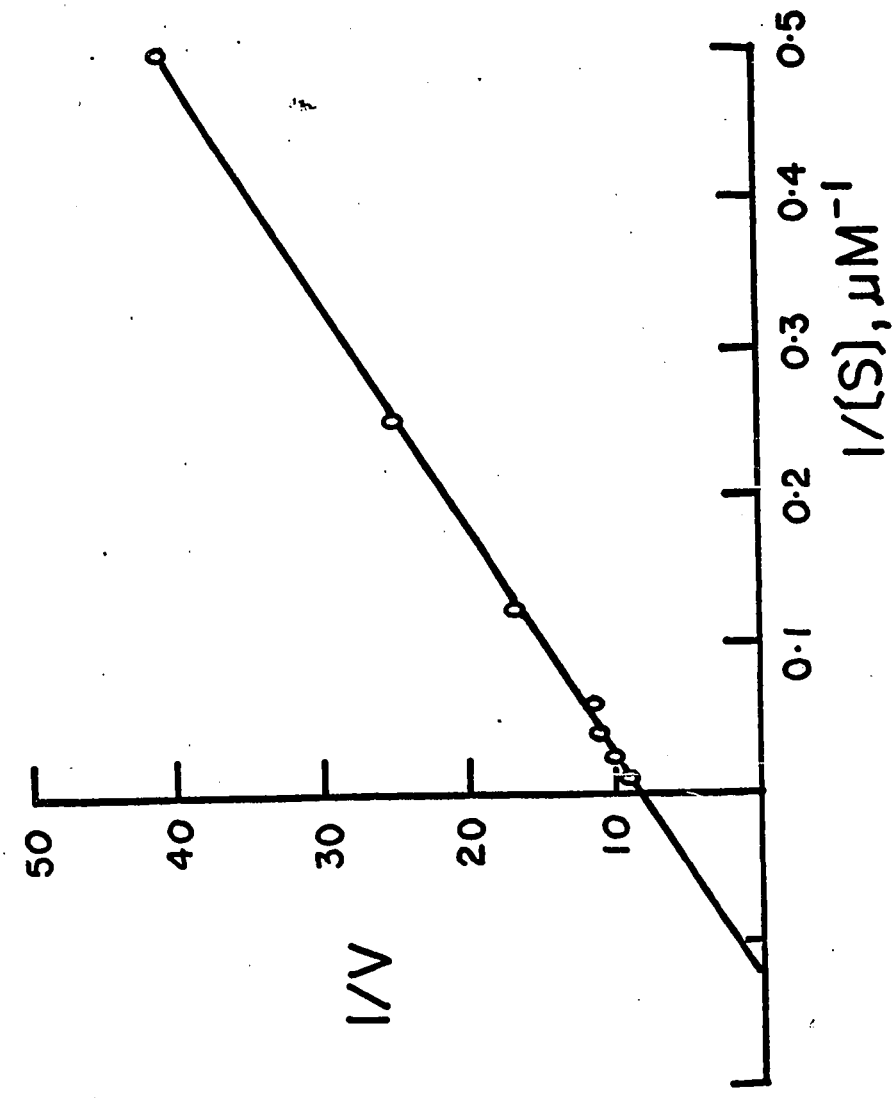

0·12
0·10
0·08
0·06
0·04
0·02
ΔO·D· 340 mμ/min
10
20
30
PHLOROGLUCINOL (μM)

Fig.14 Lineweaver-Burk plot of the reciprocal phloroglucinol concentration vs. reciprocal of its velocity of oxidation of NADPH by phloroglucinol reductase


1/V
50
40
30
20
10
0·1
0·2
0·3
0·4
0·5
1/[S], μM⁻¹

culated as 6.25×10^{-6}M and indicates a high affinity between enzyme and substrate.

Effect of pH- The effect of pH on the enzymatic reaction was determined by the use of various buffers at suitable pH values. Fig.15 indicates that phloroglucinol reductase has a sharp pH optimum at 7.4 in phosphate buffer. The stability of the enzyme at various pH values was optimal at pH 7.0 (Fig.16).

Thermostability of Enzyme- When the enzyme was heated for 10 minutes at different temperatures, its survival activity was found to be 100%, 100%, 61%, 8% and 0% at 4° C, room temperature, 37° C, 50° C and 60° C, respectively. No loss of activity was observed when the enzyme had been stored at -20° C for a week.

Effect of Cofactors,Activators and Inhibitors- The addition of FMN,FAD or cytochrome c to the phloroglucinol reductase reaction mixture neither replaced NADPH nor resulted in an increase in the rate of NADPH oxidation by phloroglucinol. No stimulation was obtained with cations such as Mg^{+2} , Fe^{+2} , Fe^{+3} and Mn^{+2} .

Phloroglucinol reductase was inhibited by mercuric chloride, sodium iodoacetate and p-chloromercuribenzoate. Chelating agents such as EDTA and 2,2'-bipyridine were innocuous (Table VII).

The data obtained above thus indicate that phloroglucinol reductase does not require cations for maximum activity and its activity depends upon free -SH groups at the active center of the enzyme molecule.

Fig.15 Effect of pH on enzyme activity

Reaction mixture (1.8 ml) contained, in micromoles: NADPH; 0.5; phloroglucinol, 5; enzyme, 8.9 units and buffer as indicated, 100.

o—— o phosphate buffer, Δ—— Δ Tris buffer

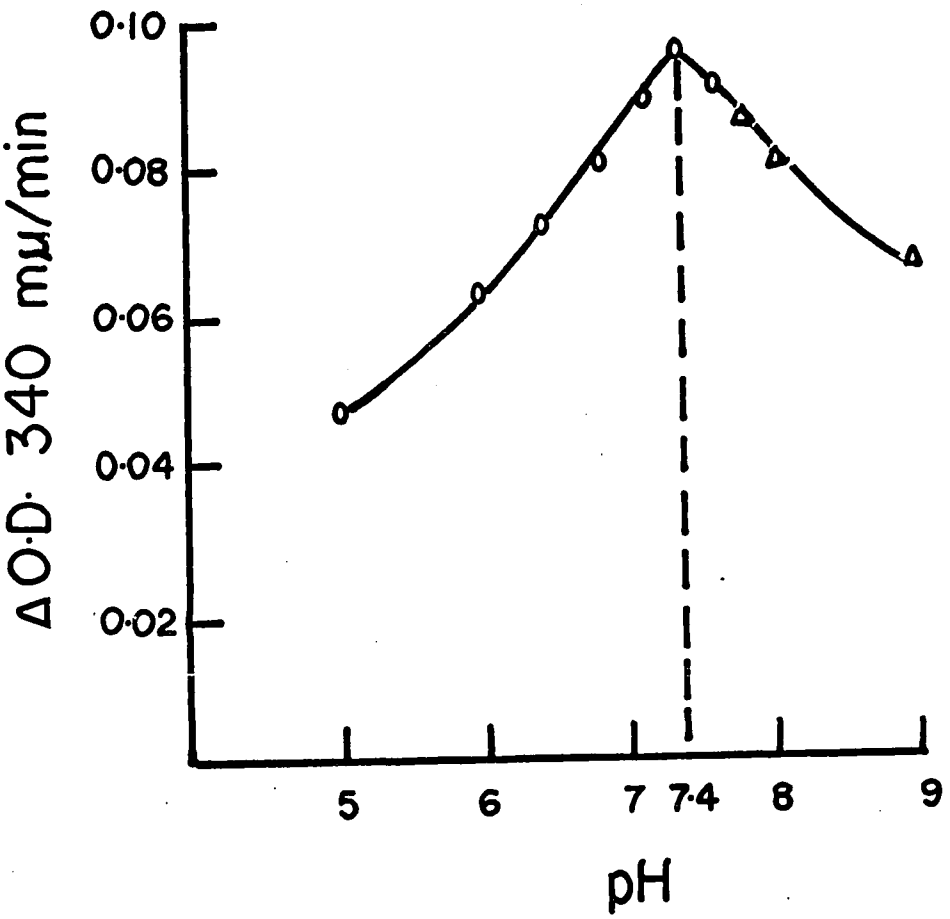

0·10
0·08
0·06
0·04
0·02
ΔO·D· 340 mμ/min
5
6
7
7·4
8
9
pH

Fig.16 pH stability of phloroglucinol reductase Enzyme dilutions were made in 0.1 M phosphate buffer at various pH values and incubated at room temperature for 30 minutes. Dilutions were then adjusted to pH 7.2, and assayed

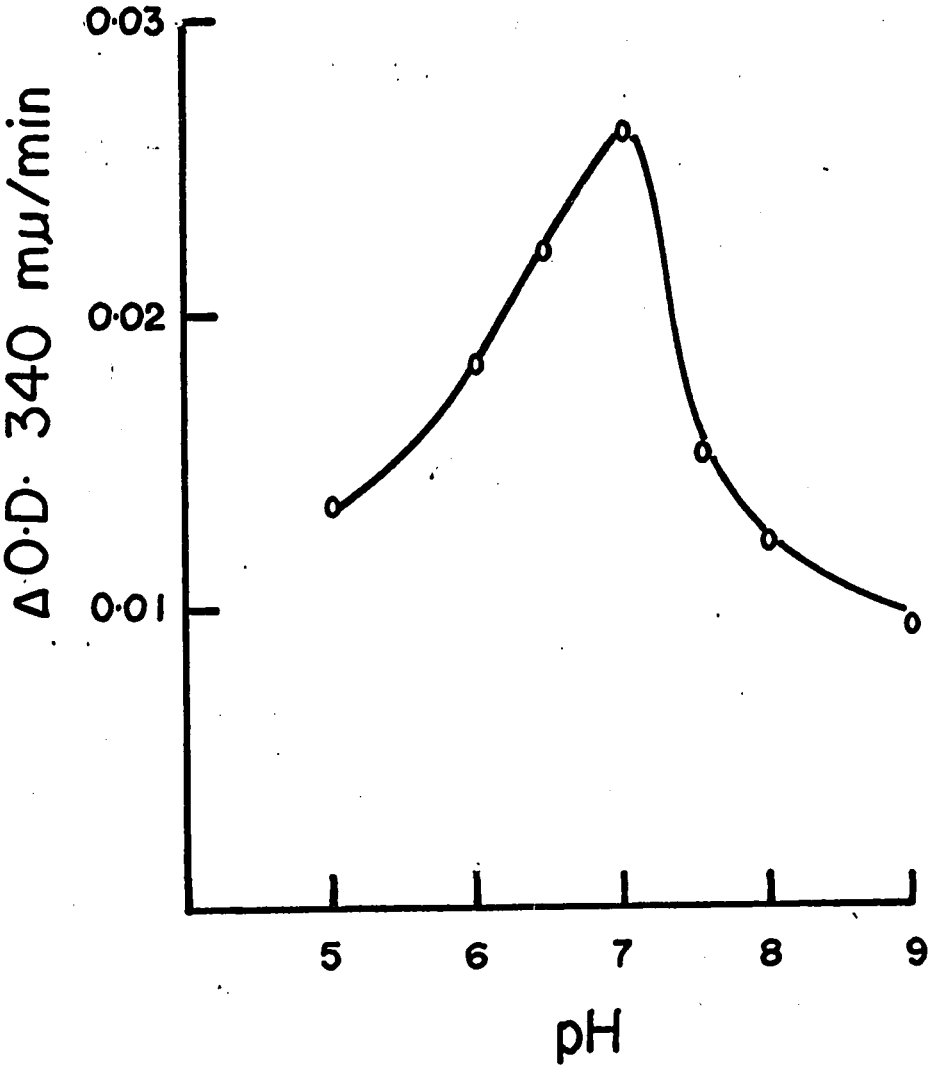

0·03
0·02
0·01
ΔO·D· 340 mμ/min
5
6
7
8
9
pH

Table VII Effect of inhibitors on enzyme activity

Inhibitor	Concentration M	Inhibition %
EDTA	10^{-3}	0
2,2'-bipyridine	10^{-3}	0
Iodoacetate	10^{-3}	32
	10^{-2}	86
Mercuric chloride	10^{-3}	100
p-Chloromercuribenzoate	10^{-3}	100

Reaction mixture (1.8 ml) contained, in micromoles: NADPH, 0.5; potassium phosphate buffer (pH 7.2), 100; phloroglucinol,5; enzyme, 5.4 units and inhibitor as indicated.

DISCUSSION

Robern (1965) reported that cell-free extracts prepared from a Pseudomonas sp. required NADPH in the decomposition of phloroglucinol. Substituting NADH for NADPH in the reaction mixture resulted in about 50% decrease in the rate of enzyme activity. The partially purified enzyme obtained in this work was found to be NADPH-specific; no activity occurred with NADH. This was possibly due to the absence of NADH-oxidase and NADH:NADP transhydrogenase from the partially purified enzyme preparation.

Wosilait and Nason (1954b) found no flavin requirement for menadione reductase from E.coli; similarly no exogenous flavin was required for phloroglucinol reductase from Pseudomonas sp. Mac 451. However, quinone reductase from E. coli (Wosilait and Nason,1954a) and other menadione reductases from H.eutropha (Raspaske and Lizotte,1965), A.fischeri (Cormier and Totter,1954) and S.faecalis (Dolin,1954) were activated by added flavin.

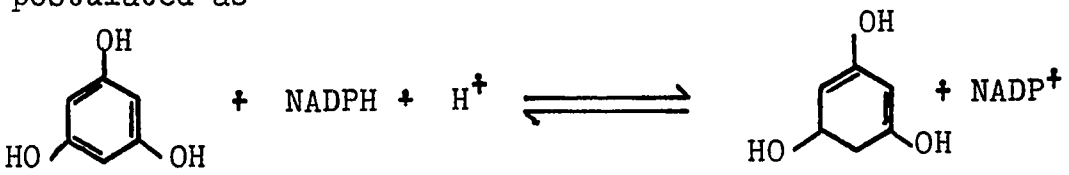
Phloroglucinol reductase was able to catalyse an rapid oxidation of NADPH with phloroglucinol as electron acceptor but phloroglucinolcarboxylic acid, various quinones and potassium ferricyanide could replace phloroglucinol as the electron acceptor. However, phenol, catechol, resorcinol, pyrogallol, orcinol and phloroglucinaldehyde were unable to act as electron acceptors for phloroglucinol reductase. This

confirms Robern's (1965) earlier report that cell-free extracts of Pseudomonas sp. catalysed the oxidation of NADPH with either phloroglucinol or phloroglucinolcarboxylic acid as the substrate and no activity occurred with resorcinol, catechol and pyrogallol.

Respaske and Lizotte (1965) reported that menadione reductase from H.eutropha was inhibited by p-chloromercuribenzoate, phloroglucinol reductase from Pseudomonas sp. Mac 451 was similarly inhibited. Chelating agents such as EDTA and 2,2'-bipyridine had no effect which indicates that phloroglucinol reductase does not require inorganic ions for maximum activity and its activity depends upon free -SH groups at the active center of the enzyme molecule.

When aromatic compounds serve as carbon and energy sources for some microorganisms, the benzene nucleus usually is degraded to compounds that can be oxidized in Krebs' cycle. The nucleus is hydroxylated, usually give rise to catechol, and is then cleaved by the oxygenase (Dagley,1965). However, Robern (1965) demonstrated that cell-free extracts prepared from a Pseudomonas sp. was not active oxidatively, but was NADPH dependent producing a conjugated phenolic compound when incubated in the presence of phloroglucinol. No evidence was thus obtained that hydroxylation of phloroglucinol occurred. This was further confirmed by the results obtained in the present work using partially purified enzyme. The product of enzymatic reduction of phloroglucinol, tentatively identified as dihydrophloroglucinol, was a result of reduction of

the substrate with NADPH as hydrogen donor. Stoichiometry of phloroglucinol reductase reaction gave evidence that the oxidation of NADPH was equivalent to the disappearance of phloroglucinol and confirmed the earlier findings that the product of phloroglucinol reductase reaction was dihydrophloroglucinol which was a result of addition of two atoms of hydrogen to phloroglucinol with NADPH as donor. The reaction catalysed by phloroglucinol reductase was postulated as



Phloroglucinol

Dihydrophloroglucinol

When incubated with $NADP^+$ and phloroglucinol reductase, dihydrophloroglucinol was enzymatically oxidized to phloroglucinol by $NADP^+$ in the reverse direction.

Robern (1965) reported that the first enzymatic process of phloroglucinol degradation was NADPH-dependent and reductive in nature. The reductive process was thought to be a result of removal of a hydroxyl group from phloroglucinol. The results obtained in this work gave the evidence that the first enzyme, phloroglucinol reductase, involved in the pathway of phloroglucinol degradation was NADPH-dependent and confirmed Robern's (1965) earlier report. However, the

reductive process was a result of the addition of two atoms of hydrogen to phloroglucinol with NADPH as donor. No evidence was obtained that a hydroxyl group was removed from phloroglucinol. This reductive process involved in the course of phloroglucinol degradation is therefore in distinct constrast to the pathway of the metabolism of other aromatic compounds in which hydroxylation of these compounds usually occurs first.

GENERAL DISCUSSION

Based on manometric studies, chemical analyses and enzymatic assays, Robern (1965) proposed a pathway of phloroglucinol degradation by a Pseudomonas sp. as follows. Phloroglucinol ⟶ conjugated phenolic compound ⟶ β-hydroxymuconic semialdehyde ⟶ β-hydroxymuconic acid ⟶ β-ketoadipic acid ⟶ acids of the tricarboxylic acid cycle. In this work, the first enzyme, phloroglucinol reductase, involved in the pathway of phloroglucinol degradation was successfully isolated and purified approximately 23-fold. The enzyme is able to catalyse a rapid oxidation of NADPH with phloroglucinol as the acceptor and the product of enzymatic reduction of phloroglucinol, tentatively identified as dihydrophloroglucinol, is the result of the addition of two atoms of hydrogen to the substrate as NADPH as donor. These data thus confirm Robern's (1965) earlier postulation that the first enzymatic step of phloroglucinol degradation was NADPH-dependent and reductive in nature. Dagley (1965) reported that aromatic compounds served as carbon and energy sources for microorganisms and in the course of metabolism the benzene nucleus was degraded to compounds that were oxidized through Krebs' cycle. The nucleus was hydroxylated, usually gave rise to catechol, and was then cleaved by the oxygenase. In his studies on the metabolism of phloroglucinol by a Pseudomonas sp., Robern (1965), however, reported that incubation of phloroglucinol with cell-free extracts and NADPH did not exhibit

any oxidative activity and no evidence was obtained that hydroxylation of phloroglucinol occurred. This was further confirmed by the results obtained in the present investigation using partially purified enzyme. The reductive process involved in the first step of phloroglucinol degradation is therefore in distinct contrast to the pathway of the metabolism of the other aromatic compounds in which a process of hydroxylation usually occurs first.

Resorcinol was also successfully isolated and definitely identified as one of the key intermediates involved in the pathway of phloroglucinol degradation by Pseudomonas sp. Mac 451. The formation of this dihydroxy phenol is possibly the result of the dehydration of dihydrophloroglucinol, the first product of phloroglucinol degradation and the enzyme responsible for this conversion is possibly a dihydrophloroglucinol dehydratase. Preliminary attempts to isolate the enzyme, however, were not successful. Larway and Evans (1965) reported that resorcinol was metabolized by a crude extract of resorcinol grown cells in the presence of NADH or NADPH through hydroxylation at the C-4 position, with subsequent ring fission between the o-dihydroxy groups, giving rise to a di-basic acid. The ring fission enzyme required Fe^{+2} and was also capable of splitting catechol and protocatechuic acid between the

o-dihydroxy groups with the final production of β-keto-adipic acid. In this work, attempts to detect the first intermediate of resorcinol degradation, hydroxyquinol, were not successful, but an aliphatic acid which is possibly identical to the di-basic acid reported by Larway and Evans (1965) was observed on the thin-layer chromatograms of samples obtained from a growing cell fermentation liquor when sprayed with bromocresol purple. The unsuccessful detection of hydroxyquinol is due possibly to the instability of the trihydroxy phenol.

Robern (1965) successfully isolated and identified β-ketoadipic acid as one of the key intermediates involved in the pathway of phloroglucinol degradation by a Pseudomonas sp. In this work, this keto acid was not observed.

The data obtained in this work gives evidence that the first enzymatic step of phloroglucinol degradation is NADPH-dependent and the product of enzymatic reduction of phloroglucinol is dihydrophloroglucinol. Resorcinol, the second intermediate of phloroglucinol degradation, is possibly the result of the dehydration of dihydrophloroglucinol. An aliphatic acid, possibly a product of resorcinol degradation, is also observed, but β-ketoadipic acid was not detected.

On the basis of the data given above, a new pathway of phloroglucinol degradation is postulated. This pathway in-

involves the reduction of phloroglucinol to dihydrophloroglucinol which is dehydrated then to resorcinol. The dihydroxy phenol is possibly metabolized according to the pathway proposed by Larway and Evans (1965).

SUMMARY

The aims of this study were to isolate and characterize the intermediates involved in the pathway of phloroglucinol degradation by Pseudomonas sp. Mac 451.

Studies on the detection of intermediates of phloroglucinol degradation by thin-layer chromatography revealed the presence of two phenolic compounds, two fluorescent compounds and an aliphatic acid. Experiments were undertaken then to confirm this finding by the direct isolation of these intermediates from a growing cell fermentation liquor. Dihydrophloroglucinol, resorcinol and a fluorescent compound were separated successfully by column chromatography on Sephadex G-10. Identification of the isolated intermediates were confirmed by melting point determinations, thin-layer chromatography, ultraviolet absorption spectrophotometry and where possible by mass spectrometry.

Phloroglucinol reductase, the first enzyme involved in the pathway of phloroglucinol degradation by Pseudomonas sp. Mac 451 was isolated successfully and purified approximately 23-fold. The enzyme is able to catalyse a rapid oxidation of NADPH with phloroglucinol as the acceptor. Phloroglucinolcarboxylic acid, various quinones and potassium ferricyanide can replace phloroglucinol as the acceptor. Phenol, catechol, resorcinol, orcinol, pyrogallol and phloroglucinaldehyde are not active. The enzyme is NADPH-specific and no activity occurs with added NADH. No stimulation is obtained with FAD, FMN, cytochrome c or cations such as Mg^{+2}, Fe^{+2}, Fe^{+3} and

Mn^{+2}. The enzyme is most stable at pH 7.0 and has an optimum pH at 7.4 in phosphate buffer. The Km for phloroglucinol is 6.25×10^{-6} M. It is inhibited by mercuric chloride, iodoacetate and p-chloromercuribenzoate. Chelating agents such as EDTA and 2,2'-bipryridine have no effect. These data indicate that the enzyme does not require inorganic ions for maximum activity and the activity depends upon free -SH groups at the active center of the enzyme molecule. The product of enzymatic reduction of phloroglucinol, tentatively identified as dihydrophloroglucinol, is the result of the addition of two atoms of hydrogen to the substrate with NADPH as donor. The reaction catalysed by phloroglucinol reductase is postulated as

$$\text{Phloroglucinol} + \text{NADPH} + H^{+} \rightleftharpoons \text{Dihydrophloroglucinol} + NADP^{+}$$

The reduced compound was prepared by chemical reduction of phloroglucinol with sodium borohydride. This product has the same R_f value, color reaction, ultraviolet absorption spectrum and similar mobility through Sephadex G-10 as the enzymatically produced product. On this basis the product is tentatively identified as dihydrophloroglucinol.

On the basis of the data obtained in this work, a new pathway of phloroglucinol degradation by Pseudomonas sp. Mac 451 is postulated. This pathway involves the reduction of phloroglucinol to dihydrophloroglucinol which is dehydrated then to resorcinol. The dihydroxy phenol is further metabolized.

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