A B S T R A C T

Ph.D. Dhanbir K. Mathur Microbiology

ENZYMATIC DEGRADATION OF PHLOROGLUCINOL BY *Penicillium* SP. MAC M-47

Utilization of phloroglucinol by *Penicillium* sp. Mac M-47 was demonstrated in both growing-cell and resting-cell fermentations, by colorimetric analysis, ultraviolet absorption spectroscopy, and thin-layer chromatography. No intermediates were detected. Cell-free extracts prepared after growth on phloroglucinol or resorcinol, but not on glucose, catalyzed a rapid oxidation of NADPH with the substrates phloroglucinol and resorcinol. Phloroglucinol plays a significant and unique role in controlling the levels of enzyme which disappears with concomitant disappearance of the substrate from the fermentation medium. The presence of a minimal amount of phloroglucinol at harvesting time and throughout further processing of the harvested mycelia is an obligatory requirement for enzyme stabilization. Preliminary evidence suggested that phloroglucinol enzyme and resorcinol enzyme activities are closely related and form a part of an enzyme complex involved in the degradation of phloroglucinol by the *Penicillium* sp. Significant data supporting this concept are presented. Purification and properties of the enzyme complex are described in detail. The proposed pathway of phloroglucinol degradation by *Penicillium* sp. Mac M-47 involves the reductive dehydroxylation of phloroglucinol to resorcinol which is further metabolized by hydroxylation and subsequent ring cleavage.

Résumé

Ph.D. Dhanbir K. Mathur Microbiologie

LA DEGRADATION ENZYMATIC DU PHLOROGLUCINOL PAR *Penicillium* SP. MAC M-47

L'utilisation du phloroglucinol par *Penicillium* sp. Mac M-47 a été démontrée à la fois dans des fermentations de cellules proliférantes et de cellules au repos par analyse colorimétrique, par absorption spectroscopique en ultraviolet, et par chromatographie sur couche mince. Aucun produit intermédiaire ne fut détecté. Les extraits de cellules, préparés après croissance sur le phloroglucinol ou le résorcinol mais non sur le glucose, catalysèrent une oxidation rapide du NADPH avec les substrats phloroglucinol et résorcinol. Le phloroglucinol joue un rôle particulier et unique en contrôlant le niveau d'enzyme qui disparait du milieu de fermentation en même temps que le substrat. La présence d'une quantité minimale de phloroglucinol au moment de la récolte et tout au long du traitement subséquent du mycélium est nécessaire pour stabiliser l'enzyme. Des études préliminaires ont suggéré que les activités des enzyme du phloroglucinol et du résorcinol sont intimement liées et font partie d'un complexe enzymatique impliqué dans la dégradation du phlotoglucinol par *Penicillium* sp. Des résultats confirmant cet avancé sont présentés en même temps qu'une description détaillée de la purification et des propriétés du complexe enzymatique. Le schème proposé pour la dégradation du phloroglucinol par *Penicillium* sp. Mac M-47 comprend une déshydroxylation réductive du phloroglucinol au résorcinol qui est ensuite métabolisé par hydroxylation puis par la clive de l'anneau de benzène.

A suggested short title:

PHLOROGLUCINOL DEGRADATION BY A *Penicillium* SP.

D.K. Mathur

D E D I C A T E D

T O

M Y P A R E N T S

ENZYMATIC DEGRADATION OF PHLOROGLUCINOL

BY A *Penicillium* SP. MAC M-47

by

Dhanbir K. Mathur

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.

Department of Microbiology
Macdonald College of McGill University
Montreal, Quebec, Canada.

May 1971

A suggested short title:

PHLOROGLUCINOL DEGRADATION BY A *Penicillium* SP.

D.K. Mathur

A B S T R A C T

Ph.D. Dhanbir K. Mathur Microbiology

ENZYMATIC DEGRADATION OF PHLOROGLUCINOL BY *Penicillium* SP. MAC M-47

Utilization of phloroglucinol by *Penicillium* sp. Mac M-47 was demonstrated in both growing-cell and resting-cell fermentations, by colorimetric analysis, ultraviolet absorption spectroscopy, and thin-layer chromatography. No intermediates were detected. Cell-free extracts prepared after growth on phloroglucinol or resorcinol, but not on glucose, catalyzed a rapid oxidation of NADPH with the substrates phloroglucinol and resorcinol. Phloroglucinol plays a significant and unique role in controlling the levels of enzyme which disappears with concomitant disappearance of the substrate from the fermentation medium. The presence of a minimal amount of phloroglucinol at harvesting time and throughout further processing of the harvested mycelia is an obligatory requirement for enzyme stabilization. Preliminary evidence suggested that phloroglucinol enzyme and resorcinol enzyme activities are closely related and form a part of an enzyme complex involved in the degradation of phloroglucinol by the *Penicillium* sp. Significant data supporting this concept are presented. Purification and properties of the enzyme complex are described in detail. The proposed pathway of phloroglucinol degradation by *Penicillium* sp. Mac M-47 involves the reductive dehydroxylation of phloroglucinol to resorcinol which is further metabolized by hydroxylation and subsequent ring cleavage.

Résumé

Ph.D. Dhanbir K. Mathur Microbiologie

LA DEGRADATION ENZYMATIC DU PHLOROGLUCINOL PAR *Penicillium* SP. MAC M-47

L'utilisation du phloroglucinol par *Penicillium* sp. Mac M-47 a été démontrée à la fois dans des fermentations de cellules proliférantes et de cellules au repos par analyse colorimétrique, par absorption spectroscopique en ultraviolet, et par chromatographie sur couche mince. Aucun produit intermédiaire ne fut détecté. Les extraits de cellules, préparés après croissance sur le phloroglucinol ou le résorcinol mais non sur le glucose, catalysèrent une oxidation rapide du NADPH avec les substrats phloroglucinol et résorcinol. Le phloroglucinol joue un rôle particulier et unique en contrôlant le niveau d'enzyme qui disparait du milieu de fermentation en même temps que le substrat. La présence d'une quantité minimale de phloroglucinol au moment de la récolte et tout au long du traitement subséquent du mycélium est nécessaire pour stabiliser l'enzyme. Des études préliminaires ont suggéré que les activités des enzyme du phloroglucinol et du résorcinol sont intimement liées et font partie d'un complexe enzymatique impliqué dans la dégradation du phlotoglucinol par *Penicillium* sp. Des résultats confirmant cet avancé sont présentés en même temps qu'une description détaillée de la purification et des propriétés du complexe enzymatique. Le schème proposé pour la dégradation du phloroglucinol par *Penicillium* sp. Mac M-47 comprend une déshydroxylation réductive du phloroglucinol au résorcinol qui est ensuite métabolisé par hydroxylation puis par la clive de l'anneau de benzène.

TABLE OF CONTENTS

TABLE OF CONTENTS (cont'd.)

TABLE OF CONTENTS (cont'd.)

TABLE OF CONTENTS (cont'd.)

ACKNOWLEDGEMENTS

The author expresses his sincere gratitude to Dr. A.C. Blackwood, Professor of the Department of Microbiology, Macdonald College, for suggesting this study and his continuous interest and guidance throughout the span of this investigation, and in the preparation of this manuscript.

I am deeply indebted to Dr. Jordan M. Ingram, Department of Microbiology, for his generous discussion of the problems encountered, valuable suggestions offered, and critical appraisal of parts of this manuscript.

Sincere appreciation is extended to Mr. James Gilchrist for the expertise in drafting the graphs presented in this thesis. Thanks are also due to other colleagues for the helping hand at odd hours. I wish to thank Mrs. E. Rowell for skilfully typing the manuscript.

The financial assistance for this investigation from the National Research Council of Canada is gratefully acknowledged.

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont'd.)

CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. Utilization of phloroglucinol by *Penicillium* sp. Mac M-47 was demonstrated in both growing-cell and resting-cell fermentations, by colorimetric analysis, ultraviolet absorption spectroscopy, and thin-layer chromatography.

2. Cell-free extracts prepared after growth on phloroglucinol or resorcinol, but not on glucose, catalyzed a rapid oxidation of NADPH with the substrates phloroglucinol and resorcinol.

3. Phloroglucinol was shown to play a significant and unique role of stabilizing and thus controlling the levels of enzyme which disappears with concomitant disappearance of the substrate from the fermentation medium.

4. Evidence was obtained which supported the concept that the phloroglucinol and resorcinol enzyme activities are closely related and form a part of an enzyme complex involved in the degradation of phloroglucinol by the *Penicillium* sp.

5. The enzyme complex has been purified approximately 20-fold for the phloroglucinol enzyme and 21-fold with respect to resorcinol enzyme activity. The properties of the complex for the component enzyme activities were studied.

6. A pathway for the degradation of phloroglucinol by *Penicillium* sp. Mac M-47 has been proposed. The postulated pathway involves the reductive dehydroxylation of phloroglucinol to resorcinol, possibly through the transitory formation of dihydrophloroglucinol. Resorcinol is then further metabolized by hydroxylation and subsequent ring cleavage.

INTRODUCTION

The degradation of biosynthesized organic molecules is obligatory to maintain the biochemical cycle of nature. Microorganisms play an important role in the transformation of aromatic compounds by virtue of their versatility in degrading the complex molecules not metabolized by plants and consequently accumulated in soil through dead plants. The carbon atoms released in the process are then incorporated by plants into the benzene rings of lignins and flavonoid compounds. Although microbes capable of metabolizing phenolic compounds have been isolated, most, naturally occurring processes function in mixed cultures, and several microbial species participate in the turnover of aromatic organic matter in a heterogenous medium like soil.

Phloroglucinol (1,3,5-trihydroxybenzene) forms the A-ring moiety of a wide variety of flavonoids, and occurs in most plants as part of the complex molecule. The limited occurrence of free phloroglucinol in soil and in waste water results from biological and chemical decomposition of the complex molecules. Degradation of phloridzin and tannins by microorganisms in the soil, biodegradation of rutin in the rumen, and chemical decomposition of industrial wastes, all yield phloroglucinol as one of the products. The subject of its occurrence and importance, application in

industry, its bactericidal and fungicidal properties, has been dealt with in the review by Robern (1965).

Phloroglucinol is prepared from s-triamino benzene (Kalle, 1965; Kastens and Kaplans, 1950) which is available as a reduction product of the corresponding trinitro compound which in turn is prepared from trinitrotoluene. Alkaline cleavage of hesperetin and naringenin has also been reported to give phloroglucinol on hydrolysis (Horowitz, 1961). Because of the cost and importance of phloroglucinol, a preparative, alkaline fusion procedure was developed by Newhall and Ting (1967) for the production of phloroglucinol from hesperetin and naringenin. Separation of phloroglucinol from the accompanying aromatic acid derivatives was best accomplished in each case by column chromatography on Dowex-1 resin.

A number of reports have appeared in literature showing the utilization of phloroglucinol by microorganisms. However, very little effort has been devoted on the metabolic pathway of phloroglucinol in isolated cultures. Robern (1965) first studied the degradation of phloroglucinol by a pseudomonad and proposed a pathway of degradation which was later elucidated by Hang (1967). The utilization of phloroglucinol by a *Penicillium* sp., isolated from soil, was also examined by Robern (1965).

The objectives of the present investigation were to study the

enzymatic degradation of phloroglucinol by *Penicillium* sp. Mac M-47, and to purify and characterize the enzyme(s) involved in the metabolic pathway of phloroglucinol.

This thesis is organized in two parts. The first part deals with the utilization of phloroglucinol and the physiological conditions necessary for enzyme production. The second part describes the purification and properties of an enzyme complex catalyzing the reactions involved in the pathway of phloroglucinol degradation.

LITERATURE REVIEW

Microorganisms are well known for their ability to breakdown and utilize complex aromatic compounds as a source of carbon and energy. A wide variety of soil bacteria and fungi, and a few yeasts possess this ability to degrade phenolic compounds, an aspect of metabolism which is of considerable importance in the carbon cycle. The microbial degradation of aromatic compounds has been comprehensively reviewed by Evans (1956, 1958, 1969), Rogoff (1961), Towers (1964), Ribbons (1965) and Dagley (1967). Of the two recent specific reviews, Robern (1965) surveyed the literature on the metabolism of phloroglucinol and other simple phenolic compounds by microorganisms and animals, while Hang (1967) reviewed only the degradation of phloroglucinol by microorganisms. The present literature review is essentially an extension of the earlier summaries with emphasis on more recent developments.

From extensive studies on the oxidative metabolism of aromatic compounds by aerobic microorganisms, many of the pathways and mechanisms involved have been described (Evans, 1963; Ribbons, 1965; Dagley, 1967). All aromatic structures must be capable of being modified into either *ortho* or *para*-dihydroxyphenols before ring cleavage can occur. Two methods of *ortho*-diphenol (catechol) cleavage have been demonstrated in bacteria: (1) *ortho* cleavage -

fission of the bond between carbon atoms bearing the hydroxyl groups (MacDonald *et al.*, 1954; Ribbons, 1965), and (2) *meta* cleavage - rupture of the aromatic structure at C - C bond adjacent to the *ortho*-diphenol group (Dagley *et al.*, 1960, 1964; Gibson *et al.*, 1967; Ribbons, 1965). Completely different metabolic pathways result from these two modes of ring cleavage. It is generally accepted that a particular species of *Pseudomonas* employs either *ortho* or *meta* cleavage for a given aromatic substrate and that both types of oxygenase are not derepressed simultaneously by one inducer (Stanier *et al.*, 1966), as is found in some other microorganisms (Farr and Cain, 1968; Griffiths *et al.*, 1964). In the case of *Pseudomonas fluorescens*, the electron donating or electron withdrawing capacity of the side chain substituents of the benzene ring was shown to determine whether *ortho* or *meta* cleaving enzymes are derepressed, the whole molecule seemingly being unimportant (Seidman *et al.*, 1969).

In the case of *para*-diphenols, rupture of the bond occurs between carbon atoms bearing a hydroxyl and an adjacent carbon atom carrying a hydrogen, carboxyl or side chain. Thus quinol was shown by Larway and Evans (1965) to give rise to γ-hydroxy-muconic semialdehyde; gentisate gives maleylpyruvate (Lack, 1959) and homogentisate affords maleylacetoacetate (Chapman and Dagley, 1962).

In contrast to *ortho* and *para*-diphenols, the metabolism of

meta-diphenol (resorcinol) has received very little attention. Larway and Evans (1965) reported that resorcinol was metabolized, by a soil pseudomonad, through hydroxylation at the C-4 position to give 1,2,4-trihydroxybenzene with subsequent ring fission between the *ortho*-dihydroxy group to yield a dibasic acid.

Bacteria that dissimilate aromatic compounds under anaerobic conditions have also been reported. Tarvin and Buswell (1934), and Barker (1956) described several aromatic acids that were utilized under strictly anaerobic conditions by the methanogenic bacteria. Scher and Proctor (1960) isolated several strains of non-sulfur photosynthetic bacteria that utilized benzoate anaerobically. A new, reductive pathway of anaerobic degradation of benzoate by *Rhodopseudomonas palustris* has recently been partially elucidated (Dutton and Evans, 1969; Guyer and Hegeman, 1969). Benzoate is photometabolized with reduction to cyclohex-1-ene-carboxylate followed by hydration to 2-hydroxycyclohexane carboxylate. Dehydrogenation then yields 2-ketocyclohexane carboxylate which is thiolytically cleaved to pimelate. The reducing power involved in such a mechanism was thought to be generated photochemically. Taylor *et al.* (1970) suggested a modified mechanism of benzoate by a facultatively anaerobic *Pseudomonas* sp., since the organism did not grow on cyclohexane carboxylate and neither was this compound oxidized in the presence of KNO_3, by cell suspensions grown

anaerobically on p-hydroxybenzoate. The alternate mechanism involves the addition of water which forms a polyhydroxy derivative of cyclohexane carboxylate that could then be dehydrogenated and thiolytically cleaved as postulated in the pathway for *Rhodopseudomonas palustris* (Dutton and Evans, 1969; Guyer and Hegeman, 1969).

Generally, the metabolic pathway of phenolic compounds in yeasts is not well known (Dagley *et al.*, 1964; Harris and Ricketts, 1962). Hashimoto (1970), recently investigated the oxidative metabolism of p-cresol by an isolated strain of yeast. In the phenol-adapted culture, an enzyme sequence is formed by which p-cresol is oxidized to 4-methylcatechol and the benzene ring is cleaved between carbon atoms 1 and 6 to give 5-formyl-2-hydroxy-2,4-pentadienoic acid.

Stanier (1947), working with *Pseudomonas* strains, developed the valuable technique of "sequential induction" to determine the nature of the intermediates involved in the microbial metabolic pathway of aromatic compounds. The Berkeley school has significantly contributed to this topic, especially by purifying and crystallizing some of the enzymes involved (Ornston, 1966[1-4]), the reconstitution of reaction sequences and their metabolic control (Canovas *et al.*, 1967). The Japanese group led by Hayaishi have also made notable contributions, both in the identification of intermediates, crystallization of oxygenases and elucidation of

their reaction mechanisms (Hayaishi, 1966; Hayaishi and Nozaki, 1969; Hayaishi, 1969).

The results of various investigations have shown that the ability to degrade phloridzin is more frequent in fungi than in bacteria. While Jayasankar *et al.* (1969) noted that, of 54 species of fungi studied, 31 produced phloretic acid and phloroglucinol from phloridzin, only one of the 13 species of bacteria examined by Chatterjee and Gibbins (1969) was able to degrade phloridzin. Chatterjee and Gibbins (1969) showed that *Erwinia herbicola* degrades phloridzin by initial cleavage to phloretin followed by further hydrolytic cleavage to phloroglucinol and phloretic acid by the purified enzyme phloretin hydrolase. The purification and properties of the enzyme from *Aspergillus niger* was later described by Minamikawa (1970). The subsequent degradation of phloroglucinol and phloretic acid was not elucidated by these workers. Neither of these compounds supported the growth of *E. herbicola* or *A. niger*.

Westlake *et al.* (1959) screened a large number of molds, streptomycetes and bacteria for their ability to degrade rutin. The molds, particularly *A. flavis* and *A. niger*, were more active than the streptomycetes and bacteria. Anaerobic degradation of bioflavonoids such as rutin, quercitrin and naringin by rumen microflora was observed by Simpson *et al.* (1969). Phlorogiucinol, detected as a transitory intermediate and utilized by the mixed anaerobic microflora, yielded no degradation products as determined by paper

chromatography. However, in subsequent studies (Krishnamurty *et al.*, 1970) using the rumen isolate *Butyrivibrio* sp. C_3, phloroglucinol was not further metabolized even in the presence of glucose. Nine strains of obligately anaerobic Gram-positive streptococci, capable of degrading phloroglucinol were isolated from rumen by Jones *et al.* (1970). Rumen fluid was, however, required for the utilization of phloroglucinol.

Although phloroglucinol, which forms the A-ring of the flavonoids discussed above, has been reported to be a transitory intermediate in the biodegradation of those flavonoids, very little is known about its degradative pathway. Since large amounts of flavonoids are returned to the soil and not accumulated there, Towers (1964) envisaged the existence of microorganisms in soil, capable of cleaving the ring of phloroglucinol. On this premise, Robern (1965) isolated a *Pseudomonas* sp. and a *Penicillium* sp., by soil perfusion technique, capable of utilizing phloroglucinol as the sole source of carbon in an otherwise mineral salts medium. The effects of various physiological factors on the metabolism of phloroglucinol by the *Pseudomonas* sp. was studied by Robern (1965) and the pathway of phloroglucinol degradation proposed was elucidated by Hang (1967). This pathway involves the reduction of phloroglucinol to dihydrophloroglucinol which is dehydrated to resorcinol and the dihydroxyphenol is further metabolized. Purification and properties of phloroglucinol reductase catalyzing the first step

in the degradation of phloroglucinol was also reported by Hang (1967). In his preliminary studies, Robern (1965) showed the utilization of phloroglucinol by the isolated *Penicillium* sp., but no intermediates were detected.

Metabolites either invoke or suppress the synthesis of those enzymes involved in aromatic pathways. A previous view (Stanier, 1947) was that each enzyme is induced (or derepressed) as soon as its substrate begins to accumulate as a result of the newly synthesized enzyme responsible for the previous step. Working with systems for which a detailed knowledge of the enzymology is known, Stanier and his colleagues (1963) have shown that several of them may be induced together (that is, "block-induced") by the presence of a single metabolite in the cell. The use of mutants in studying the multienzyme sequences of mandelate (Stanier *et al.*, 1963), β-ketoadipate (Ornston and Stanier, 1964) and the tryptophan pathways (Palleroni and Stanier, 1964) have increased considerably our understanding of the metabolic control of such systems.

Multienzyme complexes such as α-keto acid dehydrogenase complexes, fatty acid synthetase complexes and tryptophan synthetase, the multienzyme sequence of the tryptophan pathway, are the most extensively studied. A multienzyme complex has been defined as an organized aggregate of different, functionally related enzymes that catalyze successive steps in a reaction sequence. The

subject has been comprehensively reviewed by Henning (1966), Reed and Cox (1966), and Ginsburg and Stadtman (1970). Gaertner and DeMoss (1969) have recently purified and characterized an enzyme aggregate which catalyzes three reactions involved in tryptophan biosynthesis in *Neurospora crassa* : anthranilate synthetase, phosphoribosyl-anthranilate isomerase, and indole-3-glycerol phosphate synthetase. Kochetov *et al.* (1970) suggested, on the basis of data obtained by ion-exchange chromatography and polyacrylamide disc gel electrophoresis, that together with the free forms of transketolase and glyceraldehydephosphate dehydrogenase from baker's yeast, there is also a complex of these enzymes. With enzyme purification procedures becoming more sophisticated, more multiple enzyme activities likely would be isolated in a natural complexed state. As pointed out by Reed and Cox (1966), successful isolation of multienzyme aggregates may be an accident of the purification procedure employed.

P A R T I.

UTILIZATION OF SUBSTRATE AND PHYSIOLOGICAL CONDITIONS

FOR THE PRODUCTION OF ENZYME

INTRODUCTION

Although fungi have been isolated from soils where phloroglucinol and its derivatives are present (Miller, 1961), and degradation of phloridzin to phloroglucinol by isolated strains of *Aspergillus* and *Penicillium* has been reported (Jayasankar *et al.*, 1969), the utilization of phloroglucinol by a pure culture was not shown until 1965 when Robern first demonstrated the utilization of phloroglucinol by a *Penicillium* sp. In his preliminary investigation, Robern (1965) studied the effects of inorganic ions on the growth of the fungus, and on the pH and coloration of the phloroglucinol medium. Manganese ions were found to cause a browning of the medium and to lower the pH as compared to the uninoculated controls. This was attributed to the catalytic effect of Mn^{++} on the oxidation of phloroglucinol by air. Excluding Mg^{++} and $PO_4^{\equiv}$ from the medium resulted in negligible growth and a marked decrease in pH. The addition of Fe^{++} to the medium eliminated these variations in colour and pH but had no stimulatory effect on growth. Although utilization of phloroglucinol was shown by colorimetric and paper chromatographic techniques, no intermediates were detected either from a growing-cell culture or from a resting-cell suspension. Spectrophotometric analysis of the resting-cell fermentation liquor showed the accumulation of an unidentified compound which was not further metabolized.

Robern (1965) limited his brief investigation with *Penicillium* sp. Mac M-47 to the whole cells only; the enzymological aspects of the problem were not explored. The aims of the work reported here were to study the utilization of phloroglucinol in growing-cell and resting-cell fermentations, attempt to detect the intermediate(s) of phloroglucinol degradation, and to study the physiological conditions ideal for the production of enzyme(s) involved in the pathway of phloroglucinol degradation.

MATERIALS AND METHODS

ORGANISM

The test organism - *Penicillium* sp. Mac M-47, used in this study, was originally isolated and described by Robern (1965).

PREPARATION OF MEDIA

The medium employed for cultivating the fungal culture was the same as used by Robern (1965), and contained 0.1% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.001% $FeCl_2$, and 0.25% phloroglucinol as the sole carbon source. The basal medium containing only the ammonium and potassium salts was sterilized separately from the rest of the medium components which were individually sterilized and then combined with the basal medium just prior to use. The agar slants were made by adding 1.5% agar to the liquid medium.

GROWTH AND MAINTENANCE OF CULTURE

The fungus was grown at 30°C, in the liquid medium for 36 hours on a rotary shaker and on agar slants for a period of 3 - 5 days. The culture was stored both on agar slants and in liquid medium at 4°C, and transferred periodically.

PREPARATION OF GROWING-CELL AND RESTING-CELL SUSPENSIONS

A 50 ml Erlenmeyer flask containing 25 ml medium was inoculated from a phloroglucinol agar slant (or with 1 ml of liquid culture) and incubated at 30°C on a rotary shaker. After an incubation of 36 hours, a second transfer was made and subsequently the growth medium was transferred to a 2 litre Erlenmeyer flask containing 500 ml of the phloroglucinol medium. The flask was incubated at 30°C on the rotary shaker.

For resting-cell suspension, the fungal hyphae from one litre of the 24 hours old culture were collected on 8 folds of cheese cloth, washed with saline, and transferred to a 2 litre Erlenmeyer flask containing 500 ml of phloroglucinol medium without $(NH_4)_2SO_4$. The culture was incubated on a rotary shaker at 30°C.

PREPARATION OF SAMPLES FOR ANALYSIS

Samples in aliquots of 20 ml each, were removed at appropriate time intervals from growing-cell and resting-cell fermentations, both to study the rate of phloroglucinol utilization and to detect any intermediates in the fermentation liquor. The fungal hyphae were removed by filtration through a Whatman No. 1 filter paper, and the clear filtrate used for analysis. One millilitre and 0.5 ml aliquots were used for quantitative determination of phloroglucinol and spectral analysis respectively, and the remaining filtrate

was acidified to pH 2.0 with conc. HCl. The acidified sample was extracted with 10 ml aliquots of ethyl ether five times, the ethereal extracts were pooled and then evaporated to dryness under vacuum. The residue was dissolved in 1 ml of 95% ethanol, and 50 λ aliquots used for thin-layer chromatography.

THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography was used to follow the disappearance of phloroglucinol from the fermentation medium and to detect the presence of any intermediates. Glass plates of 20 x 20 cm were acid-cleaned, thoroughly rinsed in distilled water, and dried with methanol. Using a Quickfit apparatus, four plates were uniformly coated to a thickness of 500 μ with a slurry made by homogenizing 45 g of Silica gel G (E. Merckag, Darmstadt, Germany) in 90 ml distilled water. The plates were air-dried overnight, stored in a desiccator and were activated at 105°C for 30 minutes prior to use.

The chromatograms were run at room temperature, using the ascending technique, in rectangular glass chromatography tanks. The solvent systems used in this study were - (1) benzene : methanol : acetic acid :: 45 : 8 : 4 (Randerath, 1963); (2) benzene : dioxane : acetic acid :: 90 : 25 : 4 (Randerath, 1963) and (3) 95% ethanol : conc. NH_4OH :: 100 : 1 (Kennedy and Barker, 1951).

The colour developing reagents used for spraying the chromatograms included vanillin-toluene-p-sulfonic acid (Roux and Maihs, 1960), tetrazotized-benzidine (Randerath, 1963), and bromocresol purple (Reid and Lederer, 1951).

ULTRA-VIOLET ABSORPTION SPECTROPHOTOMETRY

A 0.5 ml aliquot of the sample and 2.5 ml distilled water were added to a quartz cuvette of 3 ml capacity and 10 mm light path. The diluted sample was scanned against distilled water, kept in the reference cuvette, in a recording UNICAM SP. 800 ultra-violet spectrophotometer.

QUANTITATIVE DETERMINATION OF PHLOROGLUCINOL

Phloroglucinol was determined by the method of Jayasankar and Bhat (1966), using a Gilford 300-N spectrophotometer. The determinations were recorded directly from the concentration scale after calibrating the machine with a known standard and using samples appropriately diluted to fall within the limits of linearity of the method.

LARGE-SCALE FERMENTATION OF PHLOROGLUCINOL

A MicroFerm Laboratory Fermenter (New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.) was used for the large-scale

fermentation of phloroglucinol. Ten litres of 0.25% phloroglucinol medium was prepared in the fermentation jar of 14 litre capacity, cooled down to 30°C and maintained at that temperature until inoculation.

The inoculum was prepared by harvesting the mycelial growth from 10 x 500 ml of a 24 hours old culture. The fungal hyphae were collected on cheese cloth and washed with 2 volumes (10 litres) of normal saline. The mycelial mat thus obtained was resuspended in 125 ml saline and homogenized in a Sorval Omni Mixer to obtain a uniform sample for the dry weight determinations. The medium in the fermentation jar was inoculated with 100 ml of this suspension.

The following fermentation conditions were maintained throughout the duration of the run:

Temperature: 30°C

Aeration: Air pressure : 15 lbs/sq. inch

Air flow : 5000 cc/min (1/2 vol. of med.)

Agitation: 300 r.p.m.

A silicone anti-foam A spray (Dow Corning Corp., Midland, Michigan, U.S.A.) was used whenever necessary.

The level of phloroglucinol was monitored by analyzing samples

periodically, and the fermentation was stopped when it reached about 10 - 15% of the amount originally added. The fermentation jar was disconnected from the fermenter and transferred to the cold room.

PREPARATION OF FREEZE-DRIED MYCELIAL POWDER

The mycelial powder, serving as the source of crude enzyme extract, was prepared by a modified technique of Ingram and Hockster (1967).

The mycelial growth, at the end of the 10 litre fermentation run, was harvested on 8 folds of 90 gauge cheese cloth and washed with one volume (10 litres) of saline. The washed mycelial mat was transferred between several folds of cheese cloth, and after squeezing out the excess moisture, was subjected to a hydraulic pressure of 5000 lbs/sq. inch on a French Press fitted with an adapter. The semi-dry mycelial cake thus obtained was crumbled into small pieces, and freeze-dried for a period of about 48 hours. The freeze-dried material was ground in a mortar and the resultant powder stored in a screw-capped bottle at -20°C.

PREPARATION OF CELL-FREE EXTRACT

Forty milligrams of the freeze-dried mycelial powder was weighed in a 15 ml nalgene centrifuge tube to which was added 2 ml

of 0.05 M potassium phosphate buffer (pH 7.5) containing 5 mM EDTA, 1 mM Cleland's reagent, and 100 µM phenyl-methyl sulfonyl fluoride (inhibitor of proteolytic enzymes). The suspension was shaken for a period of 2 hours on a reciprocal shaker at 4°C, and then centrifuged in a Sorvall RC-2B centrifuge at 48000 x g for 20 minutes. The cell debris was discarded and the supernatant used as the crude enzyme extract.

ENZYME ASSAY

All enzymatic assays were run at room temperature in 1.8 ml of reaction mixture containing 100 µmoles potassium phosphate buffer (pH 7.5), 0.5 µmole NADPH, 1 µmole phloroglucinol, and a suitable amount of enzyme. The reaction, started by the addition of phloroglucinol, was followed in a recording UNICAM SP. 825 spectrophotometer by measuring a decrease in optical density at 340 nm which corresponds to the oxidation of NADPH to NADP. The enzyme unit is defined as an optical density change of 0.01 per minute at 340 nm.

DETERMINATION OF PROTEIN

Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. The developed colour was read at 660 nm on a Gilford 300-N spectrophotometer on the concentration scale, after calibrating the machine with a known standard.

R E S U L T S

UTILIZATION OF PHLOROGLUCINOL IN GROWING-CELL AND RESTING-CELL FERMENTATIONS

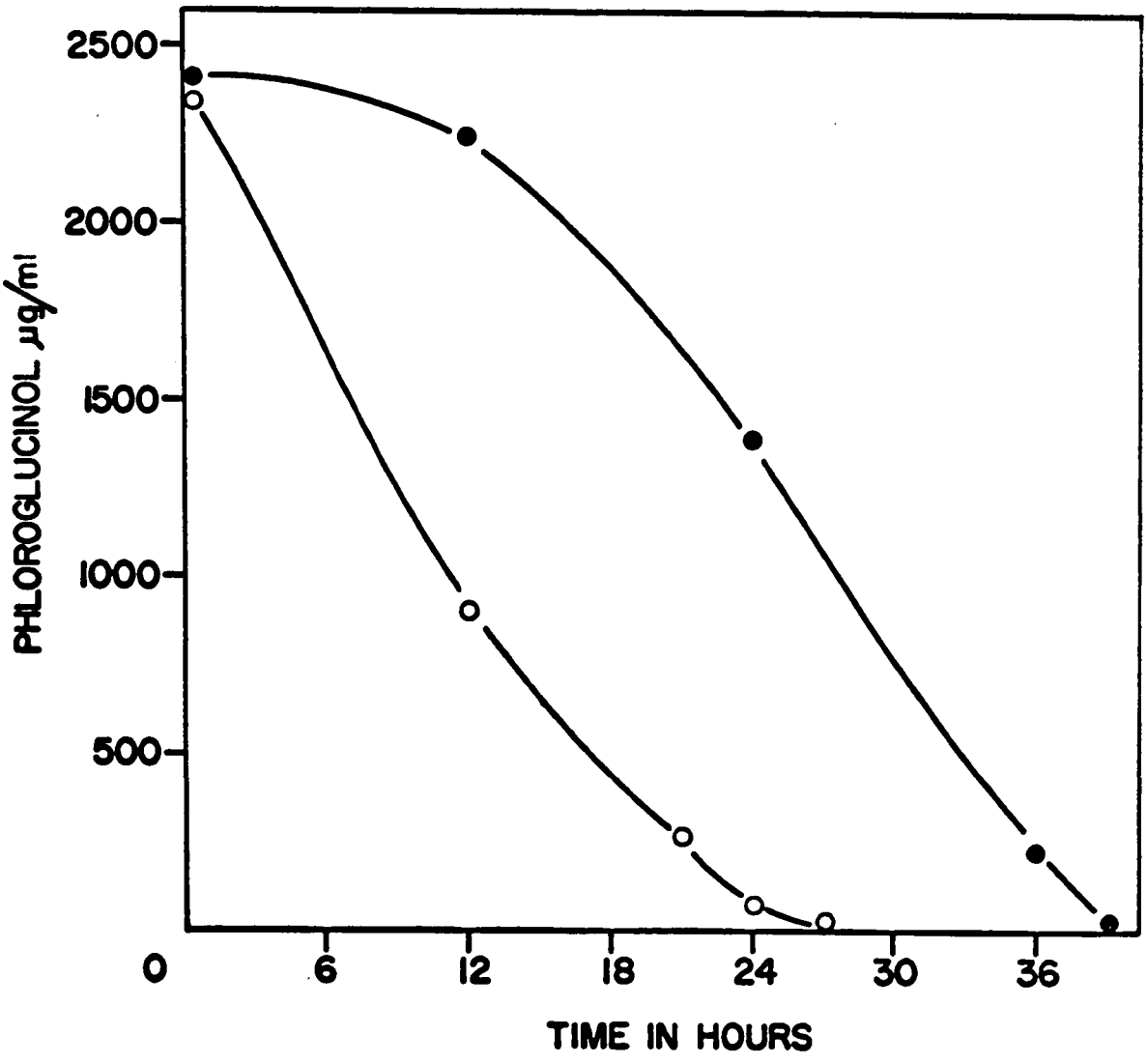
The rate of utilization of the substrate in growing-cell fermentation was followed by sampling at various time intervals, from the fermentation flask containing the complete phloroglucinol medium inoculated with the fungal culture. The colorimetric analyses for the residual level of phloroglucinol showed (Fig. 1) that there was an initial lag of about 12 hours preceding the linear rate of substrate utilization, and complete disappearance of the substrate shortly after 36 hours.

In the resting-cell fermentation, the rate of phloroglucinol utilization was studied in a medium devoid of the nitrogen source $(NH_4)_2SO_4$. The results of the colorimetric analyses of the time sequence samples revealed (Fig. 1) that the substrate was utilized without any lag, and the complete disappearance of 0.25% phloroglucinol required 24 - 27 hours compared to 39 hours for the growing-cell fermentation.

CHROMATOGRAPHY OF FERMENTATION LIQUOR

Fermentation samples from growing-cell and resting-cell fermentations were chromatographed on thin-layer plates to study the

Fig. 1. Rate of phloroglucinol utilization in a growing-cell fermentation (●——●) and a resting-cell fermentation (○——○).


2500
2000
1500
1000
500
PHLOROGLUCINOL µg/ml
0
6
12
18
24
30
36
TIME IN HOURS

utilization of phloroglucinol and also to detect the production of any intermediates of phloroglucinol metabolism. Although the disappearance of phloroglucinol was easily shown, no other compounds were detected. Figure 2 shows the tracings of the thin-layer chromatogram of samples from a resting-cell fermentation. The chromatogram was developed in benzene-methanol-acetic acid, and vanillin-toluene-p-sulfonic acid was used as the detecting spray reagent. Only a reddish-brown spot with R_f value of 0.28, and corresponding to the authentic phloroglucinol spot, was detected in the initial samples. The size and colour intensity of the spot decreased with time, disappearing completely after 21 hours.

ULTRA-VIOLET ABSORPTION SPECTROPHOTOMETRY OF FERMENTATION LIQUOR

Both the utilization of the substrate phloroglucinol and the production of any intermediates were also studied by ultra-violet spectrophotometric analyses. The results of a typical experiment, with samples from a resting-cell fermentation, are shown in Fig. 3. Note the gradual shift in the absorption peak from 268 nm to 285 nm between 18 and 24 hours. No further shift in the 285 nm peak was observed even after prolonged incubation. Attempts to detect the compound absorbing at 285 nm, by thin-layer chromatography, were without any success.

Fig. 2. Tracings of thin-layer chromatograms of a resting-cell fermentation liquor.

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

Colour developing reagent: Vanillin-toluene-p-sulfonic acid

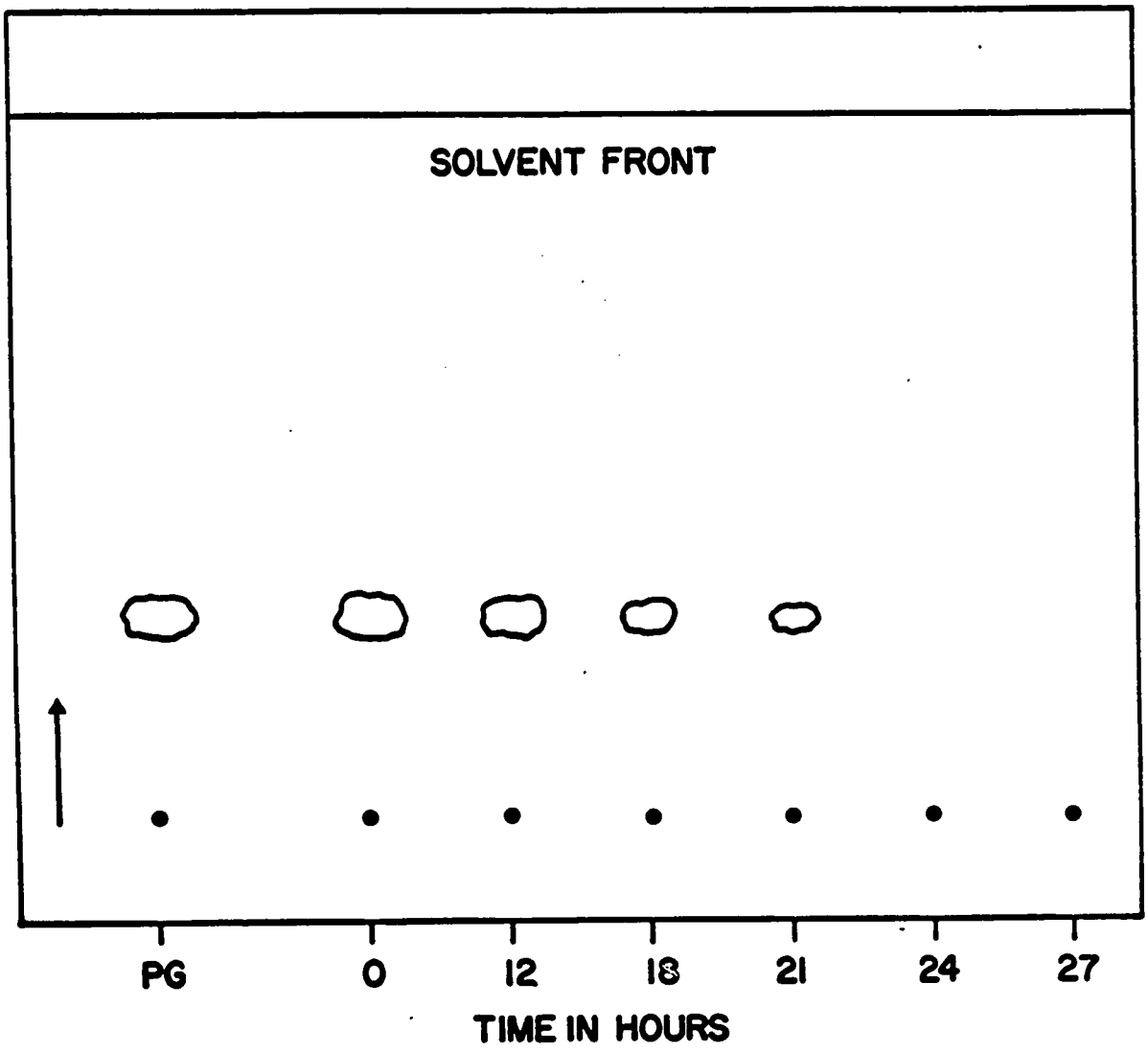
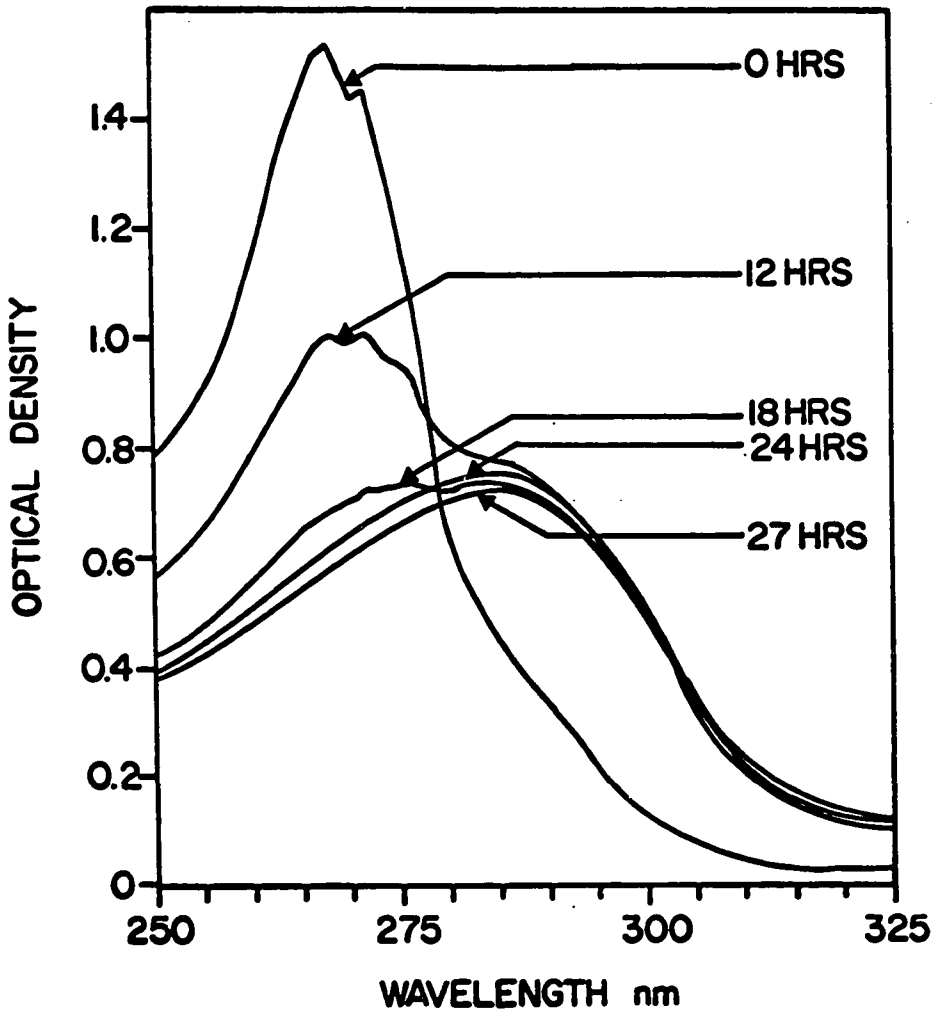

SOLVENT FRONT
PG
0
12
18
21
24
27
TIME IN HOURS

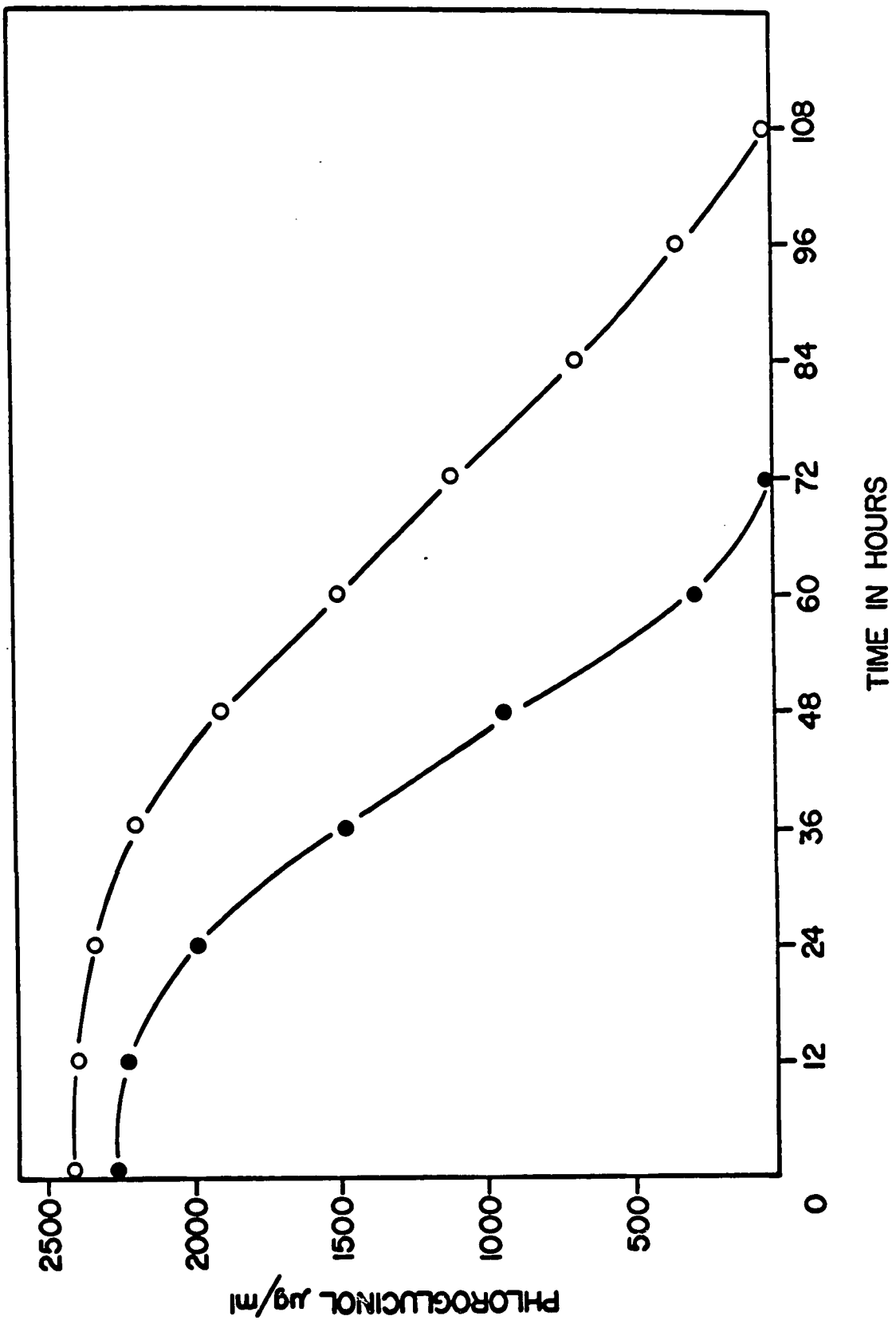
Fig. 3. Ultraviolet absorption spectra of the resting-cell fermentation liquor at different time intervals.


0 HRS
12 HRS
18 HRS
24 HRS
27 HRS
OPTICAL DENSITY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
250
275
300
325
WAVELENGTH nm

UTILIZATION OF PHLOROGLUCINOL BY GLUCOSE-GROWN CELLS

The rate of phloroglucinol utilization was compared on phloroglucinol-grown cells and glucose-grown cells, in a growing-cell fermentation. The culture was transferred on potato dextrose agar slants twice before inoculating a 50 ml Erlenmeyer flask containing 25 ml of the basic mineral salts medium (see Materials and Methods) containing 0.25% glucose as the sole carbon source. After an incubation of 36 hours at 30°C, a second transfer was made in the glucose medium, and subsequently the fungal hyphae were collected on cheese cloth, washed with saline, and transferred to a 2 litre Erlenmeyer flask containing 500 ml of 0.25% phloroglucinol medium. The phloroglucinol-grown cells were prepared as previously described under Materials and Methods. The two flasks were incubated at 30°C on the rotary shaker, and 10 ml samples were removed at appropriate time intervals. Figure 4 shows that the glucose-grown cells start utilizing phloroglucinol after a much longer lag period compared to the cells which had previously been adapted to phloroglucinol. The total time required for complete utilization of 0.25% phloroglucinol was 108 hours by the glucose-grown cells compared to 72 hours by the phloroglucinol-grown cells. Clearly the results indicate that the enzyme system involved in the degradation of phloroglucinol is an inducible one and was further confirmed by a lack of enzyme activity in the glucose-grown cells.

Fig. 4. Comparative rates of phloroglucinol utilization by phloroglucinol-grown cells (●——●) and glucose-grown cells (O——O), in a growing-cell fermentation.


PHLOROGLUCINOL µg/ml
2500
2000
1500
1000
500
0
12
24
36
48
60
72
84
96
108
TIME IN HOURS

EFFECT OF INCUBATION TIME ON SUBSTRATE UTILIZATION, MYCELIAL YIELD, AND ENZYME ACTIVITY

A systematic study of the optimum length of incubation was made in relation to the substrate utilization, yield of the freeze-dried mycelial powder, and the corresponding enzyme activity of the crude extract. The mycelial growth from 4 x 500 ml of 24 hours old culture medium was collected on cheese cloth, washed with 2 volumes of saline, and resuspended in 50 ml saline. The suspension was homogenized, and 5 ml of this suspension (equivalent of 83 mg dry weight)was inoculated into each of the eight 2 litre Erlenmeyer flasks containing 500 ml of the complete 0.25% phloroglucinol medium. The flasks were incubated at 30°C on the rotary shaker. Every 12 hours, one flask was removed, and after analyzing an aliquot for the residual level of phloroglucinol, the mycelial growth from each flask was harvested and freeze-dried as described previously. The crude extracts, prepared from these freeze-dried mycelial powders, were analzed for protein and enzyme activity. The results are presented in Table 1.

As previously observed, the substrate phloroglucinol was metabolized after an initial lag of about 18 hours and was completely utilized on 48 hours incubation which also corresponded to the maximum build-up of the mycelial mass. On prolonged incubation, there was a gradual decrease in the mycelial yield suggesting an autolysis

T A B L E 1.

Effect of incubation time on the rate of substrate utilization, mycelial yield and enzyme activity.

Incubation time (hrs)	Residual substrate (µg/ml)	Mycelial yield (mg)	Crude extract	
			Protein (mg/ml)	Enzyme (units/ml)
0	2500	-*	-	-
12	2250	-*	-	-
24	1650	88.0	2.7	72
36	350	244.5	2.8	60
48	<10	263.0	2.0	0
60	0	219.0	1.7	0
72	0	220.0	1.6	0
84	0	195.0	1.2	0

*Yield of freeze-dried mycelial powder was too little to recover after 12 hrs incubation.

of the fungal hyphae in the absence of a ready source of carbon and energy, as also evidenced by a decrease in protein concentration.

The enzyme activity was observed only in the crude extracts prepared from 24 hours and 36 hours old cultures, when the substrate phloroglucinol was still present in the culture medium. The crude extracts, prepared from cultures after the complete utilization of the substrate, showed no enzyme activity at all. These observations were substantiated in subsequent experiments which showed a very rapid depletion of the enzyme level only a few hours after the disappearance of the substrate phloroglucinol from the culture medium. These unique findings strongly suggest that phloroglucinol exerts a stabilizing and/or protective effect on the enzyme protein.

In all subsequent experiments, the fermentation was stopped when the residual level of phloroglucinol was about 300 - 500 µg per ml, and the harvested mycelial mass was washed with 0.25% phloroglucinol, instead of saline, to avoid total depletion of the substrate during the time lapse before lyophilization.

EFFECT OF SUBSTRATE INCREMENTS ON MYCELIAL YIELD AND ENZYME ACTIVITY

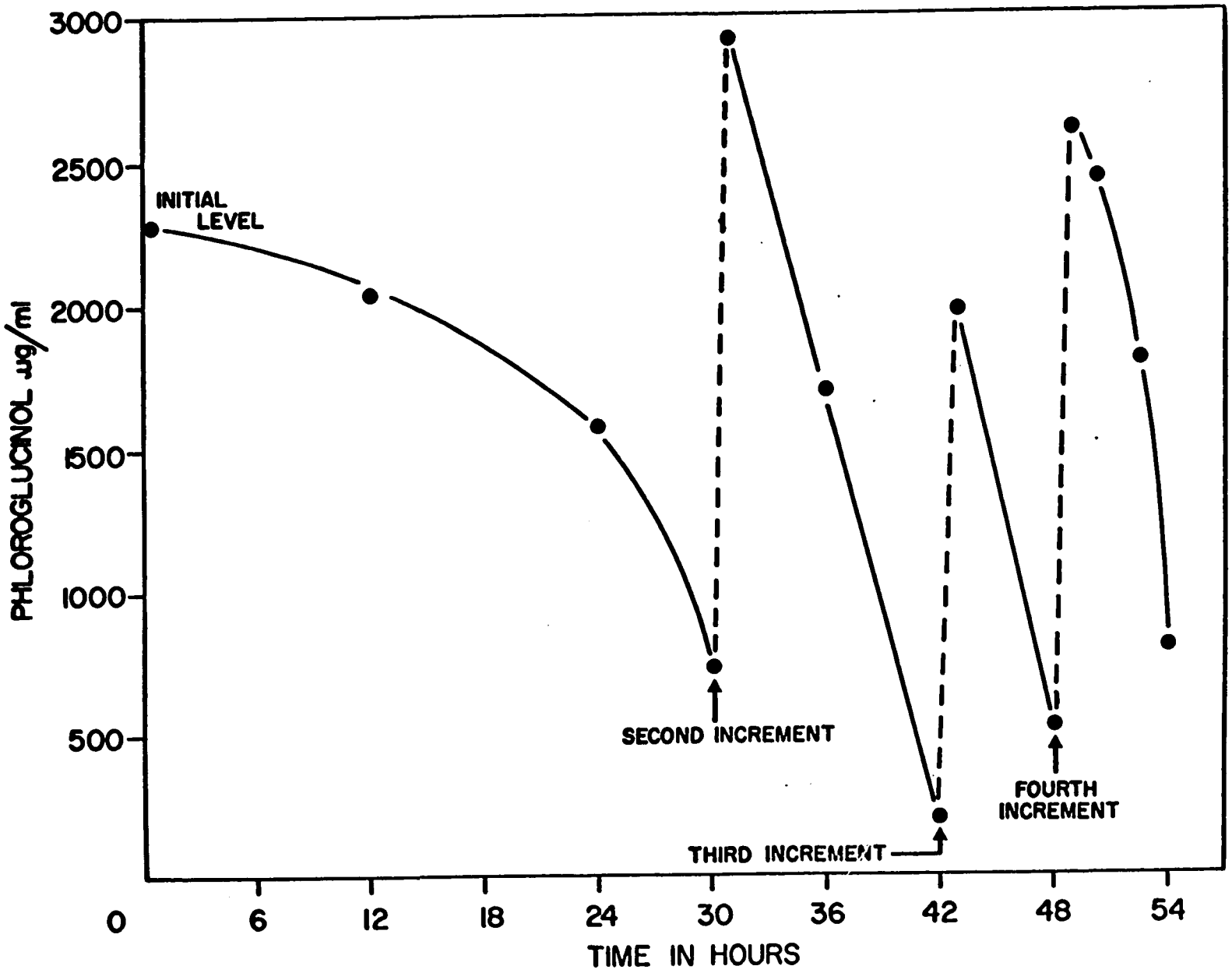
From a large scale fermentation of phloroglucinol in 10 litre medium in the MicroFerm Laboratory Fermenter, the average yield of

the freeze-dried mycelial powder was found to be only about 6 gms. To improve the yield, the effects were studied of adding the substrate phloroglucinol in more than one increment to the fermentation medium at a time when most of the substrate from a previous addition had been utilized (Fig. 5). Three additional increments, of 25 g each (to give 0.25% conc.), of phloroglucinol were added to the 10 litre fermentation medium after 30, 42, and 48 hours incubation, and the mycelial growth was harvested after a total incubation of 54 hours. Although the mycelial yield increased three-fold, the enzyme activity in the crude extract dropped to less than 30% of the activity from a normal single step fermentation. Addition of $(NH_4)_2SO_4$ along with the phloroglucinol increments did not restore the enzyme activity to normal. All subsequent batches of freeze-dried mycelial powder were, therefore, prepared from fermentations in medium containing only the initial 0.25% phloroglucinol.

EFFECT OF SIZE AND PHYSIOLOGICAL STATE OF THE INOCULUM ON THE RATE OF SUBSTRATE UTILIZATION

The size of the inoculum, used in the fermenter, was shown to be very significant in arriving at the time of harvesting of the mycelial growth. The larger the inoculum, the faster the rate of substrate utilization, and consequently shorter the incubation time before harvesting when a minimal amount of the substrate was still

Fig. 5. Pattern of phloroglucinol utilization in a growing-cell fermentation, with multiple step-wise additions of the substrate.


3000
2500
2000
1500
1000
500
PHLOROGLUCINOL µg/ml
INITIAL LEVEL
SECOND INCREMENT
THIRD INCREMENT
FOURTH INCREMENT
0
6
12
18
24
30
36
42
48
54
TIME IN HOURS

present in the medium. To keep the harvesting time relatively constant, and to avoid the total depletion of the substrate and hence the enzyme level, the inoculum was homogenized for dry weight determination on an aliquot, and a calculated amount of the homogenized suspension, equivalent of 1.6 $\pm$ 0.2 g dry weight, was used as the inoculum for all large scale fermentation experiments.

However, a relatively long lag period was consistently observed before any significant utilization of the substrate. To explain this long lag, two different possibilities were examined. As described previously, preparation of the inoculum, involving harvesting the mycelial growth and washing with 2 volumes of saline, required approximately three hours. If the phloroglucinol concentration and hence the enzyme level is depleted during this time, the observed lag period could then be explained due to re-adaptation of the cells to the substrate. To check this possibility the inoculum was washed with 0.25% phloroglucinol solution instead of saline, but it failed to eliminate or even reduce the lag period.

The second possibility was the homogenization step being responsible for the lag by causing cellular damage in the inoculum. If true, the level of viable cells in the inoculum would be much lower than anticipated. When the homogenization step in the preparation of inoculum was omitted, the lag period was almost completely

eliminated (Fig. 6). The complete utilization of 0.25% phloroglucinol required only 15 hours, as against 36 hours when the inoculum was homogenized. Moreover, the mycelial yield was relatively better than before and the enzyme activity was found to be nearly double over the previous preparations.

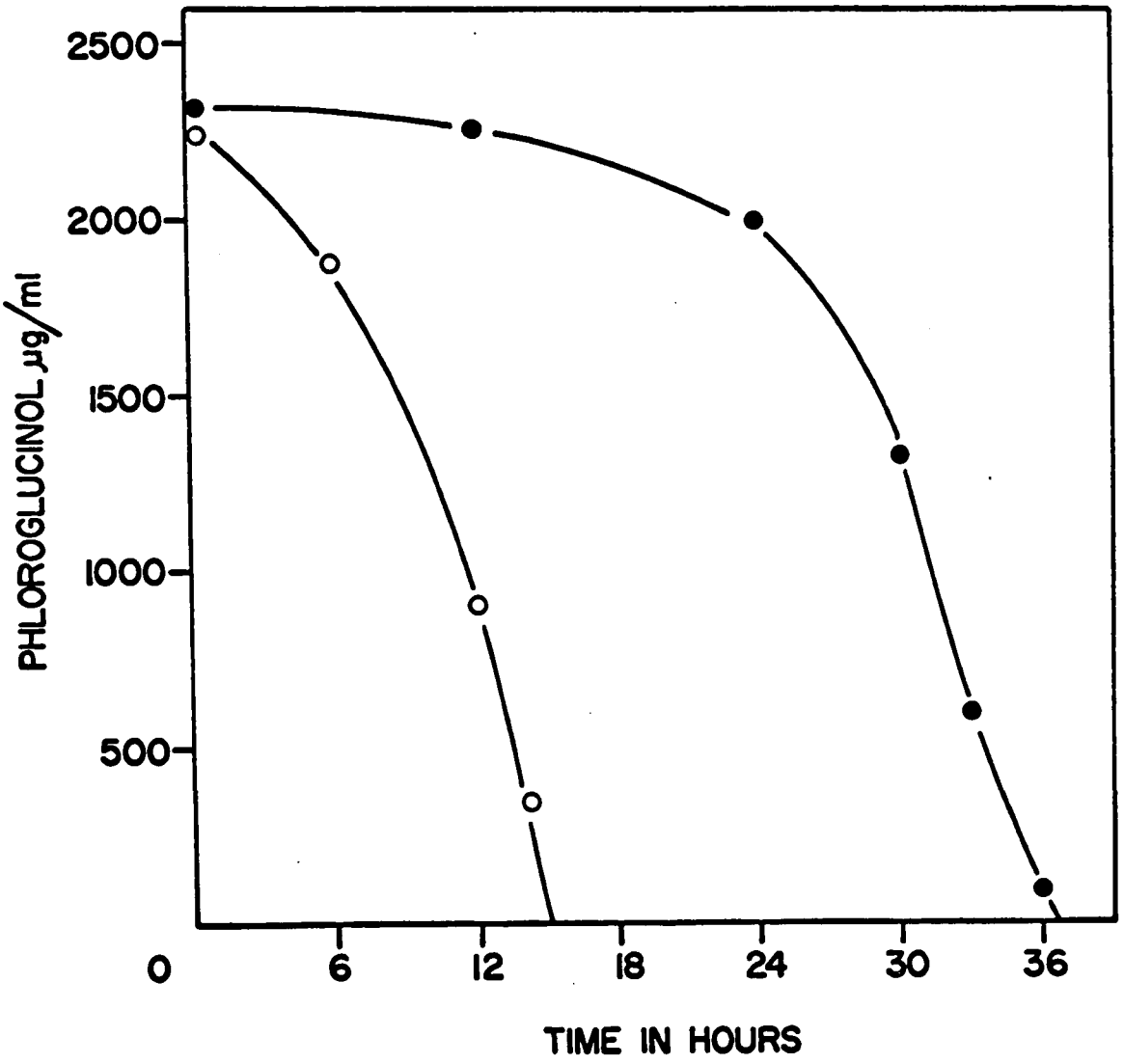
STABILITY OF THE FREEZE-DRIED MYCELIAL POWDER AT -20°C

The freeze-dried mycelial powder was found to be fairly stable at -20°C. Crude extracts were prepared from different batches of the freeze-dried mycelial powder stored at -20°C for a period ranging from one month to 22 months. An activity loss of only 20% was observed in preparations stored up to ten months. Mycelial powder stored for a period of 22 months still contained 50% of its original enzyme activity.

Fig. 6. Effect of physiological state of the inoculum on the rate of substrate utilization.

●—● Inoculum homogenized

○—○ Inoculum not homogenized


2500
2000
1500
1000
500
PHLOROGLUCINOL µg/ml
0
6
12
18
24
30
36
TIME IN HOURS

DISCUSSION

Robern (1965) investigated the fermentative pathway of phloroglucinol metabolism using resting cells of the *Penicillium* sp. Mac M-47, and reported the accumulation of a 278 nm absorbing compound in the culture medium. This compound was not further metabolized by the fungus and could not be detected by paper chromatographic techniques. A similar shift in the absorption peak from 268 nm to 278 nm was also reported by Hang (1967) with a *Pseudomonas* sp., and the new compound was tentatively identified as dihydrophloroglucinol on the basis of chromatographic and enzymatic data. The identification was later confirmed (Jamieson *et al.*, 1970) by mass spectroscopic analysis. In the present work, both the rate of utilization of phloroglucinol and the production of intermediates were followed spectrophotometrically and by thin-layer chromatography. A shift in the absorption peak was observed, but the peak shifted from 268 nm (phloroglucinol) to 285 nm. The compound absorbing at the higher wavelength was not further metabolized and could not be detected by chromatographic techniques, as reported by Robern (1965). The inability to detect any intermediates in the fermentation medium was interpreted as an indication for the existence of an enzyme complex. This hypothesis is supported by subsequent studies (PART II). The initial breakdown products of phloroglucinol are, presumably, further metabolized by a sequence of enzymatic reactions without being released from the postulated enzyme complex.

The enzyme was shown to be inducible by phloroglucinol and was not detected in glucose-grown cells. The inducing growth substrate phloroglucinol exhibits a significant and rather unique role in controlling the level of enzyme which rapidly disappears with concomitant disappearance of the substrate from the fermentation medium. Various workers have reported that an induced enzyme activity can be caused to decay by removal of the inducing conditions. In contrast to the general finding of stability of induced enzymes in bacteria (Mandelstam, 1960), the universal finding in adult or differentiated mammalian tissue has been that the enzyme activity returns to a basal constitutive level along an exponential time course once the stimulus is removed (Schimke, 1969, 1970). Rat liver tryptophan pyrrolase is stabilized *in vitro* by the substrate tryptophan (Schimke *et al.*, 1965). Yeast hexokinase is stabilized against trypsin attack by glucose (Berger *et al.*, 1946). There are no documented reports of this phenomenon in molds. In view of the widespread ability of substrates to protect enzymes against various forms of denaturation, and the known ability of proteolytic enzymes to degrade denatured proteins (Linderstrøm-Lang, 1950), it may be that the conformation of the enzyme-substrate complex is so different from that of the free enzyme, that the enzyme is resistant to proteolysis (Schimke, 1964; Green and Neurath, 1954).

While comparing the efficiency of disruption of mycelia for the release of enzymes,by various techniques, Zetelaki (1969) failed to

make any mention of the technique of freeze-drying the mycelial mat (Ingram and Hochster, 1967) and subsequently preparing a crude extract by buffer extraction of the mycelial powder. A modification of this technique was used in the present studies, and was found to offer an excellent base material for preparing the crude extracts. The freeze-dried mycelial powder lost little enzymatic activity when stored at -20°C for about one year.

While the mycelial yield increased three-fold by the addition of multiple increments of the substrate phloroglucinol to the fermentation medium, the crude extracts derived from such mycelial powder had only 25 - 30% of the enzyme activity as compared to control preparations. This anomaly can be explained as follows: (a) the second, third and subsequent increments of the substrate bring about a 3-fold increase in the mycelial mass; (b) the specific activity decreases about 3 to 4-fold; (c) apparently, the observed phenomenon is not a case of simple dilution of the enzyme protein, and can be accounted for, in addition, by specific degradation of the enzyme protein in preference to, and faster than the other protein species in the aging hyphae.

P A R T II.

PURIFICATION AND PROPERTIES OF THE ENZYME COMPLEX

INTRODUCTION

Many of the enzymes mediating the individual reactions in the well-mapped metabolic pathways of aromatic compounds have been purified and, in some cases, crystallized (Ornston, 1966[1-4]; Ono *et al.*, 1970). Studies with purified enzymes have been mainly responsible for a clear understanding of the metabolic pathways of various aromatic compounds. Data obtained from experiments with whole cells may reveal only part of a metabolic sequence, whereas enzymological studies elucidate the individual reactions involved in a metabolic pathway.

The intermediate role of β-ketoadipate in the oxidative metabolism of aromatic compounds by bacteria was first suggested by Kilby (1948) who found the accumulation of this compound in the culture medium. This was confirmed by the ability of cell-free extracts of *Pseudomonas putida*, induced by growth on aromatic precursors, to convert catechol and protocatechuate quantitatively to β-ketoadipate. Subsequent enzymatic studies (Ornston and Stanier, 1964; Ornston, 1966[1-4]) elegantly elucidated the steps in the convergent catabolic pathways leading from catechol and protocatechuate to β-ketoadipate.

In his studies on the metabolism of phloroglucinol by a *Pseudomonas* sp., Robern (1965) observed that a conjugated phenolic

compound 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. Hang (1967) subsequently isolated and purified phloroglucinol reductase catalyzing the first step in the pathway of phloroglucinol degradation by the bacterium. Studies with the purified enzyme, however, showed the formation of dihydrophloroglucinol (Hang, 1967; Jamieson *et al.*, 1970), the reductive product of phloroglucinol, and this compound then dehydrates to give resorcinol.

Furthermore, in the case of metabolic schemes wherein the whole or part of the sequence of reactions is catalyzed by an enzyme complex, detection and isolation of the intermediates is a formidable task when working with whole cells. Such a situation was obtained in the present investigation. The inability to detect any intermediate in the growing-cell or resting-cell fermentations of phloroglucinol by *Penicillium* sp. Mac M-47 was instrumental in the concept of an enzyme complex proposed. This report describes the purification and properties of the enzyme complex mediating the reactions in the degradation of phloroglucinol by the fungus. A pathway of phloroglucinol degradation by the *Penicillium* sp. is proposed.

MATERIALS AND METHODS

PREPARATION OF GLASSWARE AND SOLUTIONS

All the essential glassware was precleaned in chromic acid and all solutions were made in deionized water.

SOURCE OF CHEMICALS

Reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), cytochrome C, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), protamine sulfate, iodoacetic acid, N-ethylmaleimide (NEM), ethylene-diamine-tetraacetic acid (EDTA), and tris (hydroxymethyl) aminomethane (TRIS) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Biogel P-300, phloroglucinol (PG), Cleland's reagent, p-chloromercuribenzoate (p-CMB), and 2-(N-morpholino)-ethanesulfonic acid (MES) were obtained from Calbiochem, Los Angeles, California, U.S.A. Benzoquinone (quinone), 1,4-naphthoquinone, and 2-methyl-1, 4-naphthoquinone (menadione) were purchased from Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A. Resorcinol (RES), pyrogallol,orcinol, phenol, mercuric chloride, and potassium ferricyanide were obtained from Fisher Scientific Company, Montreal, Quebec,

Canada. 1,2,4-trihydroxybenzene was bought from K & K Laboratories Inc., N.Y., U.S.A. Phloroglucinol carboxylic acid was purchased from Morton Chemical Co., Ringwood, Ill., U.S.A. Phloroglucinaldehyde was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. All types of Sephadex gels, including the diethylaminoethyl (DEAE) Sephadex A-50 were from Pharmacia (Canada) Ltd., Montreal, P.Q., Canada. All other chemicals, not mentioned above, were of reagent grade, and bought from available commercial sources.

METHODS OF ANALYSES

Protein determinations on all samples were done after the method of Lowry *et al.* (1951). Assay conditions for the enzyme activities were the same as described under Materials and Methods (Part I), unless otherwise stated.

PURIFICATION OF THE ENZYME

All purification procedures were carried out at 4°C in a cold room, and all centrifugations were done in a Sorvall RC-2B centrifuge at 48000 x g for 20 minutes.

STEP 1. *Preparation of crude extract*

Two grams of the freeze-dried mycelial powder (see Materials

and Methods, Part I) were suspended in 50 ml of 0.05 M potassium phosphate buffer (pH 7.5) containing 5 mM EDTA, 1 mM Cleland's reagent and 100 μM phenyl-methyl-sulfonyl-fluoride (extraction buffer) in a 250 ml Erlenmeyer flask, and shaken on a reciprocal shaker for 3 hours. The suspension was centrifuged, and the brownish pellet was discarded.

STEP 2. *Membrane filtration*

The crude extract was filtered through XM-50 membrane in a Diaflo ultra-filtration apparatus (Amicon Corp., Lexington, Mass., U.S.A.), according to the instructions of the manufacturer. The operation was carried out under a flow of N_2 gas at 40 lbs/sq. inch pressure and with constant stirring. When about 5 ml was left on top of the membrane, the filtration was stopped by simultaneously closing the effluent line and the in-flowing N_2 gas, to avoid back flow. The concentrated material was recovered, made up to 50 ml with the extraction buffer and clarified by centrifugation.

STEP 3. *Protamine sulfate treatment*

A 2% protamine sulfate solution was prepared in 0.05 M potassium phosphate buffer (pH 7.5), and 5 ml of this solution was added to 50 ml of the enzyme solution from Step 2, in three increments of 2 ml, 2 ml, and 1 ml respectively. The addition was

dropwise, and after each increment, the mixture was allowed to stir for an additional 30 minutes preceding centrifugation and the precipitate was discarded.

STEP 4. $(NH_4)_2SO_4$ *fractionation*

The protamine sulfate-treated supernatant solution was made 45% saturated with $(NH_4)_2SO_4$ by the addition of 14.5 g of the solid salt to 55 ml of the solution with stirring. After an additional 15 minutes stirring, the solution was centrifuged, and the pellet was discarded. To the supernatant solution (61 ml) were added 5.6 g $(NH_4)_2SO_4$ to obtain 60% saturation and stirred for an additional 15 minutes preceding centrifugation. The precipitate was dissolved in 5 ml of extraction buffer, and dialyzed against 4 x 500 ml 0.01 M potassium phosphate buffer (pH 7.5) containing 5 mM EDTA and 1 mM Cleland's reagent over a period of about 18 hours.

STEP 5. *Column chromatography on DEAE-Sephadex*

DEAE-Sephadex A-50 was precycled and equilibrated according to the instructions of the manufacturer. Fifty grams of the exchanger gel were gently stirred into 7 litres of deionized water, and allowed to swell for 3 hours at room temperature. The supernatant containing the fine particles was decanted, and the swollen gel was resuspended in 0.1 N NaOH. After three changes in NaOH, the exchanger was washed with deionized water until the effluent

reached a pH of about 7. At this time, the exchanger was washed in 3 changes of 0.1 N HCl followed by deionized water washing until the pH was near neutral. The DEAE-Sephadex was then washed in 3 changes of 0.05 M Tris-HCl buffer (pH 7.5) and finally suspended in the same buffer to a volume of 2 litres. It was stored at 4°C and used for packing all the columns required in different purification experiments.

One hundred ml of the DEAE-Sephadex slurry was made 5 mM with respect to EDTA and 1 mM Cleland's reagent and allowed to stir at 4°C for about 2 hours. A Sephadex K 15/30 column was fitted with the column reservoir, mounted on a stand, and filled about 2/3 with 25 ml of the starting buffer, 0.05 M Tris-HCl (pH 7.5) containing 5 mM EDTA and 1 mM Cleland's reagent, keeping the effluent line at a higher level. The gel slurry was poured in along the side of the column reservoir to avoid any air bubbles, and allowed to stand for about 5 minutes. The column effluent line was subsequently lowered and the column was packed at a pressure head of 20 cm. When all the gel was settled, the excess buffer on top was siphoned out, and the bed was topped with about 2 cm layer of Sephadex G-25 (Medium) for stability. The packed column was equilibrated with about 150 ml of the starting buffer, before use.

The dialyzed sample of 45 - 60% saturated $(NH_4)_2SO_4$ fractionation was run into the equilibrated column (1.5 cm x 22 cm), eluted first with 100 ml 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM

EDTA and 1 mM Cleland's reagent, and subsequently a linear gradient, of 0.0 M to 0.15 M KCl in the same buffer, was applied. The elution was carried out with 20 cm pressure head, at an average rate of 13 ml/hour. A total number of 80 fractions of 5 ml each were collected using an LKB fraction collector. After analyzing for enzyme activity and protein, the peak fractions were pooled and concentrated to 5 ml by ultrafiltration on XM-50 membrane filter.

STEP 6. *Column chromatography on Sephadex G-200*

Two grams of Sephadex G-200 was suspended in about 200 ml 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 1 mM Cleland's reagent, under constant stirring, and allowed to swell for 72 hours at room temperature, changing the buffer several times in between by decantation and thus removing the fine particles. The slurry was finally made up to 200 ml in the same buffer and stored at 4°C. A slurry of 100 ml was used for packing a Sephadex K 15/30 column, at a pressure head of 15 cm. The soft gel in the column was stabilized by applying a 1.5 cm layer of Sephadex G-25 (Medium) at the top of the column bed. The column was equilibrated with 150 ml of the same buffer.

The material from the DEAE-Sephadex step was applied on the Sephadex G-200 column, and eluted at the rate of 15 ml/hour, under positive pressure,with 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 1 mM Cleland's reagent. Thirty fractions of 2 ml

each were collected on the LKB automatic fraction collector. The fractions with high specific activity were pooled and concentrated to 5 ml by ultrafiltration.

The enzyme purification steps were normally completed over a period of 5 days. The material obtained at the end of the purification scheme was dialyzed against 0.01 M potassium phosphate buffer (pH 7.5), and stored frozen at -20°C.

RESULTS

PRELIMINARY PURIFICATION OF THE ENZYME

Ultrafiltration through a Diaflo filtration unit has been used by various workers for concentrating column eluates (Chatterjee and Gibbins, 1969; Wang *et al.*, 1968; Blat *et al.*, 1965). In addition, ultrafiltration can be used with advantage to remove low molecular weight proteins and other substances in crude enzyme extracts. In the present work, the enzyme activity was found to be retained by a XM-50 membrane but not by a XM-100 membrane.

Hang (1967) employed the method of Burchall and Hitchings (1965) for precipitating the nucleic acids with streptomycin sulfate from crude extracts of a *Pseudomonas* sp., but failed to get any significant increase in specific activity of phloroglucinol reductase, although it was reported to be efficient in removing the nucleic acids, and thus facilitating further purification. In the present investigation,better results were obtained by using protamine sulfate as the precipitating agent for nucleic acids.

Depending on the heat sensitivity of the enzyme protein, brief exposures of crude extracts to increasing temperatures has been used by some workers (Winnacker and Barker, 1970) to precipitate proteins and thus to obtain an increase in specific activity for a

particular enzyme. Heat treatment could not be employed as a purification step in the present scheme since momentary exposure to 45°C resulted in total loss of enzyme activity.

Ammonium sulfate fractionation of the protamine sulfate-treated solution precipitated the enzyme activity between 45% and 60% of saturation. In the preliminary fractionation experiments, 50% of the enzyme activity was lost when the crude extract was dissolved in potassium phosphate buffer alone. Fortifying the extraction buffer with EDTA, Cleland's reagent and phenyl-methyl-sulfonyl fluoride increased the percentage recovery.

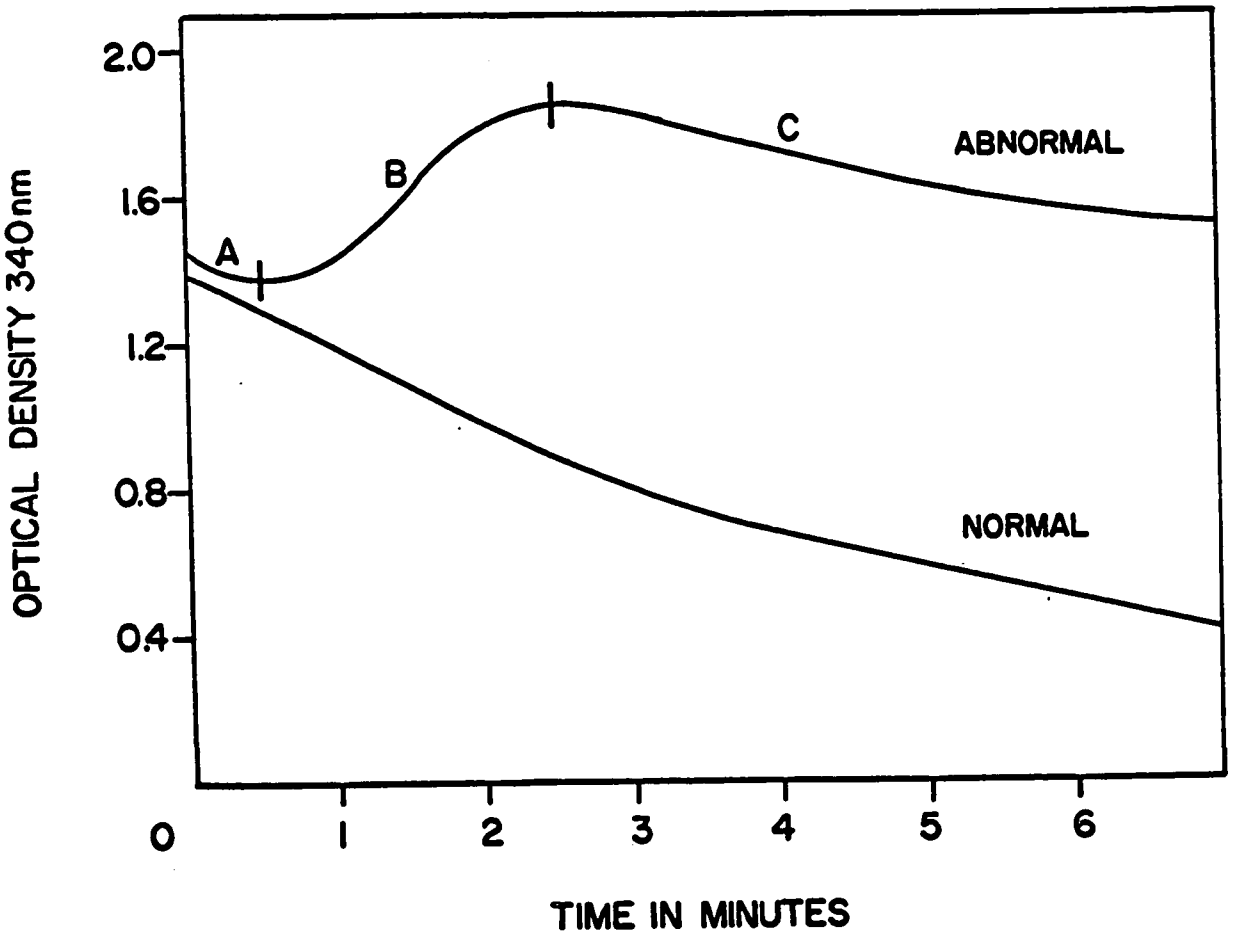
Attempts were made to purify the enzyme both by positive and negative absorption on calcium phosphate gel (Colowick, 1955). Since a significant increase in specific activity was not obtained, the technique was not pursued further.

The results obtained with gel filtration showed that the enzyme activity was excluded from a column of Sephadex G-100 but was eluted after the void volume on Sephadex G-200 and Bio gel P-300, thus indicating the molecular weight to be greater than 100,000. Since the eluates obtained from a Sephadex G-200 column showed a higher specific activity than those obtained from a column of Bio gel P-300, the former was the obvious choice in subsequent purification experiments.

Linear gradient elution (0 to 0.3 M KCl) chromatography of 45% to 60% of saturation $(NH_4)_2SO_4$ fraction on DEAE-cellulose or DEAE-Sephadex resulted in about 2-fold purification. Since the activity was eluted at about 0.1 M KCl concentration, a smaller but broader gradient of 0 to 0.15 M KCl was employed to achieve a better purification. Column eluates from such a gradient elution resulted in an "abnormal" profile for enzyme activity (Fig. 7). A normal assay profile is defined as a linear decrease in optical density at 340 nm with time. The enzyme assays with fractions from the DEAE-Sephadex column resulted in a profile which deviated from the normal. There was an initial decrease in optical density (A) in the first 20 to 30 seconds followed by an increase in absorbance (B) and then a second phase of decreased optical density (C) similar to, but slower than the normal assay. Various attempts were made to explain this "abnormal" behaviour of the column eluates obtained by DEAE-Sephadex fractionation. Although a complete explanation cannot be offered, the results of these experiments revealed the presence of a second enzyme in these peculiar fractions and in fact, in the crude extract and at each stage of the purification scheme.

Assuming that the product of the initial reaction is accepted as substrate by a second enzyme - either entirely different from the first enzyme or a part of an enzyme complex, and that the rate of formation of the product limits the second reaction, the addition

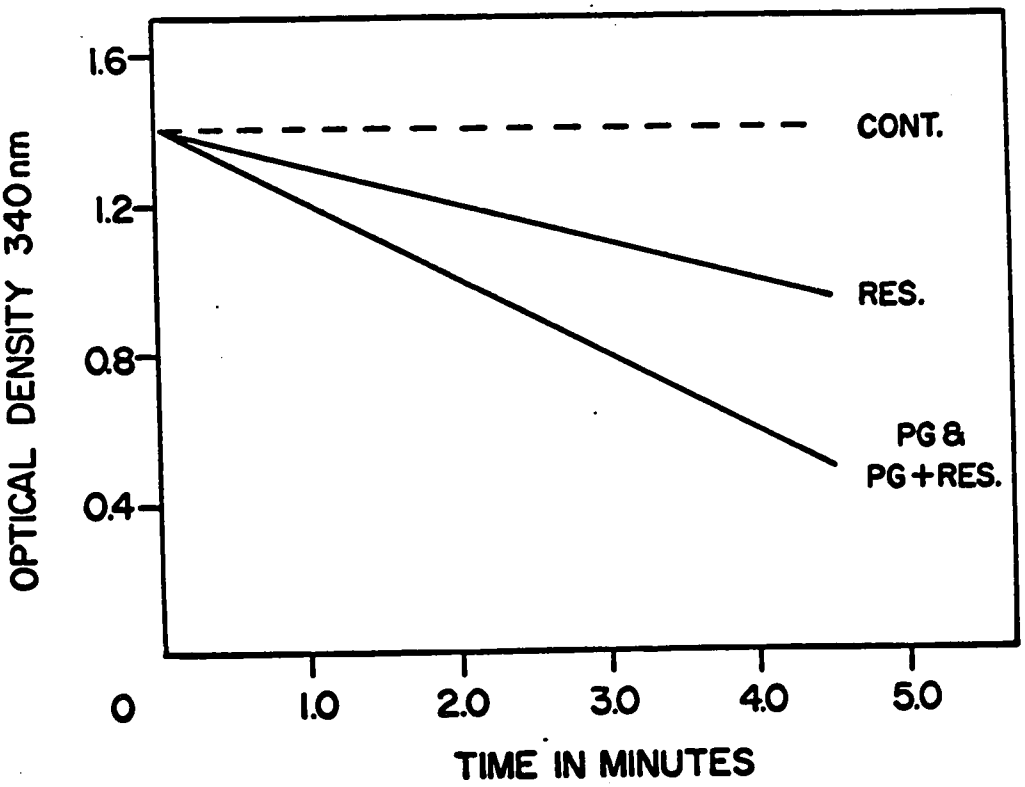
Fig. 7. Profiles of normal and abnormal assays of PG enzyme activity.


OPTICAL DENSITY 340nm
2.0
1.6
1.2
0.8
0.4
0
1
2
3
4
5
6
TIME IN MINUTES
A
B
C
ABNORMAL
NORMAL

of that compound should restore the abnormal profile to the normal assay profile. Hang (1967) in his studies on the pathway of phloroglucinol degradation by a *Pseudomonas* sp. reported that dihydrophloroglucinol was the first degradation product, which dehydrates to yield resorcinol. Addition of the substrate PG or the possible products, either dihydrophloroglucinol or resorcinol to the reaction mixture at stage C (Fig. 7), failed to restore normal activity. Dihydrophloroglucinol did not substitute for PG as the substrate in the enzyme assays of the abnormal fractions. When resorcinol (RES) was used as substrate at the beginning of the reaction, a normal assay profile with significant activity was obtained. A similar activity for RES was then shown to exist in the crude extract. These results suggested strongly that RES is a metabolic intermediate of PG degradation by *Penicillium* sp.

RES enzyme activity is approximately 50% of the PG enzyme activity (Fig. 8). When both PG and RES were added together in the enzyme assay, the activity was identical as when PG was the only substrate. This result eliminates the possibility that the two enzyme activities are identical since there was no inhibition by RES of PG activity in the mixed assay. Since the activities were not additive when both the substrates were included in the assay, it is suggested that the two enzymes are distinct from each other but not entirely independent. These observations, and other data presented

Fig. 8. Comparative profiles of enzyme activities in single and mixed substrate assays.


OPTICAL DENSITY 340nm
1.6
1.2
0.8
0.4
0
1.0
2.0
3.0
4.0
5.0
TIME IN MINUTES
CONT.
RES.
PG &
PG+RES.

elsewhere, strongly support the concept that the two enzymes are closely related and form a part of an overall enzyme complex.

The fact that neither PG nor RES initiated further reaction when added to the terminated assay suggested inactivation of both the first enzyme, PG activity, and the second enzyme, RES activity. The inactivation may be due to either the formation of some reaction product or by removal of a factor during chromatography on DEAE-Sephadex. Since both enzyme activities are dependent upon the cofactor NADPH, the essential reaction product NADP was examined as a possible inhibitor. With crude enzyme preparations, no inhibition of either enzyme activity was observed even when NADP concentration was 10 times the concentration of NADPH normally used in reaction mixture. Addition of divalent cations, Mg^{++}, Mn^{++}, Ca^{++}, Cu^{++}, Co^{++}, and Zn^{++}, separately or together, or changing the pH of the reaction mixture from the normal 7.5, had no effect on the normal assay of the crude enzyme extract or the abnormal assay profile of the DEAE-Sephadex fractions. Substituting $(NH_4)_2SO_4$ or NH_4Cl for KCl in the gradient, or by excluding EDTA from the buffer used to elute the DEAE-Sephadex column failed to alter the abnormal assay profile of the column eluates.

The application of substrate elution technique has been reported by various workers (Sarngadharan *et al.*, 1969; Pogell,

1966; Fernando *et al.*, 1968) to give fold purifications, orders of magnitude greater than those obtained by more classical fractionation steps. Eluting the enzyme from DEAE-Sephadex columns with either PG or NADPH resulted in neither higher purifications of the enzyme as compared to the conventional eluting procedure, nor in restoration of the abnormal enzyme assay profile of the column eluates to the normal pattern.

Other than the characteristic abnormal profile of the enzyme assays with the DEAE-Sephadex eluates, the reaction mixture developed a faint purplish-pink colour which slowly disappeared with time. If the increase in optical density at stage B (Fig. 7) was due to the absorption of some reaction product at the assay wavelength of 340 nm, and the same compound accounted for the absorption towards the end of stage C, then the original reaction should be terminated and there should be no residual PG substrate. This possibility was examined by chromatographically examining the final products of the enzyme assay on thin-layer plates. PG spots were detected up to 20 minutes after starting the reaction, and the colour spots were of relatively the same intensity as at the outset, suggesting that the reaction had not gone to completion at point C.

These observations were further examined by performing a coupled enzyme reaction. If the optical density at stage C (Fig. 7) was in part due to the absorption of residual NADPH, it should be possible

to oxidize the residual NADPH with an appropriate NADPH-requiring enzyme in the presence of its substrate. The PG enzyme assay was allowed to proceed to the stage when the reaction terminated. Lactic acid dehydrogenase and sodium pyruvate were then added to the reaction assay. The second observed reaction proceeded at a rate comparable to a control assay of lactic acid dehydrogenase in the presence of lactic acid dehydrogenase, NADPH, and the substrate pyruvate. This result confirms the postulate that the initial PG enzyme reaction did not proceed to completion and that the NADPH was not completely oxidized even though PG was in excess.

As previously mentioned, the change in assay profile of PG enzyme from normal to abnormal occurred after the $(NH_4)_2SO_4$ fractionation step. If the enzyme protein is partially dissociated by $(NH_4)_2SO_4$ fractionation and loses a protein or non-protein component to the supernatant, then addition of the supernatant, obtained after centrifugation of the $(NH_4)_2SO_4$ precipitated protein, to the DEAE-Sephadex eluate should restore the abnormal assay profile to the linear normal profile. The addition of this supernatant not only restored the assay profile to normal but also stimulated the PG enzyme activity as compared to a control assay. However, the RES enzyme activity was slightly inhibited by the $(NH_4)_2SO_4$ supernatant. The ability to restore the normal activity was attributed to ammonium ion (NH_4^+), since a dialyzed supernatant lost the

property while a heat treated preparation still possessed activation properties. This conclusion was confirmed when $(NH_4)_2SO_4$ and NH_4Cl, but not K_2SO_4 and KCl, were found to correct the abnormal activity of the DEAE-Sephadex eluates to the normal activity. No stimulation in PG activity was observed with the ammonium salts indicating that the activation phenomenon is the property of another component of the $(NH_4)_2SO_4$ supernatant. The ammonium salts were, however, shown to possess slightly inhibitory properties with respect to the RES activity.

Thiol reagents were also studied for their effect on the abnormal activity of the eluates from the DEAE-Sephadex column chromatography. Both Cleland's reagent and β-mercaptoethanol converted the abnormal assay profile to normal when they were added, singly, to assay mixtures. β-Mercaptoethanol at a final concentration of 1×10^{-2} M in reaction mixture, stimulated both PG and RES enzyme activities (Table 2). Cleland's reagent, on the other hand, was effective in enhancing the PG enzyme activity only.

The foregoing experiments indicated that ammonium salts (NH_4^+) and the thiol reagents, β-mercaptoethanol and Cleland's reagent, linearized the abnormal assays obtained with the DEAE-Sephadex eluates. β-Mercaptoethanol (BME) was selected as the reagent of choice and unless otherwise stated, in all subsequent studies, it was included at a concentration of 1×10^{-2} M in the assay mixture for both PG and RES enzyme activities.

TABLE 2.

Effect of thiol reagents on the assay profile and activity of DEAE-Sephadex eluates.

Addition	Concentration M	Assay profile (PG)	Activity %	
			PG Enzyme	RES Enzyme
None	–	abnormal	100	100
β-Mercaptoethanol	1×10^{-1}	normal	138	42
	1×10^{-2}	normal	163	110
	1×10^{-3}	abnormal	63	95
Cleland's Reagent	1×10^{-1}	normal	138	44
	1×10^{-2}	normal	163	74
	1×10^{-3}	normal	93	60

ENZYME INDUCTION

Both PG and RES enzyme activities are coinduced by growing the fungal culture on either PG or RES. Cells grown in a basal mineral medium containing glucose as the sole source of carbon and energy had no enzymatic activity on the test substrates.

PURIFICATION OF THE ENZYME COMPLEX

The purification procedures for a typical preparation are summarized in Table 3. For the RES enzyme, the initial activity was recorded after membrane filtration since the crude extract contained PG as a stabilizer, thus obscuring the activity due to RES reduction. The initial specific activities of the PG and RES enzymes were calculated, therefore, after the membrane filtration step so that a uniform assessment of the purification of each of the two component enzymes was possible. With respect to PG activity, the crude extract, after membrane filtration through a XM-50 membrane, had an initial specific activity of 42. The removal of nucleic acids by protamine sulfate treatment enhanced the specific activity to 57, without any significant loss in total activity. A three-step addition of protamine sulfate solution precipitated the nucleic acids better than the conventional single step addition of the reagent. The enzyme activity was fractionated between 45% and

TABLE 3.

Purification of the enzyme complex.

Treatment	Total protein (mg)	PG Enzyme			RES Enzyme		
		Total units	Specific* activity	Recovery %	Total units	Specific* activity	Recovery %
1. Crude extract	310.0	14500	--	--	--	--	--
2. Membrane filtration	250.0	10500	42.0	100.0	5000	20.0	100.0
3. Protamine sulfate	183.7	10450	56.9	99.5	4950	27.0	99.0
4. Ammonium sulfate 45-60%	62.3	8800	141.3	83.8	4125	66.2	82.5
5. DEAE-Sephadex	6.5	3250	500.0	31.0	1750	269.2	35.0
6. Sephadex G-200	2.9	2400	827.6	22.9	1200	413.8	24.0

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

60% $(NH_4)_2SO_4$ saturation. This procedure gave approximately a 3-fold purification as compared to the crude extract. Chromatography on DEAE-Sephadex resulted in a 12-fold purification with an overall 31% recovery. Filtration through Sephadex G-200 yielded a preparation 20-fold purified as compared to the activity in the crude extract. By the same procedure, an overall 21-fold purification was achieved with respect to the RES enzyme.

The elution profile of the protein from DEAE-Sephadex is presented in Figure 9. The column was washed initially with 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 1 mM Cleland's reagent, and a linear gradient of 0 to 0.15 M KCl in the same buffer was applied. The enzyme activity was eluted at approximately 0.12 M KCl. It may be noted that the enzyme activities for PG and RES were eluted coincidentally, further supporting the concept of the enzyme complex. The pooled fractions (62 - 72) were concentrated and chromatographed on Sephadex G-200. Figure 10 shows a typical elution pattern of the enzyme from Sephadex G-200. The PG and RES enzyme activities were recovered in the same fractions.

PROPERTIES OF THE ENZYME COMPLEX

Homogeneity

The degree of homogeneity of the purified enzyme complex was determined by polyacrylamide disc gel electrophoresis. Electrophoresis

Fig. 9. Linear gradient elution of the enzyme complex from DEAE-Sephadex. The gradient was 0 to 0.15 M KCl dissolved in 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 1 mM Cleland's reagent.

●—● PG - enzyme

○—○ RES - enzyme

△—△ Protein

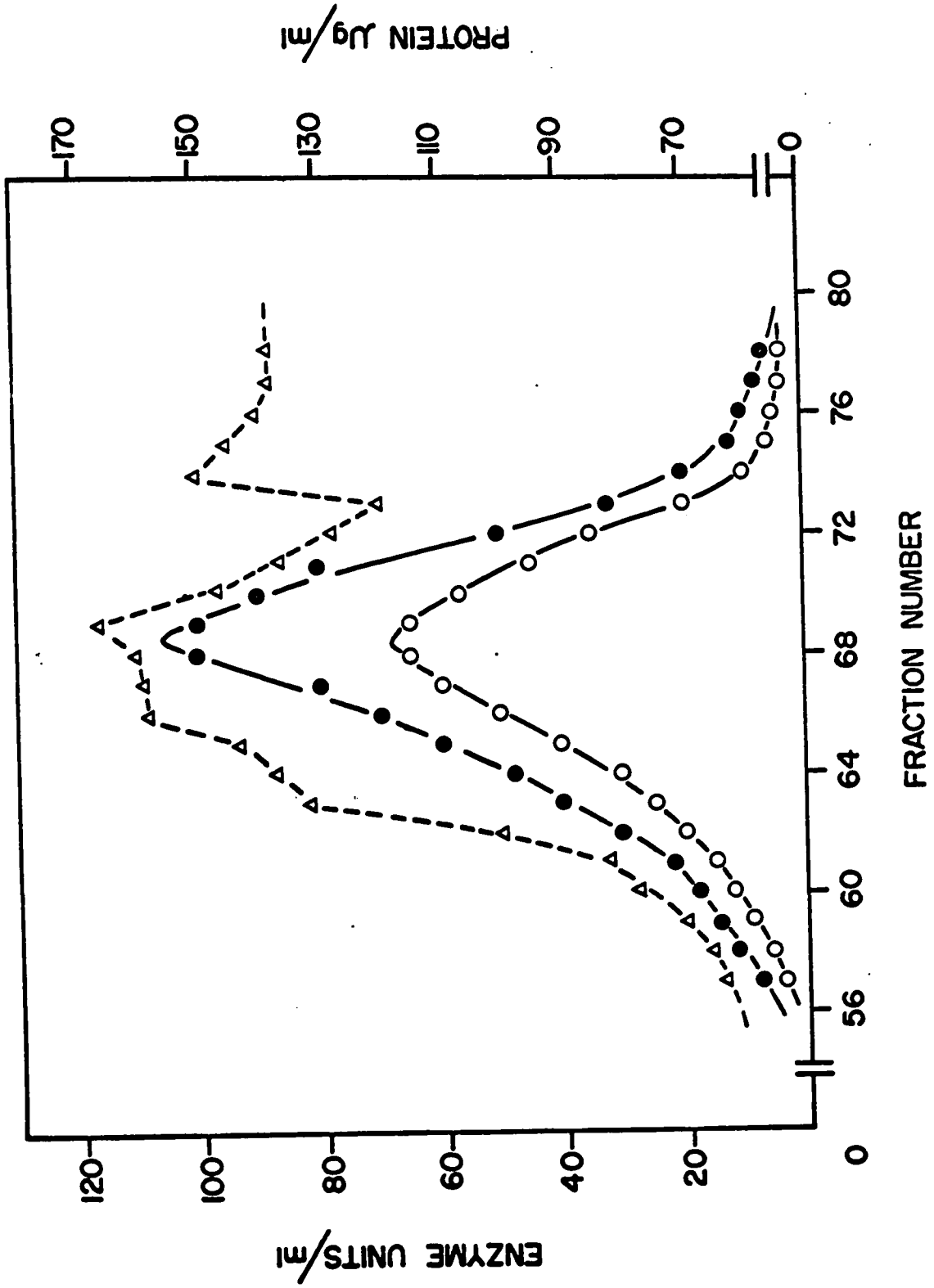
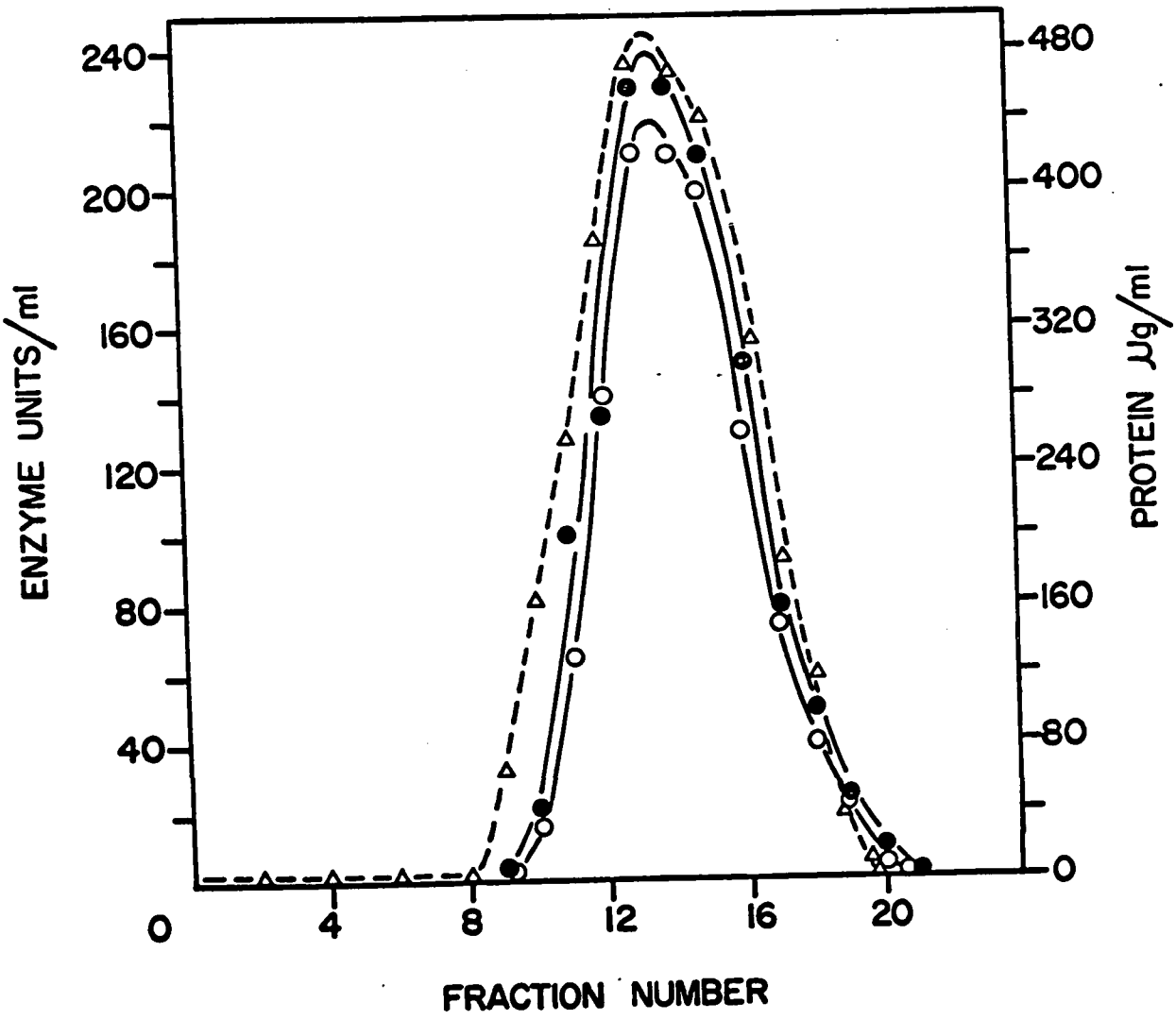

PROTEIN µg/ml
170
150
130
110
90
70
0
ENZYME UNITS/ml
120
100
80
60
40
20
0
56
60
64
68
72
76
80
FRACTION NUMBER

Fig. 10. Elution pattern of the enzyme complex on a Sephadex G-200 column by 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 1 mM Cleland's reagent.

●——● PG - Enzyme

○——○ RES - Enzyme

△——△ Protein


ENZYME UNITS/ml
PROTEIN µg/ml
FRACTION NUMBER
240
200
160
120
80
40
0
480
400
320
240
160
80
0
4
8
12
16
20

was conducted at pH 8.3, according to the method of Davis (1964), at room temperature (23 - 25°C) and a constant current of 3 mA per sample tube. Gels were stained for protein overnight in a 0.5% solution of amido black dissolved in 7% acetic acid. The gels were destained electrophoretically and stored at 4°C in 7% acetic acid. One major protein band and a minor band were detected after the analytical gel electrophoresis (Fig. 11). The enzyme preparation was estimated to be approximately 90% pure on this basis.

Nucleotide specificity

The enzyme complex is dependent upon NADPH for both PG and RES activities but may accept NADH as an alternate electron donor at 50% efficiency. No activity could be obtained when NADPH was replaced by FAD, FMN, cytochrome C, ATP, ADP or AMP. The addition of FMN, FAD, and cytochrome C to the reaction mixture supplemented to NADPH, resulted in an appreciable increase in the enzyme activities, the stimulation of the RES enzyme activity being more pronounced than the PG enzyme activity (Table 4). Supplementing with ATP, ADP or AMP had no influence upon the normal enzyme activities with NADPH as cofactor.

Substrate specificity

The crude extract and the purified enzyme preparations were compared for activities upon a variety of phenolic compounds as substrate. The data in Table 5 shows the relative rates of the

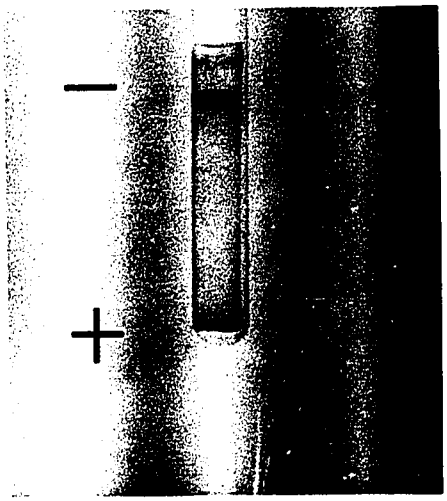


Fig. 11. Analytical polyacrylamide disc gel electrophoresis of the purified enzyme complex. Gels containing 7.5% acrylamide were loaded with 50 µg of the enzyme protein and run in 0.005 M Tris-glycine buffer (pH 8.3) at 3 mA per tube for 2.5 hours. Protein migration is towards the anode, as indicated.

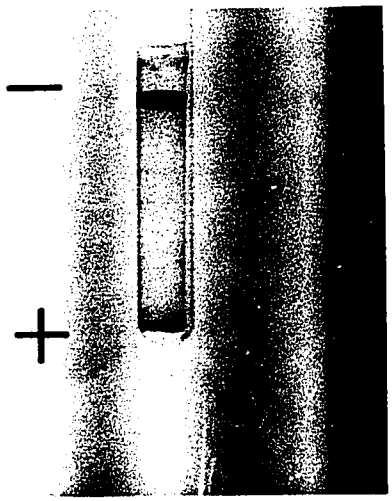


Fig. 11. Analytical polyacrylamide disc gel electrophoresis of the purified enzyme complex. Gels containing 7.5% acrylamide were loaded with 50 µg of the enzyme protein and run in 0.005 M Tris-glycine buffer (pH 8.3) at 3 mA per tube for 2.5 hours. Protein migration is towards the anode, as indicated.

TABLE 4.

Effect of various nucleotides as a supplement to NADPH on enzyme activities.

Nucleotide	Concentration M	Activity %	
		PG Enzyme	RES Enzyme
None	---	100	100
NADH	1×10^{-4}	100	100
FAD	1×10^{-4}	132	150
FMN	1×10^{-4}	126	133
Cyt.C	5×10^{-5}	111	142
ATP	1×10^{-4}	100	100
ADP	1×10^{-4}	100	100
AMP	1×10^{-4}	100	100

Reaction mixture (1.8 ml) contained, in µMoles: NADPH, 0.5; potassium phosphate buffer (pH 7.5), 87.5; BME, 20 mMoles; substrate, 1 (PG) or 10 (RES); enzyme, 9.5 units (PG) or 6 units (RES); nucleotides, as indicated.

TABLE 5.

Comparative rates with various acceptors for the enzyme complex with NADPH as the electron donor.

Acceptor	Activity %	
	Crude enzyme	Purified enzyme
Phloroglucinol	100	100
Resorcinol	53	64
Catechol	11	0
Hydroquinone	13	0
Quinone	337	0
Pyrogallol	18	0
1,2,4-Trihydroxybenzene	0	0
Phenol	---	0
Phloroglucinolcarboxylic acid	21	14
Phloroglucinaldehyde	0	0
Orcinol	5	0
1,2-Naphthoquinone	50	0
1,4-Naphthoquinone	516	0
2-Methylnaphthoquinone	442	0
Potassium ferricyanide	621	0

Reaction mixture (1.8 ml) contained, in µMoles: NADPH, 0.5; potassium phosphate buffer (pH 7.5) 97.5; BME (with pure enzyme only), 20 mMoles; enzyme, 19 units (crude) and 9.5 units (pure); acceptor, 1 (except 10 RES).

various electron acceptors with NADPH as the donor. Using a crude enzyme extract, various quinones, catechol, and potassium ferricyanide, in addition to PG and RES, were found to act as acceptors of electrons from NADPH. In reactions catalyzed by the purified enzyme complex, however, phloroglucinol carboxylic acid was the only compound, in addition to PG and RES, capable of acting as the electron acceptor. The rates of oxidation of NADPH with RES and phloroglucinol carboxylic acid were shown to be 64% and 14%, respectively, of the PG activity.

Effect of enzyme concentration

The effect of enzyme concentration on the rate of NADPH oxidation is presented in Figure 12. The oxidation rate of NADPH in the presence of either PG or RES is proportional to the enzyme concentration over a 4 to 5-fold concentration range, and deviates from linearity only at high protein concentration.

Effect of substrate concentration

Initial velocity measurements of the oxidation of NADPH were performed with varying concentrations of PG and RES. The data plotted according to the method of Lineweaver and Burk (1934) are shown in Figures 13 and 14. At pH 7.5 and in the presence of BME, the K_m values were 2×10^{-5} M for PG and 1.43×10^{-3} M for RES. Substrate inhibition was observed at concentrations higher than 1.1×10^{-3} M PG or 5.5×10^{-2} M RES.

Fig. 12. Effect of enzyme concentration on the rate of NADPH oxidation by PG and RES. Reaction mixture (1.8 ml) contained: NADPH, 0.5 μMole; potassium phosphate buffer (pH 7.5), 100 μMoles; BME, 20 mMoles; substrate, 1 μMole PG or 10 μMoles RES; and enzyme as indicated.

●——● PG - enzyme

○——○ RES - enzyme

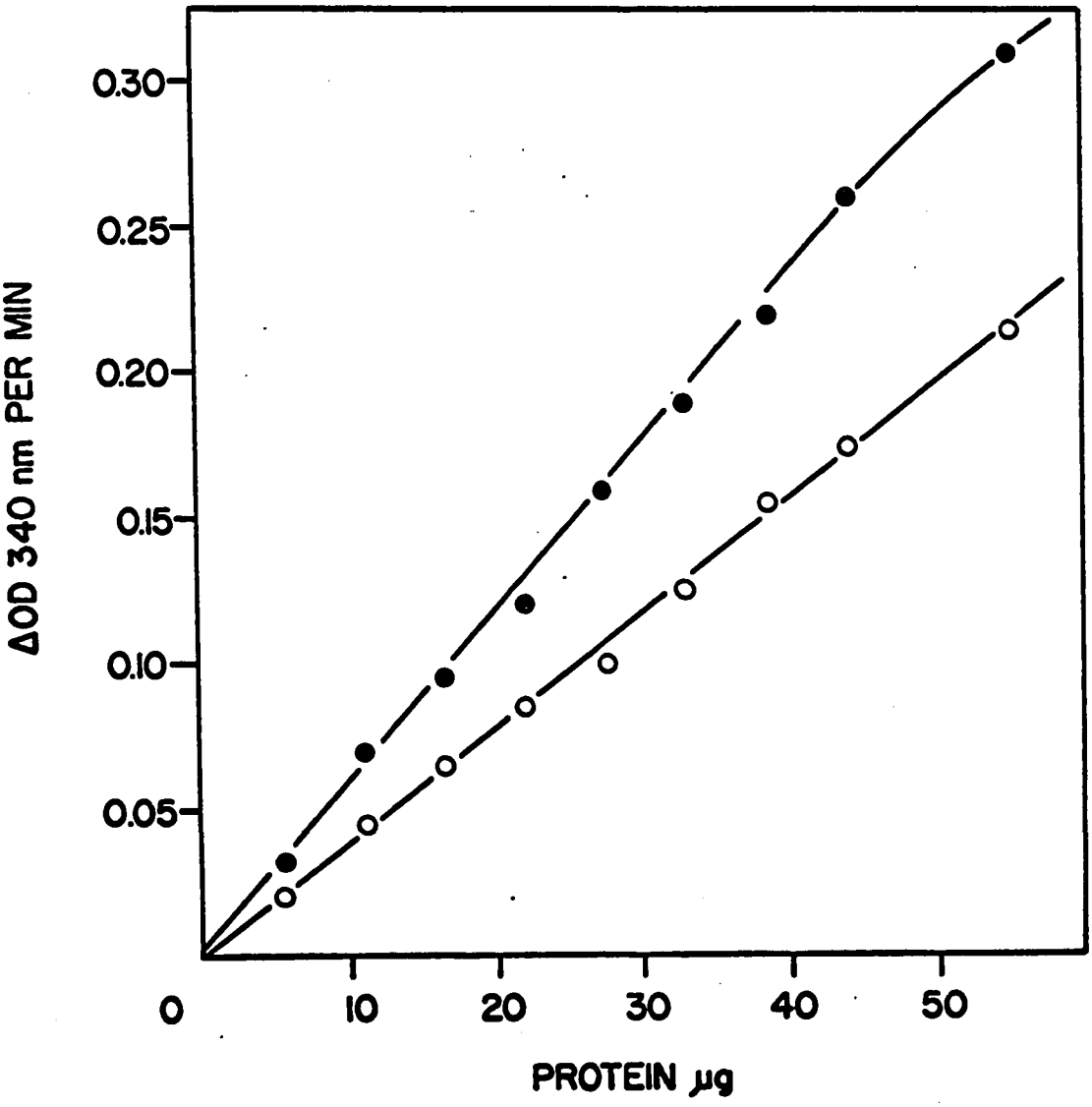

0.30
0.25
0.20
0.15
0.10
0.05
ΔOD 340 nm PER MIN
0
10
20
30
40
50
PROTEIN µg

Fig. 13. Lineweaver-Burk plot of the effect of PG concentration on the rate of NADPH oxidation by the enzyme complex.

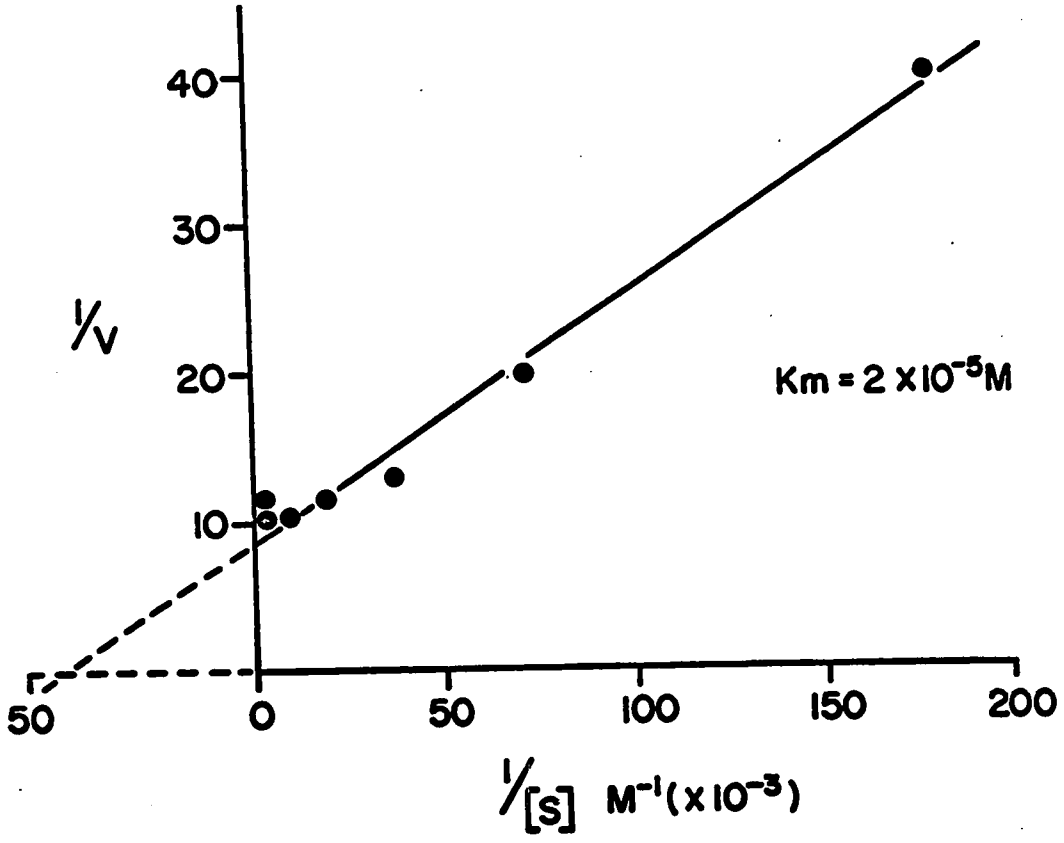
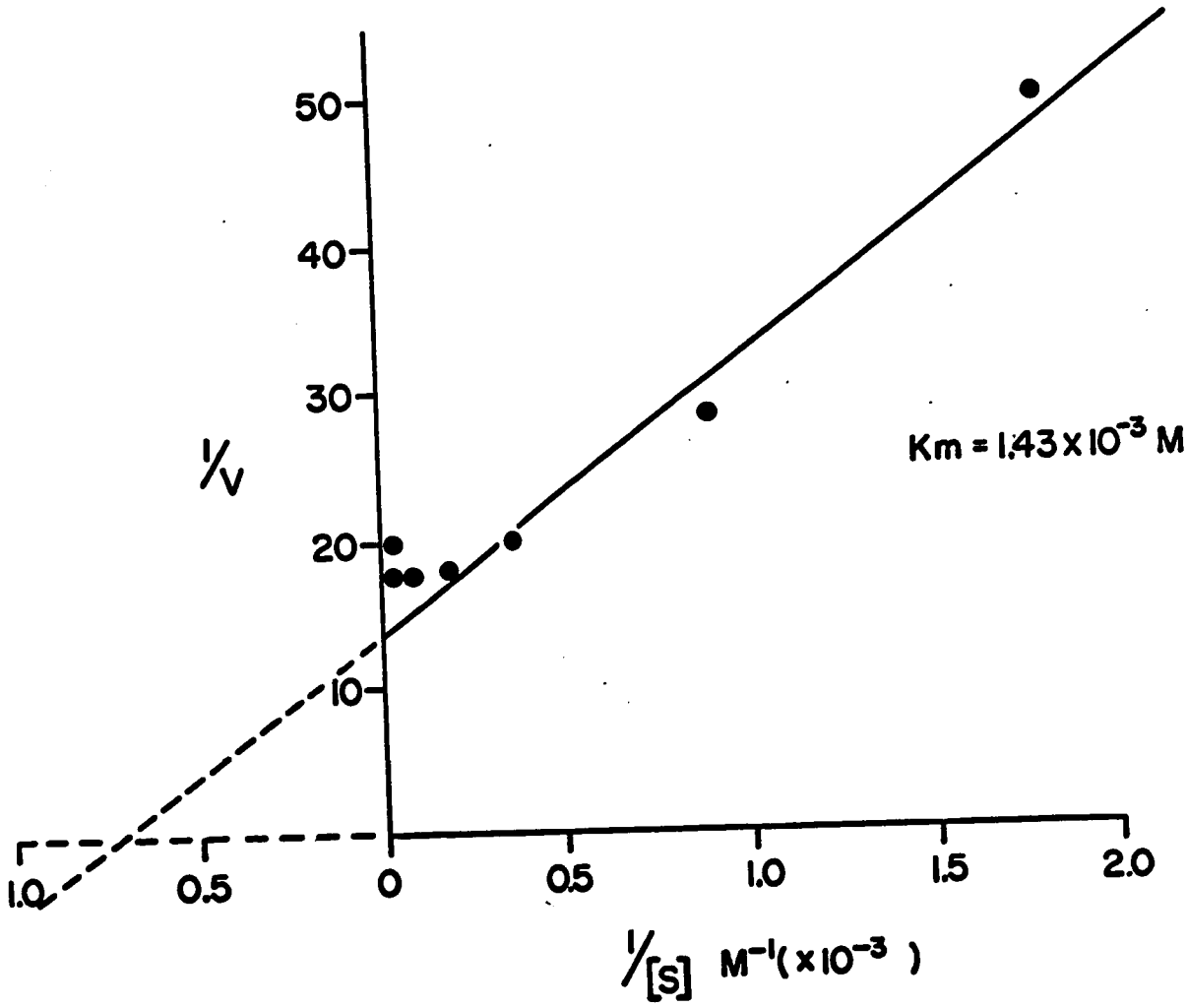

40
30
20
10
1/V
Km = 2 X10⁻⁵M
50
0
50
100
150
200
1/[S] M⁻¹(x10⁻³)

Fig. 14. Lineweaver-Burk plot of the effect of RES concentration on the rate of NADPH oxidation by the enzyme complex.


50
40
30
1/V
20
10
Km = 1.43 x 10⁻³ M
1.0
0.5
0
0.5
1.0
1.5
2.0
1/[S] M⁻¹(x10⁻³)

Influence of pH

The optimum pH for both the PG and the RES enzyme activities was approximately pH 7.3 (Fig. 15), the reaction velocities being almost identical at pH 7.0 and pH 7.5. There was no change in the initial rate of reaction with RES over a pH range of 5.0 to 6.5, unlike the enzyme activity with PG which showed a typical pH optimum curve.

The pH optimum for the stability of the enzyme complex was found to be pH 7.0 and pH 7.5 for the PG activity and the RES activity respectively (Fig. 16).

Effect of heat treatment

When the enzyme solution was exposed to different temperatures for 10 minutes, a total loss of activity occurred at 45° and 50°C, while inactivation was 30 to 40% at 37°C (Table 6). The enzyme activities for PG and RES were unaltered at 4°C and 24°C (room temperature).

Stability of enzyme complex

The effect of storage temperature was studied on the stability of the enzyme complex over an extended period of time. The results of storage at 4°C and -20°C are compared in Table 7. No differences were observed in the enzyme activities for PG and RES at the two storage temperatures in the first week. However, after 2 weeks,

Fig. 15. Effect of pH on enzyme activity. Reaction mixture (1.8 ml) contained: NADPH, 0.5 μMole; BME, 20 mMoles; enzyme, 9 units (PG) or 6 units (RES); substrate, 1 μMole PG or 10 μMoles RES; and 100 μMoles buffer as indicated.

—△— MES - buffer

—●— Potassium phosphate buffer

—○— Tris-HCl buffer

——— PG - enzyme

– – – RES - enzyme

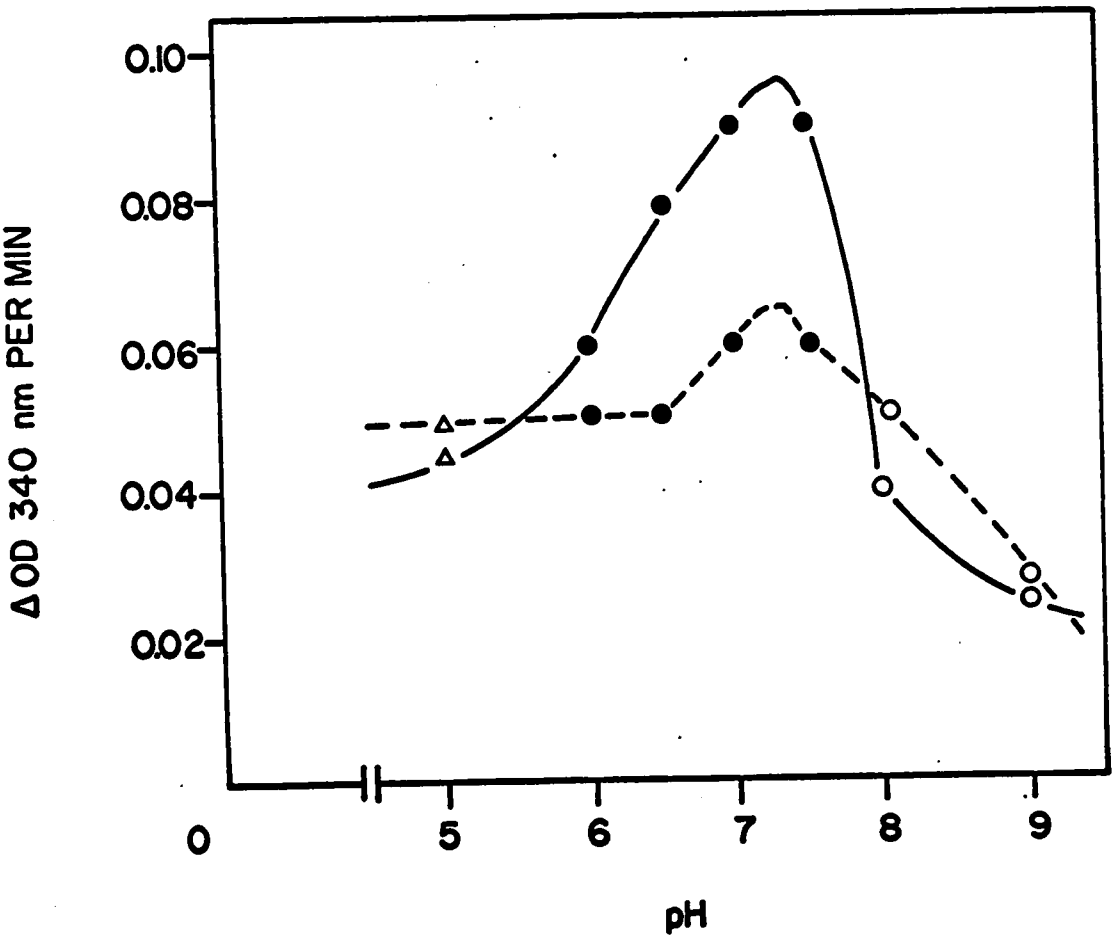

ΔOD 340 nm PER MIN
0.10
0.08
0.06
0.04
0.02
0
5
6
7
8
9
pH

Fig. 16. Effect of pH on enzyme stability. Enzyme dilutions were made in 0.1 M buffer, as indicated, at various pH values and incubated at room temperature for 30 minutes. Dilutions were then adjusted to pH 7.5 with 0.5 M potassium phosphate buffer, and assayed for activity.

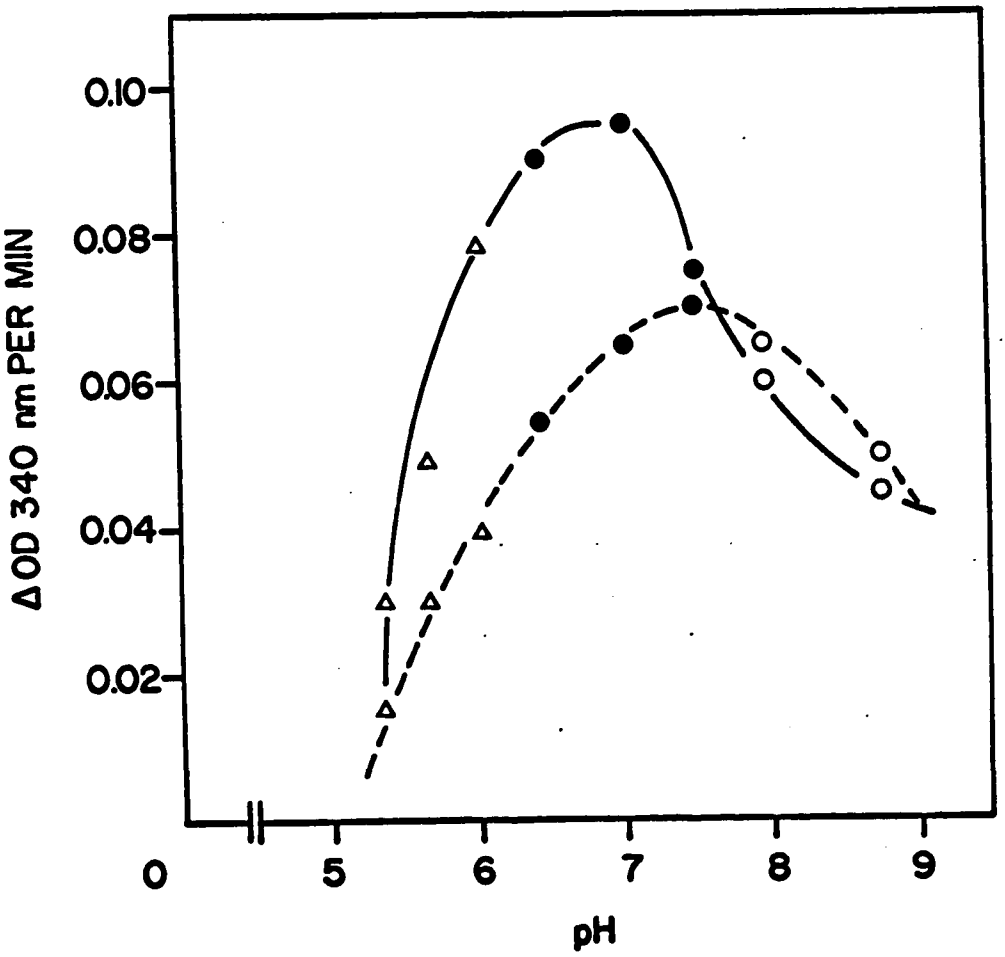
—△— MES - buffer

—●— Potassium phosphate buffer

—○— Tris-HCl buffer

——— PG - enzyme

– – – RES - enzyme


0.10
0.08
0.06
0.04
0.02
0
5
6
7
8
9
ΔOD 340 nm PER MIN
pH

T A B L E 6.

Thermostability of the enzyme complex.

Temperature °C	Activity %	
	PG Enzyme	RES Enzyme
4	100	100
24	100	100
37	39	29
45	0	0
50	0	0

Reaction mixture (1.8 ml) contained, in µMoles: NADPH, 0.5; potassium phosphate buffer (pH 7.5), 90; BME, 20 mMoles; substrate, 1 (PG) or 10 (RES); enzyme, 9 units (PG) or 5.5 units (RES).

TABLE 7.

Effect of storage temperature on the stability of the enzyme complex.

Storage time (days)	Activity %			
	PG Enzyme		RES Enzyme	
	4°C	-20°C	4°C	-20°C
0	100	100	100	100
1	100	100	100	100
2	95	95	100	100
3	95	90	100	92
7	90	90	92	92
15	16	90	17	92
30	0	90	0	92
75	0	90	0	92

Reaction mixture (1.8 ml) contained, in µMoles: NADPH, 0.5; potassium phosphate buffer (pH 7.5), 100; substrate, 1 (PG) or 10 (RES); enzyme, adequate amount.

about 85% of the activity was lost at 4°C. The enzyme complex was found to be 90 to 92% stable at -20°C up to 75 days.

Effect of metal ions

The effect of various cations on the enzymatic rate of NADPH oxidation with PG and RES as substrates was examined, and the results are presented in Table 8. While the enzyme activity with respect to PG was slightly stimulated in the presence of Ca^{++} and unaffected by Mg^{++}, the RES activity was unaltered by either of the two cations. All other metal ions tested inhibited the enzyme activities to some extent. The RES enzyme activity was generally inhibited to a greater extent than the PG activity, with the notable exception of Cu^{++} which caused a greater inhibition of the PG enzyme activity.

Effect of inhibitors and activators

The effect of various sulfhydryl and metal chelating agents on the rate of enzymatic oxidation of NADPH, in the presence of PG and RES, was studied, and the results are presented in Table 9. The individual response of the PG and RES enzymes to the various reagents tested was of a different magnitude. While the sulfhydryl reagent PCMB caused 89% inhibition of RES enzyme activity, only 26% loss in PG enzyme activity was recorded. On the other hand, the inhibition of NEM, which irreversibly reacts with -SH and amino groups, was more pronounced in the case of the PG enzyme activity.

TABLE 8.

Effect of cations on enzyme activities.

Cation	Concentration M	Activity %	
		PG Enzyme	RES Enzyme
None	-	100	100
NH_4^+	10^{-1}	84	67
K^+	10^{-1}	84	50
Na^+	10^{-1}	84	58
Mg^{++}	10^{-3}	100	100
Mn^{++}	10^{-3}	84	92
Fe^{++}	10^{-4}	90	83
Fe^{+++}	10^{-4}	90	75
Zn^{++}	10^{-3}	63	67
Cu^{++}	10^{-3}	58	75
Ca^{++}	10^{-3}	116	100

Reaction mixture (1.8 ml) contained, in μMoles: NADPH, 0.5; potassium phosphate buffer (pH 7.5), 87.5; BME, 20 mMoles; substrate, 1 (PG) or 10 (RES); enzyme, 9.5 units (PG) or 6 units (RES); cations as indicated.

T A B L E 9.

Effect of inhibitors and activators on enzyme activities.

Effector	*Activity %			
	PG Enzyme		RES Enzyme	
	-BME	+BME	-BME	+BME
None	100	100	100	100
p-Chloromercuribenzoate	74	39	11	50
N-Ethylmaleimide	37	57	56	56
Iodoacetate	111	82	61	78
EDTA	259	57	78	78
2,2'-Bipyridine	37	114	78	78

The enzyme was preincubated in the presence of 5×10^{-2} M potassium phosphate buffer (pH 7.5) and 1.33×10^{-3} M effector. After 10 minutes incubation the enzyme activities were assayed by the addition of 0.5 µMoles NADPH, 1 µMole PG or 10 µMoles RES, and 20 mMoles BME, where indicated, to a final volume of 1.8 ml.

*100% activity: PG Enzyme (-BME) = 2.7 units
PG Enzyme (+BME) = 14.0 units
RES Enzyme (-BME) = 9.0 units
RES Enzyme (+BME) = 9.5 units

Iodoacetate did not affect the PG enzyme significantly but caused 39% inhibition of RES enzyme activity. The latter was slightly but equally inhibited by the metal chelating agents EDTA and 2,2'-bipyridine. The PG enzyme activity, on the other hand, was significantly stimulated by EDTA and appreciably inhibited by 2,2'-bipyridine. Addition of the thiol reagent BME to the reaction mixture did not cause a significant reactivation of the enzyme activities following treatment with the various sulfhydryl reagents.

Stoichiometry of PG *and* RES *enzyme activities*

Attempts to determine a stoichiometric relationship between the amount of NADPH oxidized and the amount of PG and RES required in the enzymatic reactions were unsuccessful. BME interfered with the colorimetric determination of PG. Since NADP, one of the reaction products, has a significant absorption in the region of the λmax of PG and RES, calculations of substrate concentrations on the basis of extinction coefficients could not be employed.

Molecular weight of the enzyme complex

The molecular weight of the enzyme complex was estimated to be 76,000, based on data obtained from sucrose density gradient ultracentrifugation with rabbit muscle aldolase (molecular weight 140000) as a reference (Fig. 17).

Optical properties

The purified enzyme showed a typical protein absorption

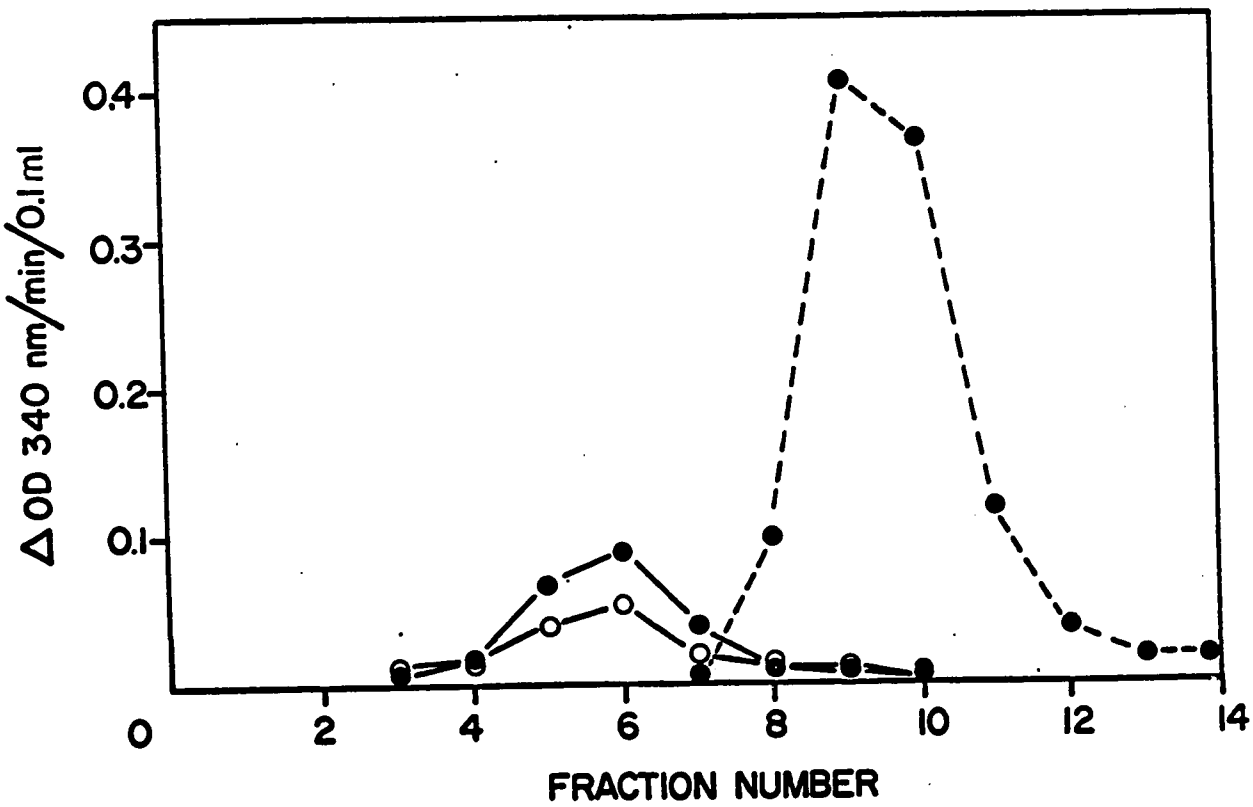
Fig. 17. Sucrose density ultracentrifugation of the enzyme complex.

The enzyme (240 µg) was centrifuged on a linear sucrose gradient (5 to 20% in 0.01 M potassium phosphate buffer, pH 7.5) for 16 hours at 36,000 rpm in SW-40 rotor of Beckman Model L2-65B. Fractions (0.5 ml) were collected on ISCO Model 180 Density Gradient Fractionator, and assayed for enzyme activities. Rabbit muscle aldolase (500 µg) was included in the gradient as reference and was assayed by the method of Ingram (1969).

●——● PG - enzyme

O——O RES - enzyme

●---● Aldolase


0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
Δ OD 340 nm/min/0.1 ml
FRACTION NUMBER

spectrum when dissolved in 0.01 M potassium phosphate buffer (pH 7.5), exhibiting an absorbance maximum at 277 nm and minimum at 250 nm. The extinction coefficient $E^{0.1\%}_{280\ nm}$ was determined to be 0.98 absorbance units. There was no absorbance in the visible range. The 280 : 260 absorbance ratio greater than 1.0 indicates that the enzyme preparation is relatively free of nucleic acids.

Manometric studies

Oxygen uptake was measured in a Gilson respirometer as described by Robern (1965) except that NADPH was added exogenously. No net CO_2 evolution was recorded in the presence of either PG or RES. There was a rapid and steady increase in oxygen uptake above the endogenous level, without a lag, in the presence of substrates PG and RES (Fig. 18). No oxygen uptake was observed when NADPH was omitted from the reaction mixture. The total oxygen uptake in the presence of PG was twice the amount observed with RES, when NADPH and the substrates were added in equimolar quantities in the reaction mixture. Attempts to relate this data stoichiometrically were unsuccessful due to the interference of BME in quantitative determination of the substrates.

Detection of reaction product(s)

Scaled-up PG and RES enzyme reactions were allowed to proceed for 0, 5, and 10 minutes and terminated by the addition of trichlor-

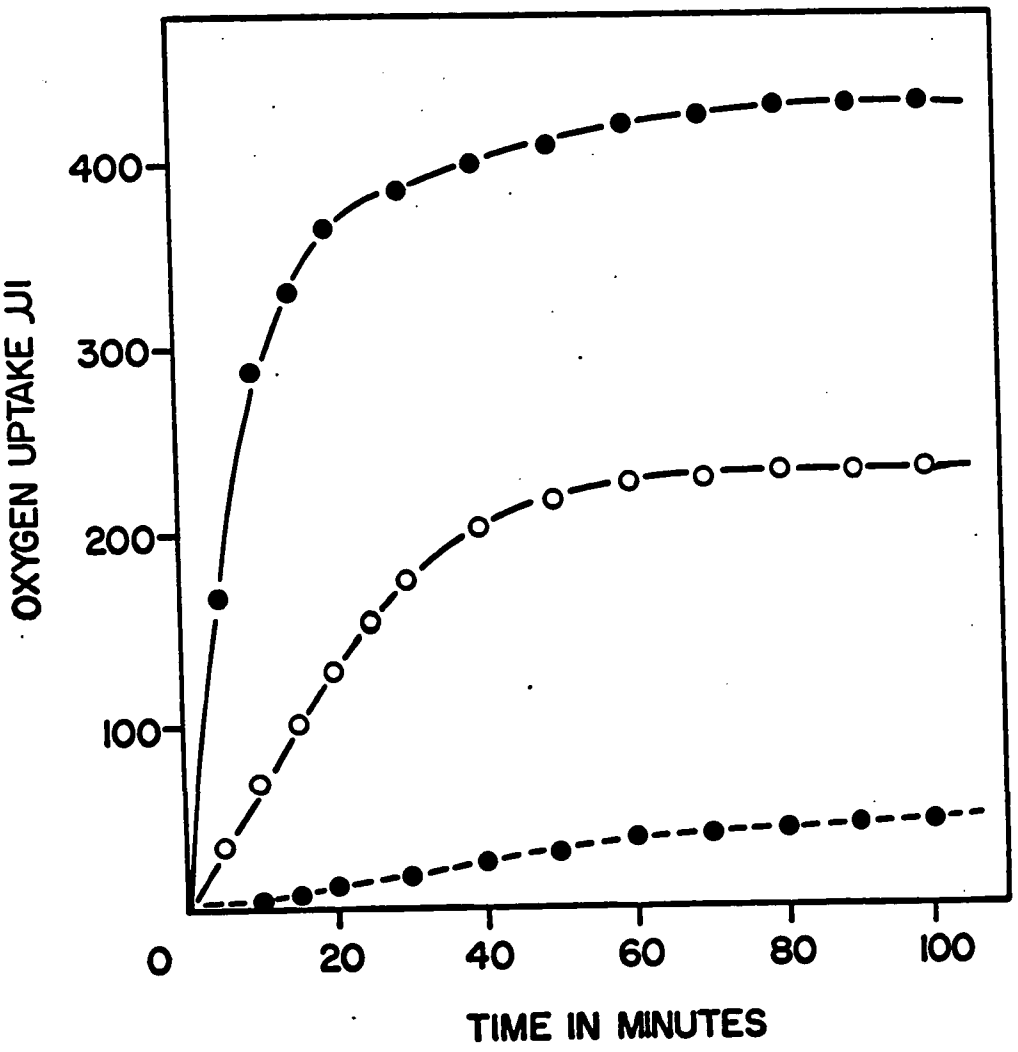
Fig. 18. Oxygen uptake by the enzyme complex in the presence of PG and RES.

Reaction mixture (4.0 ml) contained in μMoles: NADPH, 10; potassium phosphate buffer (pH 7.5), 200; substrate, 10; BME, 40 mMoles; enzyme 144 μg. The center well contained 0.2 ml of 20% KOH. Reactions were initiated by tipping the enzyme from the side arm into the main compartment of the respirometer flask.

●——● PG (not corrected for endogenous)

O——O RES (not corrected for endogenous)

●---● Endogenous


OXYGEN UPTAKE JJI
400
300
200
100
0
20
40
60
80
100
TIME IN MINUTES

acetic acid. The acidified reaction mixtures were extracted with ethyl ether, the pooled ether extract evaporated to dryness, and redissolved in a minimal volume of ethanol. The ethanol solutions were examined by thin-layer chromatography using solvent systems and spray reagents described under Materials and Methods (Part I). However, no spots in addition to the faint spots of the substrate were revealed.

DISCUSSION

In the preliminary purification of the crude enzyme extracts, DEAE-Sephadex chromatography afforded fractions that exhibited an enzyme assay profile deviating from the normal linear profile of the crude extracts. The experiments, carried out to explain the abnormal assay profile of the DEAE-Sephadex eluates, revealed the existence of an enzyme activity in the presence of RES. The RES activity was shown to be distinct from the PG enzyme activity by conducting the assay with both PG and RES in the reaction mixture. The fact that the resultant activity in the mixed assay was the same as the activity for PG alone, discounted the possibility that the two activities were identical since PG activity was not inhibited in the presence of RES. Since the two activities were not additive when both the substrates were included in the assay indicated that the two enzymes were distinct but not entirely independent - the second enzyme being dependent upon the formation of RES by the first reaction. These observations strongly suggested that the two enzymes are closely related and form a part of an enzyme complex.

Further evidence to support this concept was obtained by enzyme purification and characterization studies. The two enzyme activities were eluted coincidentally during chromatography on DEAE-Sephadex

and Sephadex G-200. The K_m for the PG enzyme was found to be 2×10^{-5} M while a corresponding value of 1.43×10^{-3} M, about 140 x greater than the K_m of PG for the PG enzyme, was obtained for the RES enzyme. This is interpreted to indicate that the RES enzyme has a very low affinity for its substrate, if K_m is in fact an expression of affinity. This seems highly improbable since with this assumption it would not be possible to measure RES activity at the concentration of PG used in the assay system. In the case of the postulated enzyme complex, resorcinol, the subsequent product of PG degradation, although in low concentration in absolute terms, would have a high local concentration at the RES enzyme site in order to maintain substrate saturation and hence a maximum velocity. In addition, the apparently poor K_m for the exogenously added RES is attributed to site unavailability within the complex, and not to the binding efficiency of the site itself.

Induction of the PG enzyme, in addition to the RES enzyme, in the RES-grown cells is explained on the basis of simultaneous induction of all the component enzymes of the aggregate by the operator gene controlling the structural genes, in the cluster, responsible for the various enzyme proteins in the degradative pathway of PG. In *Neurospora crassa*, mutant studies (Giles *et al.*, 1967; Case and Giles, 1968) have shown that there is an *arom* gene cluster of five contiguous structural genes which specifies, apparently via a single polycistronic mRNA, the synthetic enzyme aggregate in the prechorismic

acid part of the polyaromatic biosynthetic pathway.

The anomalous results obtained with the metal chelating and sulfhydryl reagents may be attributed, in part, to the high requirement of the PG enzyme activity for BME. The exact role of BME, apart from its involvment in stabilizing the -SH groups, is not clearly understood. A thorough understanding of the phenomenon can only be obtained by an extensive study of the reaction mechanism which is beyond the scope of the present investigation. The non-specificity of reagents like NEM and iodoacetate, which are known to react with the lysine ε-amino groups as well as -SH groups, of proteins, may also account for the atypical results. The distinct spectrum of inhibition and activation exhibited by the PG and RES enzyme activities, as evidenced in the data presented, further substantiates the earlier observations that the two enzyme activities are not identical but rather that they reside in two distinct regions of the postulated enzyme complex.

Koli and coworkers (1969) demonstrated three menadione reductases in hog liver - one specific for NADH, another specific for NADPH, and a third equally reactive with either NADH or NADPH. The partially purified phloroglucinol reductase obtained by Hang (1967) from a *Pseudomonas* sp. required NADPH for the degradation of PG; no activity occurred in the presence of NADH. However, the enzyme complex, purified from *Penicillium* sp. Mac M-47 in the present work,

is only partially specific for NADPH. NADH may be substituted for NADPH as an alternate electron donor at 50% efficiency. This may conceivably be due to the presence of NADH oxidase and NADH : NADP transhydrogenase activity in the enzyme preparations.

In the present studies, unlike those reported by Hang (1967) for phloroglucinol reductase, the PG and RES enzyme activities of the enzyme complex were shown to be stimulated by FAD and FMN. However, the activities were not totally dependent on flavin nucleotides as indicated by the lack of an absorption spectrum typical of the flavoproteins.

The enzyme complex also catalyzes the oxidation of NADPH in the presence of phloroglucinol carboxylic acid, as well as in the presence of PG or RES. None of the other compounds tested, including catechol, served as alternate electron acceptors in the enzymatic reaction. The phloroglucinol reductase described by Hang (1967) had a relatively wider spectrum of electron acceptors but RES, among other compounds, could not replace PG. RES was shown to be an intermediate in the degradative pathway of PG by a *Pseudomonas* sp. (Hang, 1967), however, no evidence for the existence of any RES activity in the crude enzyme extracts was presented.

Manometric experiments with the partially purified enzyme complex showed no gaseous exchange in the absence of NADPH. NADPH-dependent consumption of oxygen was noted in both PG and RES enzyme reactions.

However, no CO_2 evolution was observed even in the presence of NADPH, suggesting an incomplete metabolism of PG. Attempts to detect any reaction product(s), either from reaction mixtures in the respirometer flasks or from enzyme incubation assays, were unsuccessful. Assuming that the enzyme complex is incomplete for all the enzymes required for the complete decomposition of PG, as also indicated by the lack of CO_2 evolution in the manometric studies, it seems plausible that the product of the RES enzyme reaction remains bound to the enzyme complex and hence is not detectable.

GENERAL DISCUSSION

Generally, enzymes that channel the carbon atoms of an aromatic compound through dihydroxyphenols and thence to metabolites of Kreb's Cycle are derepressed only when growth occurs at the expense of that particular compound. They are effectively absent when cells are grown with succinate or glucose as carbon sources. A prerequisite to rapid microbial growth at the expense of any aromatic compound must, therefore, be the elaboration of a contingent of enzymes which catalyze conversions, first to a dihydroxyphenol, and thence to metabolites of the Kreb's Cycle. Microorganisms so grown provide excellent experimental material for studies of the factors that govern the synthesis of multienzyme systems. One suggestion is that these enzymes appear in sequence when their respective substrates accumulate inside the cell (Stanier, 1947). Alternatively,a family of enzymes may be synthesized together as a consequence of the depression of a single operon (Jacob and Monod, 1961) by one metabolite. The latter process was indicated to be operative in the present investigation of the degradation of phloroglucinol by *Penicillium* sp. Mac M-47. Enzyme extracts prepared from fungal mycelia, grown on resorcinol as the sole carbon source, were shown to catalyze the oxidation of NADPH in the presence of either phloroglucinol or resorcinol. This observation strongly suggests the elaboration of an enzyme complex in the *Penicillium* sp. In a similar situation an *arom*

gene cluster of five contiguous structural genes which specifies information, via single polycistronic m-RNA, for the synthetic enzyme complex of the polyaromatic biosynthetic pathway was demonstrated in a mutant strain of *Neurospora crassa* (Giles *et al.*, 1967; Case and Giles, 1968).

In addition to the simultaneous induction of the phloroglucinol enzyme and resorcinol enzyme activities in the resorcinol-grown cells, five pieces of evidence suggest that the two enzyme activities are components of an enzyme complex: (1) lack of competitive inhibition, and non-additive rates of reactions in the mixed assay (PG + RES), (2) concomitant elution of the two activities on DEAE-Sephadex and Sephadex G-200, (3) an apparently poor K_m value for the exogenously added substrate resorcinol, (4) the inability to detect any intermediates of phloroglucinol degradation in studies with either whole cells or purified enzyme preparations, and (5) a distinct spectrum of inhibition and activation exhibited by the phloroglucinol and resorcinol enzyme activities.

In an evaluation of the criteria used to establish an intermediate in the metabolic pathways, Ribbons (1966) emphasized the importance of the isolation of that compound from culture filtrates. The other criterion mentioned was the ability of both whole cells and cell-free extracts to oxidize the postulated intermediate compound without lag, after growth on the aromatic compound under study but

not after growth on glucose. In the present studies, resorcinol qualifies as an intermediate in the degradation of phloroglucinol by a fungus on the latter criterion. Although quantitative growth studies were not carried out on resorcinol, the organism was capable of growing on resorcinol as the sole carbon source. The enzyme, purified from crude extracts prepared after growth on phloroglucinol, showed a NADPH-dependent consumption of oxygen, without any lag, in the presence of resorcinol. Cell-free extracts from glucose-grown cells were not active enzymatically on resorcinol. However, resorcinol was not detected in the culture filtrates of growing cells or resting-cell suspensions in phloroglucinol medium. This does not invalidate the postulation that resorcinol is an intermediate of phloroglucinol degradation since, in the presence of the postulated enzyme complex, resorcinol will be bound to the complex for further catabolism and not released to the medium for detection and isolation. Thus, the first criterion stressed by Ribbons (1966) and exemplified with the model system of o-cresol metabolism by a pseudomonad, would seem not to be universally applicable in formulating metabolic routes.

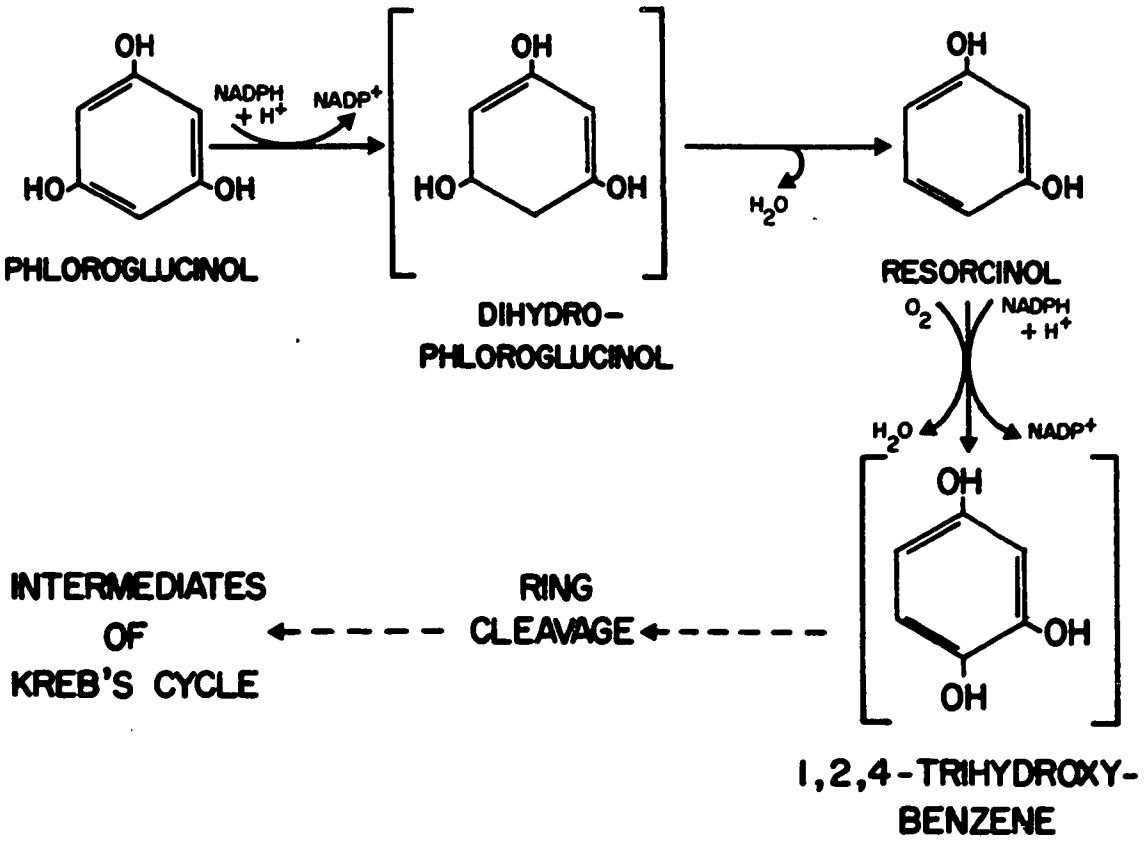
Aromatic compounds are known to be converted, through microbial manipulation, into either *ortho* or *para* dihydroxyphenols, the prerequisite structures for ring fission. This is accomplished by either elimination of substituent groups from the benzene nucleus (Dagley

and Patel, 1957; Ribbons and Evans, 1960) or hydroxylation of the benzene nucleus (Dagley, 1965; Gibson *et al.*, 1968). Hydroxylation reactions are catalyzed by monooxygenases in the presence of reduced pyridine nucleotides or a generating system thereof, with oxygen consumption. In his studies on the metabolism of phloroglucinol by a *Pseudomonas* sp.,Robern (1965) reported that incubation of phloroglucinol and NADPH with cell-free extracts did not exhibit any oxidative activity. Thus no evidence was obtained for the hydroxylation of phloroglucinol. Isolation from culture filtrates of a conjugated phenolic compound which yielded resorcinol on hydrolysis suggested, on the contrary, the dehydroxylation of phloroglucinol. Subsequent work with purified phloroglucinol reductase (Hang, 1967) showed the conversion of phloroglucinol to dihydrophloroglucinol which on dehydration yielded resorcinol. The reductive function of NADPH during dehydroxylation of phloroglucinol, as suggested by Robern (1965), was thus elucidated by Hang (1967). The reductive process, distinct in contrast to the metabolic pathway of other aromatic compounds which are first hydroxylated, was shown to be the result of the addition of two atoms of hydrogen to phloroglucinol with NADPH as donor.

In the present investigation with the purified enzyme complex, an obligate requirement of NADPH was noted for oxygen consumption at the expense of phloroglucinol and resorcinol. The NADPH-dependent consumption of oxygen in the presence of the substrate phloroglucinol, however, does not necessarily implicate hydroxylation of phloroglu-

cinol, but could be attributed to the hydroxylation of resorcinol formed by the enzymatic reduction and subsequent dehydration of phloroglucinol, as demonstrated by Hang (1967) with a *Pseudomonas* sp. In the only documented report on the metabolism of the *meta*-dihydroxyphenol, resorcinol, by a soil pseudomonad, Larway and Evans (1965) suggested the hydroxylation of the resorcinol nucleus at the C_4 position to yield 1,2,4-trihydroxybenzene; subsequent ring fission between the *ortho* dihydroxy groups, gives rise to a dibasic acid. While NADPH-dependent oxygen consumption was observed in the present experiments with resorcinol, 1,2,4-trihydroxybenzene was not detected in these studies.

Based on data obtained in the present investigation, the following pathway of phloroglucinol degradation by *Penicillium* sp. Mac M-47 is proposed:



This pathway involves reductive dehydroxylation of phloroglucinol to yield resorcinol, possibly through the transitory intermediate dihydrophloroglucinol (Hang, 1967). Resorcinol is further metabolized by hydroxylation (*ortho*-hydroxy benzene derivatives) and the subsequent ring fission between the *ortho* dihydroxy groups to give intermediates of Kreb's Cycle. The reaction sequence is envisaged to take place while substrates and products are bound to the postulated enzyme complex.

SUMMARY

This investigation was undertaken with the objectives of studying the utilization of phloroglucinol; physiological conditions necessary for optimal production of the enzyme(s) involved in the degradation; purification and characterization of the enzyme(s); and formulating the pathway of phloroglucinol degradation by *Penicillium* sp. Mac M-47.

Utilization of phloroglucinol was found with both growing-cell and resting-cell fermentations. The resting-cell suspensions required 24 - 27 hours for complete utilization of 0.25% phloroglucinol, without any lag, as compared to 36 - 39 hours, with an initial lag, with growing-cell suspensions. The glucose-grown cells, on the other hand, required much longer time and exhibited a longer lag in utilizing the substrate as compared to the cells previously adapted to phloroglucinol. Ultraviolet absorption spectrophotometric analysis of fermentation samples revealed a gradual shift in the absorption peak from 268 nm (phloroglucinol) to 285 nm which persisted even after prolonged incubation. Although the disappearance of phloroglucinol was easily shown by colorimetric and spectral analyses, and by thin-layer chromatography, all attempts to detect any intermediates, from either growing-cell or resting-cell fermentation, were unsuccessful.

Cell-free extracts prepared from phloroglucinol-grown cells were found to be enzymatically active for catalyzing a rapid oxidation of NADPH with phloroglucinol as the electron acceptor. The enzyme was shown to be substrate inducible and no activity was detected in glucose-grown cells. The inducing growth substrate phloroglucinol plays a significant role in controlling the levels of enzyme which disappears with concomitant disappearance of the substrate from the fermentation medium. The suggested enzyme stabilizing effect exhibited by phloroglucinol necessitated a strict monitoring of substrate levels during the fermentation, and harvesting of the mycelial population before complete utilization of the substrate. In large-scale fermentation experiments for enzyme production, addition of multiple increments of substrate phloroglucinol to the medium increased the mycelial yield but resulted in poor enzyme activity.

In the preliminary purification of the crude extracts, fractions obtained from column chromatography on DEAE-Sephadex exhibited an enzyme assay profile that deviated from the normal, linear profile characteristic of the enzyme assays with the crude extract. Various attempts made to explain this abnormal behaviour of the DEAE-Sephadex eluates were unsuccessful. Only ammonium salts (NH_4^+) and the thiol reagents, β-mercaptoethanol and Cleland's reagent, were found to restore the abnormal enzyme assay profile to the normal pattern. Thus β-mercaptoethanol was included in the assay mixture for all studies.

Although a satisfactory explanation for the abnormal behaviour of the DEAE-Sephadex eluates did not come forth, the experiments carried out in this direction revealed the presence of a second enzyme which catalyzes the oxidation of NADPH in the presence of resorcinol. The resorcinol enzyme activity was approximately 50% of the phloroglucinol enzyme activity. The phloroglucinol enzyme and resorcinol enzyme activities were postulated to be closely related and to form a part of an enzyme complex involved in the degradation of phloroglucinol by *Penicillium* sp. Mac M-47. Significant data supporting this concept were obtained in enzyme purification and characterization studies. The evidence for this conclusion is: (1) simultaneous induction of the phloroglucinol enzyme and resorcinol enzyme activities in the resorcinol-grown cells; (2) lack of competitive inhibition of the phloroglucinol enzyme activity by resorcinol, and the non-additive rates of reaction in enzyme assay containing both the substrates; (3) coincidental elution of the two activities in column chromatography; (4) an apparently high K_m value for the exogenously added substrate resorcinol; (5) inability to detect any intermediate of phloroglucinol degradation; and (6) a distinct spectrum of inhibition and activation depicted by the two enzyme activities.

The enzyme complex was purified approximately 20-fold for the phloroglucinol enzyme and 21-fold with respect to resorcinol enzyme activity. Analytical polyacrylamide disc gel electrophoresis

exhibited one major and one minor protein band in the purified enzyme complex. Its molecular weight is estimated to be approximately 93,000. The two component enzyme activities are NADPH-specific, however, NADH may be accepted as an alternate electron doner at 50% efficiency. The enzyme activities, though not totally dependent on flavin nucleotides, are stimulated by FAD and FMN. No stimulation is obtained with ATP, ADP or AMP. Cations are not essential for the activity of the enzyme complex. Of the various substrates examined, only phloroglucinol carboxylic acid, in addition to phloroglucinol and resorcinol, is capable of oxidizing NADPH in the presence of the enzyme complex. The K_m values for phloroglucinol and resorcinol are 2×10^{-5} M and 1.43×10^{-3} M respectively. The optimum pH for both phloroglucinol and resorcinol enzyme activity is pH 7.3 whereas, the pH optima for stability of phloroglucinol and resorcinol enzymes are pH 7.0 and pH 7.5 respectively. The involvement of thiol groups for activity was demonstrated by the heat sensitivity of the enzyme complex and the inhibitory action of sulfhydryl reagents. Manometric studies showed NADPH-dependent consumption of oxygen in both the phloroglucinol and resorcinol enzyme reactions. No net CO_2 evolution was recorded. No reaction product(s), either from reaction mixtures in the respirometer flasks or from spectrophotometric enzyme assays were detected.

Based on direct and interpretative data obtained in studies with the whole cells and the enzyme complex, a pathway of phloroglu-

cinol degradation by *Penicillium* sp. Mac M-47 is proposed. This pathway involves the reductive dehydroxylation of phloroglucinol to yield resorcinol, possibly through the transitory formation of dihydrophloroglucinol. Resorcinol is then further metabolized by hydroxylation and subsequent ring fission.

BIBLIOGRAPHY

Barker, H.A.
1956 Biological formation of methane.
Ind. Engng. Chem. 48:1438.

Berger, L., Slein, M.W., Colowick, S.P., and Cori, C.F.
1946 Isolation of hexokinase from baker's yeast.
J. Gen. Physiol. 29:379-391.

Blat, W.F., Feinberg, M.P., Hopfenberg, H.P., and Saravis, C.A.
1965 Protein solutions: concentration by a rapid method.
Science 150:224-225.

Burchall, J.J., and Hitchings, G.H.
1965 Inhibitor binding analysis of dihydrofolate reductases from various species.
Mol. Pharmacol. 1:126-136.

Canovas, J.L., Ornston, L.N., and Stanier, R.Y.
1967 Evolutionary significance of metabolic control systems.
Science 156:1695-1698.

Case, M.E., and Giles, N.H.
1968 Evidence for nonsense mutations in the arom gene cluster of *Neurospora crassa*.
Genetics 60:49-58.

Chapman, P.J., and Dagley, S.
1962 Oxidation of homogentisic acid by cell-free extracts of a Vibrio.
J. Gen. Microbiol. 28:251-256.

Chatterjee, A.K., and Gibbins, L.N.
1969 Metabolism of phloridzin by *Erwinia herbicola*: Nature of the degradation products, and the purification and properties of phloretin hydrolase.
J. Bacteriol. 100:594-600.

Colowick, S.P.
1955 Separation of proteins by use of adsorbents.
In: Methods in Enzymology.
S.P. Colowick and N.O. Kaplan, Eds. (Acad. Press, N.Y.),
vol. I, p. 90-98.

Dagley, S.
1965 Degradation of the benzene nucleus by bacteria.
Sci. Progr. 53:381-392.

Dagley, S.
1967 The microbial metabolism of phenolics.
In: Soil Biochemistry.
A.D. McLaren and G.H. Peterson, Eds.
(Marcel Dekker, Inc. N.Y.), p. 287-317.

Dagley, S., Chapman, P.J., Gibson, D.T., and Wood, J.M.
1964 Degradation of the benzene nucleus by bacteria.
Nature 202:775-778.

Dagley, S., Evans, W.C., and Ribbons, D.W.
1960 New pathways in the oxidative metabolism of aromatic compounds by microorganisms.
Nature 188:560-566.

Dagley, S., and Patel, M.D.
1957 Oxidation of p-cresol and related compounds by a *Pseudomonas* sp.
Biochem. J. 66:227-233.

Davis, B.J.
1964 Disc electrophoresis. II. Methods and application to human serum proteins.
Ann. N.Y. Acad. Sci. 121:404-427.

Dutton, P.L., and Evans, W.C.
1969 The metabolism of aromatic compounds by *Rhodopseudomonas palustris*. A new, reductive, method of aromatic ring metabolism.
Biochem. J. 113:525-536.

Evans, W.C.
1956 Biochemistry of the oxidative metabolism of aromatic compounds by microorganisms.
Chem. Soc. Ann. Rept. 53:279-294.

Evans, W.C.
1958 Metabolism of aromatic compounds by lower plants. Phenols, quinones and the problem of biogenesis of benzene nuclei.
In: Encyclopedia of Plant Physiology.
W. Ruhland, Ed. (Springer-Verlag, Berlin),
vol. X, p. 454-506.

Evans, W. C.
1963 The microbiological degradation of aromatic compounds.
J. Gen. Microbiol. 32:177-184.

Evans, W.C.
1969 Microbial transformations of aromatic compounds.
In: Fermentation Advances.
D. Perlman, Ed. (Acad. Press, N.Y.),
p. 649-687.

Farr, D.R., and Cain, R.B.
1968 Catechol oxygenase induction in *Pseudomonas aeruginosa*.
Biochem. J. 106:879-885.

Fernando, J., Enser, M., Pontremoli, S., and Horecker, B.L.
1968 Purification and properties of rabbit muscle fructose 1,6-diphosphatase.
Arch. Biochem. Biophys. 126:599-606.

Gaertner, F.H., and DeMoss, J.A.
1969 Purification and characterization of a multienzyme complex in the tryptophan pathway of *Neurospora crassa*.
J. Biol. Chem. 244:2716-2725.

Gibson, D.T., Koch, J.R., and Kallio, R.E.
1968 Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymatic formation of catechol from benzene.
Biochemistry 7:2653-2662.

Gibson, D.T., Wood, J.M., Chapman, P.J., and Dagley, S.
1967 The bacterial degradation of aromatic compounds. Biotechnol. Bioeng. 9:33-44.

Giles, N.H., Case, M.E., Partridge, C.W.H., and Ahmed, S.I.
1967 A gene cluster in *Neurospora crassa* for an aggregate of five aromatic synthetic enzymes. Proc. Nat. Acad. Sci. 58:1453-1460.

Ginsburg, A., and Stadtman, E.R.
1970 Multienzyme systems. Ann. Rev. Biochem. 39:429-472.

Green, N.M., and Neurath, H.
1954 Proteolytic enzymes. *In*: The Proteins. H. Neurath and K. Bailey Eds. (Acad. Press, N.Y.), vol. II, p. 1057.

Griffiths, E.D., Rodrique, D., Davis, J.I., and Evans, W.C.
1964 Ability of Vibrio 0/1 to synthesize either catechol 1:2 oxygenase or catechol 2:3 oxygenase depending on primary inducer. Biochem. J. 91:16p.

Guyer, M., and Hegeman, G.
1969 Evidence for reductive pathway for the anaerobic metabolism of benzoate. J. Bacteriol. 99:906-907.

Hang, Y.D.
1967 Pathway of phloroglucinol degradation by a *Pseudomonas* sp. Mac 451. Ph.D. Thesis, McGill University, Montreal, Canada.

Harris, G., and Ricketts, R.W.
1962 Metabolism of phenolic compounds by yeasts. Nature 195:473-474.

Hashimoto, K.
1970 Oxidation of phenols by yeasts. I. A new oxidation product from p-cresol by an isolated strain of yeast. J. Gen. Appl. Microbiol. 16:1-13.

Hayaishi, O.
1966 Crystalline oxygenases of pseudomonads.
Bacteriol. Rev. 30:720-731.

Hayaishi, O.
1969 Enzymatic hydroxylation.
Ann. Rev. Biochem. 38:21-44.

Hayaishi, O., and Nozaki, M.
1969 Nature and mechanisms of oxygenases.
Science 164:389-396.

Henning, U.
1966 Multienzyme complexes.
Angew. Chem. Int. Ed. 5:785-790.

Horowitz, R.M.
1961 The orange, its Biochemistry and Physiology.
W.B. Sinclair, Ed. (Univ. of California Press,
Riverside, California), p. 336-343.

Ingram, J.M.
1969 Fructose diphosphate aldolase of yeast:
Studies on the sulfhydryl groups.
Can. J. Biochem. 47:595-601.

Ingram, J.M., and Hochster, R.M.
1967 Purification and properties of fructose diphosphate
aldolase from *Fusarium oxysporum* F. Lycopersici.
Can. J. Biochem. 45:929-936.

Jacob, F., and Monod, J.
1961 On the regulation of gene activity.
Cold Spring Harbor Symp. 26:193-211.

Jamieson, W.D., Taylor, A., Mathur, D.K., and Blackwood, A.C.
1970 Identification of an intermediate of phloroglucinol
degradation by mass spectroscopic analysis.
Can. J. Biochem. 48:215-216.

Jayasankar, N.P., Bandoni, R.J., and Towers, G.H.N.
1969 Fungal degradation of phloridzin.
Phytochem. 8:379-383.

Jayasankar, N.P., and Bhat, J.V.

1966 A colorimetric method for the estimation of phloroglucinol. Anal. Biochem. 15:454-462.

Jones, G.A., Day, C.G., Blakley, E.R., and Simpson, F.J.

1970 Anaerobic degradation of phloroglucinol by rumen bacteria. Abstracts, X International Congress for Microbiology, Mexico, p. 45.

Kalle, A.G.

1965 German Patent No. 1195327. (Seigfried Pietzsch).

Kastens, M.L., and Kaplan, J.F.

1950 TNT into phloroglucinol. Ind. Eng. Chem. 42:402-413.

Kennedy, E.P., and Barker, H.A.

1951 Paper chromatography of volatile acids. Anal. Chem. 23:1033-1034.

Kilby, B.A.

1948 The bacterial oxidation of phenol to β-ketoadipate. Biochem. J. 43:5p.

Kochetov, G.A., Nikitushkina, L.I., and Chernov, N.N.

1970 A complex of functionally-bound enzymes: Transketolase and glyceraldehydephosphate dehydrogenase. Biochem. Biophys. Res. Commun. 40:873-879.

Koli, A.K., Yearby, C., Scot, W., and Donaldson, O.

1969 Purification and properties of three separate menadione reductases from hog liver. J. Biol. Chem. 244:621-629.

Krishnamurty, H.G., Cheng, K.-J., Jones, G.A., Simpson, F.J., and Watkin, J.E.

1970 Identification of products produced by the anaerobic degradation of rutin and related flavonoids by *Butyrivibrio* sp. C_3 Can. J. Microbiol. 16:759-767.

Lack, L.
1959 The enzymic oxidation of gentisic acid.
Biochim. Biophys. Acta 34:117-123.

Larway, P., and Evans, W.C.
1965 Metabolism of quinol and resorcinol by soil pseudomonads.
Biochem. J. 95: 52p.

Linderstrøm-Lang, K.
1950 Structure and enzymatic breakdown of proteins.
Cold Spring Harbor Symp. Quant. Biol. 14:117-126.

Lineweaver, H., and Burk, D.
1934 The determination of enzyme dissociation constants.
J. Amer. Chem. Soc. 56:558-666.

Lowry, O.H., Rosebrough, N.J., Farr, L.A., and Randall, J.R.
1951 Protein measurement with the Folin phenol reagent.
J. Biol. Chem. 193:265-275.

MacDonald, D.L., Stanier, R.Y., and Ingraham, J.L.
1954 The enzymatic formation of β-carboxymuconic acid.
J. Biol. Chem. 210:809-820.

Mandelstam, J.
1960 The intracellular turnover of protein and nucleic acids, and its role in biochemical differentiation.
Bacteriol. Rev. 24:289-308.

Miller, M.W.
1961 The Pfizer Handbook of Microbial Metabolites.
The Blakiston Division. (McGraw-Hill Book Co. Inc., Toronto).

Minamikawa, T., Jayasankar, N.P., Bohm, B.A., Taylor, I.E.P., and Towers, G.H.N.
1970 An inducible hydrolase from *Aspergillus niger*, acting on carbon-carbon bonds, for phlorrhizin and other C-acylated phenols.
Biochem. J. 116:889-897.

Newhall, W.F., and Ting, S.V.
1967 Degradation of hesperetin and naringenin to phloroglucinol. J. Agr. Food Chem. 15:776-777.

Ono, K., Nozaki, M., and Hayaishi, O.
1970 Purification and some properties of protocatechuate 4,5-dioxygenase. Biochim. Biophys. Acta 220:224-238.

Ornston, L.N.
1966[1] The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. I. Biochemistry. J. Biol. Chem. 241:3776-3786.

Ornston, L.N.
1966[2] The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. II. Enzymes of the protocatechuate pathway. J. Biol. Chem. 241:3787-3794.

Ornston, L.N.
1966[3] The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. III. Enzymes of the catechol pathway. J. Biol. Chem. 241:3795-3799.

Ornston, L.N.
1966[4] The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. IV. Regulation. J. Biol. Chem. 241:3800-3810.

Ornston, L.N., and Stanier, R.Y.
1964 Mechanism of β-ketoadipate formation by bacteria. Nature 204:1279-1283.

Palleroni, N.J., and Stanier, R.Y.
1964 Regulatory mechanisms governing synthesis of the enzymes for tryptophan oxidation by *Pseudomonas fluorescens*. J. Gen. Microbiol. 35:319-334.

Pogell, B.M.
1966 Enzyme purification by specific elution procedures with substrate. *In*: Methods in Enzymology. S.P. Colowick and N.O. Kaplan, Eds. (Acad. Press, N.Y.) vol. 9, p. 9-15.

Randerath, K.

1963 Thin-layer chromatography.
Acad. Press, N.Y.

Reed, L.J., and Cox, D.J.
1966 Macromolecular organization of enzyme systems.
Ann. Rev. Biochem. 35:57-84.

Reid, R.L., and Lederer, M.
1951 Separation and estimation of saturated C_2- C_7 fatty acids by partition chromatography.
Biochem. J. 50:60-67.

Ribbons, D.W.
1965 The microbial degradation of aromatic compounds.
Chem. Soc. Ann. Rept. 62:445-468.

Ribbons, D.W.
1966 Metabolism of o-cresol by *Pseudomonas aeruginosa* strain T 1.
J. Gen. Microbiol. 44:221-231.

Ribbons, D.W., and Evans, W.C.
1960 Oxidative metabolism of phthalic acid by soil pseudomonads.
Biochem. J. 76:310-318.

Robern, H.
1965 The metabolism of phloroglucinol by microorganisms.
Ph.D. Thesis, McGill University, Montreal, Canada.

Rogoff, M.H.
1961 Oxidation of aromatic compounds by bacteria.
Advanc. Appl. Microbiol. 3:193-221.

Roux, D.G., and Maihs, A.E.
1960 Selective spray reagents for the identification of flavonoid compounds associated with condensed tannins.
J. Chromatog. 4:65-74.

Sarngadharan, M.G., Watanabe, A., and Pogell, B.M.
1969 Purification of rabbit liver fructose 1,6-diphosphatase by substrate elution.
J. Biol. Chem. 245:1926-1929.

Scher, S., and Proctor, M.H.
1960 Studies with photosynthetic bacteria: Anaerobic oxidation of aromatic compounds.
In: Symposia on Comparative Biology. I. Comparative biochemistry of photoreactive systems.
M.B. Allen, Ed. (Acad. Press, N.Y.), p. 387-394.

Schimke, R.T.
1964 The importance of both synthesis and degradation in the control of arginase levels in rat liver.
J. Biol. Chem. 239:3808-3817.

Schimke, R.T.
1969 On the roles of synthesis and degradation in regulation of enzyme levels in mammalian tissues.
In: Current topics in cellular regulation.
B.L. Horecker and E.R. Stadtman, Eds. (Acad. Press, N.Y.)
vol. 1, p. 77-124.

Schimke, R.T.
1970 Regulation of protein degradation in mammalian tissues.
In: Mammalian Protein Metabolism.
H.N. Munro, Ed. (Acad. Press, N.Y.)
vol. IV, p. 177-228.

Schimke, R.T., Sweeny, E.W., and Berlin, C.M.
1965 Studies of the stability *in vivo* and *in vitro* of rat liver tryptophan pyrrolase.
J. Biol. Chem. 240:4609-4620.

Seidman, M.M., Toms, A., and Wood, J.M.
1969 Influence of side-chain substituents on the position of cleavage of the benzene ring by *Pseudomonas fluorescens*.
J. Bacteriol. 97:1192-1197.

Simpson, F.J., Jones, G.A., and Wolin, E.A.
1969 Anaerobic degradation of some bioflavonoids by microflora of the rumen.
Can. J. Microbiol. 15:972-974.

Stanier, R.Y.

1947 Simultaneous adaptation: A new technique for the study of metabolic pathways. J. Bacteriol. 54:339-348.

Stanier, R.Y., Hegeman, G., and Ornston, L.N.
1963 Colloques internationaux du Centre National de la Recherche Scientifique. No. 124,221.
Cited in: Soil Biochemistry, A.D. McLaren and G.H.Peterson, Eds. (Marcel Dekker, Inc. N.Y., 1967) p. 311.

Stanier, R.Y., Palleroni, N.J., and Douderoff, M.
1966 The aerobic Pseudomonads: A taxonomic study. J. Gen Microbiol. 43:159-271.

Tarvin, D., and Buswell, A.M.
1934 The methane fermentation of organic acids and carbohydrates. J. Amer. Chem. Soc. 56:1751-1755.

Taylor, B.F., Campbell, W.L., and Chinoy, I.
1970 Anaerobic degradation of the benzene nucleus by a facultatively anaerobic microorganism. J. Bacteriol. 102:430-437.

Towers, G.H.N.
1964 Metabolism of phenolics in higher plants and microorganisms. *In*: Biochemistry of Phenolic Compounds. J.B. Harborne, Ed. (Acad. Press, N.Y.), p. 249-294.

Wang, D.I.C., Sonoyama, T., and Mateles, R.I.
1968 Enzyme and bacteriophage concentration by membrane ultrafiltration. Anal. Biochem. 26:277-287.

Westlake, D.W.S., Talbot, G., Blakley, E. R., and Simpson, F.J.
1959 Microbial decomposition of rutin. Can. J. Microbiol. 5:621-629.

Winnacker, E.L., and Barker, H.A.
1970 Purification and properties of a NAD-dependent glutamate dehydrogenase from *Clostridium* SB_4. Biochim. Biophys. Acta, 212:225-242.

Zetelaki, K.
1969 Disruption of mycelia for enzymes. Process Biochemistry 4:19-22, 27.