INITIAL STEPS IN THE DEGRADATION OF 1,3,5-TRIHYDROXYBENZENE BY BACILLUS Sp. BPG-8

KASHMIRA ACHARYA

enzyme preparations. The Km values for PGR was 2 x 10^{-4} M and molecular weight was found to be 155,000 daltons. The PGR and RH activities from BPG-8 reached a peak in about 18 hours. The pH optimum for the enzyme activity was found to be 7.4.

Freezing and thawing had little or no effect on the PGR and RH from BPG-8. When the crude extract stored at 4°C for two days, a 90% loss of the RH activity resulted. Increasing concentrations of glycerol to (15%) offred protection to the PGR and RH.

The spectral changes observed during chemical reduction of PG by sodium borohydride indicated the formation of dihydrophloroglucinol. Evidence is presented to show that enzymic reduction of PG in the presence of NADPH forms a product with a similar spectrum.

The data presented suggest that BPG-8 may carry an enzyme complex with two separate activities, namely PGR and RH.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EU	Enzyme unit
2ME	2-mercaptoethanol
MSM	Mineral Salt Medium
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PDAB	p - Dimethylaminobenzalydehyde
PG	Phloroglucinol
PGR	Phloroglucinol reductase
PMSF	Phenylmethyl sulfonyl fluoride
RH	Resorcinol hydroxylase
TCA	Trichloacetic acid
TSA	Trypticase soy agar
TSB	Trypticase soy broth
YE	Yeast extract
MOPS	Morpholinopropane fonic acid

INTRODUCTION

Benzene and related compounds are characterized by the possession of a large (negative) resonance energy. This results in thermodynamic stability which manifests itself in chemical properties that are referred to as aromaticity.

The origin of aromatic hydrocarbons in the environment is a subject of debate. It is generally accepted that most of these compounds found in the environment are produced by the pyrolysis of organic material. The types of aromatic hydrocarbons formed depend on the pyrolysis temperature. At high temperatures (2000°C) unsubstituted polycylic aromatic hydrocarbons are the principle products. Intermediate temperatures (400° - 800°C) lead to the formation of alkyl-substituted molecules. In contrast, petroleum, which is formed at low temperatures (80° - 150°C) contains polycyclic aromatic hydrocarbons.

Many synthetic chemicals are added to our environment in the form of herbicides, pesticides and industrial effluents. Many are derivatives of benzene. If such chemicals prove recalcitrant to microbial decomposition they could accumulate in the soil. Such an accumulation could lead to serious ecological changes. Also, there is a possibility that eventually these compounds will localize in animal tissues. In order to prevent such an occurrence, it is vital that we understand how microorganisms degrade both natural and synthetic chemicals.

Degradation of Polyaromatics:

Polycyclic aromatic hydrocarbons are distributed in soil and sediment.

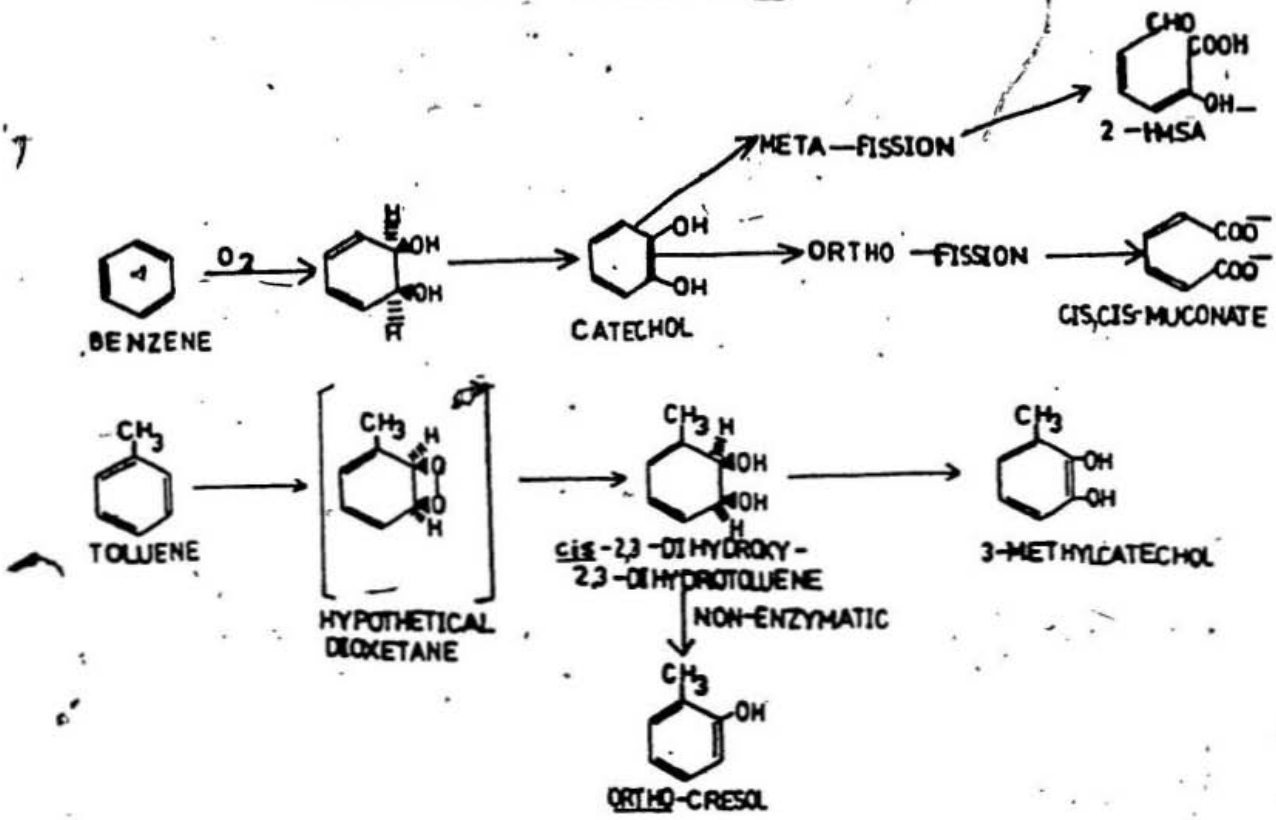
Large amounts of petroleum products are synthesized annually. (Simonarat, 1966). It is not inconceivable that these would find their way into soil and sediments. The role of microorganisms in maintaining steady-state concentrations of environmental chemicals is well estabilised. The solar energy is converted to chemical energy by the activities of photo-synthetic organisms. It is generally accepted that all biosynthetic products are subject to microbial degradation. Aromatic hydrocarbons that contain more than one ring are known as polyaromatic hydrocarbons e.g. napthalene, phenanthrene, anthracene etc. The initial reaction in the bacterial oxidation of these compounds involves the formation of a dihydrodiol intermediate. (Final, 1960; Dagley, 1965). Oxidation of dihydrodiols leads to the formation of catechols. The key intermediate in the degradation of aromatic compounds is either catechol or Protocatechute (Fig. 1) (Rogoff and Wender, 1957; Colla et al., 1959; Evans et al., 1965; Kiyohura, 1977; Gibson et al., 1971; Starovoitov et al., 1975; Kutagiri, 1966; Kuno and Akaishi, 1961). These hydroxylated compounds are subject to ring fission reactions.

Phloroglucinol Degradation Occurence

Phloroglucinol (1,3,5-trihydroxybenzene) occurs in nature as a constituent of several commonly found compounds such as flavones, anthocyanins and catechins (Robinson T., 1962). Soil microorganisms release PG in the environment by the decomposition of plant material. Certain soil microbes possess the necessary enzyme systems to utilize phloroglucinol as a sole source of carbon and energy.

PG was first prepared by a synthetic process in the laboratory by Jorden

FIGURE I METABOLISM OF AROMATIC COMPOUNDS IN PROKARYOTES GIBSON (1964)



(1897). The usual source of chemical synthesis involves the reduction of trinitrobenzoic acid or trinitrobenzene with tin and hydrochloric acid. The amine formed is neutralized and boiled for 15-20 hours forming phloroglucinol. (Clark and Hartman, 1929).

PG is used in the printing and textile dyeing industries; as a reagent for pentoses, aldehydes and lignins; in preventing sludge formation in transformer oil, in microscopy as an excellent decalcifier of bone suspension and clinically as an antimicrobial agent.

Aerobic Metabolism of PG

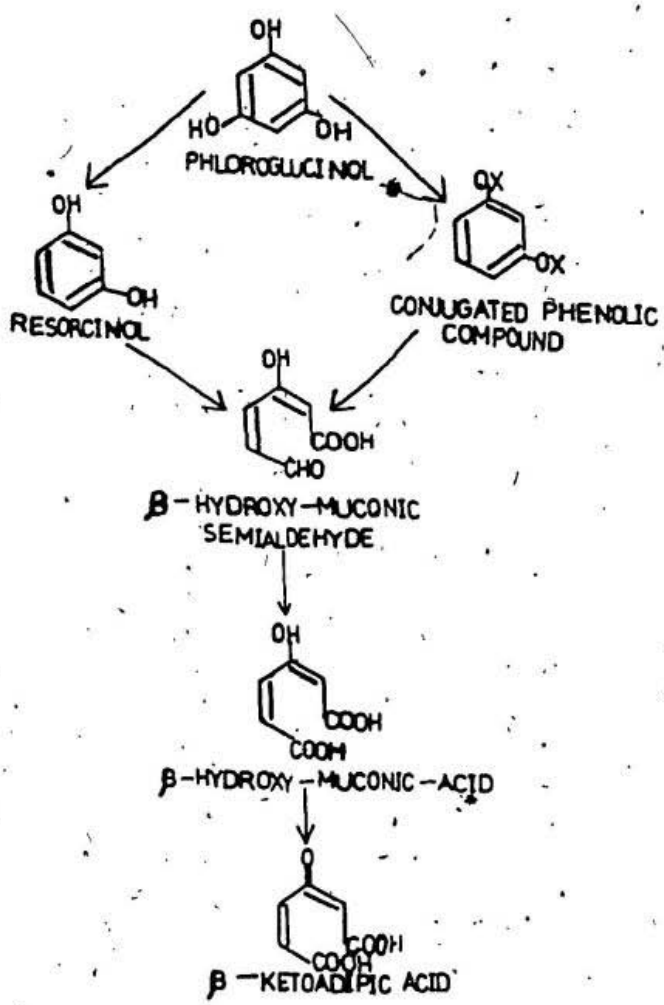
Wanger (1914) first reported the aerobic metabolism of PG by bacteria which were isolated from soil.

Thorton (1928), was able to isolate microorganisms from the soil capable of growing in pure culture which utilize PG, resorcinol, cresol and resorcyclic acid as sole sources of carbon. Bernheim (1956) demonstrated an adaptive enzyme formed in a Mycobacterium species if the organisms were preincubated with PG. Nakagwa and Takeda (1962) showed the oxidation of PG, orcinol, resorcinol, and p-, m-, and o-cresols by Brevibacterium fuscum.

Very few reports are available in the literature about the metabolic pathway of PG by microorganisms. Robern (1965) first studied the degradation of PG by a Pseudomonas species Mac 451 and proposed a pathway which was later shown to exist in this organism by Hung (1967). This pathway (Fig. 2) involves the

FIGURE 2 THE PROPOSED METABOLIC PATHWAY FOR THE OXIDATION OF PG BY PSEUDOMONAS SP. MAC 451

HUNG (1967)



reduction of PG to dihydro-PG. According to this pathway PG is converted either into resorcinol or conjugated phenol compound of unknown structure. These products are then converted into B-ketoadipate by ring-fission followed by a hydroxylation reaction. Jamieson et al. (1969), obtained further evidence for the structure of the reduction product (dihydro-PG) by mass spectral analysis. The product of enzymatic reduction of PG was compared to a chemically reduced product and it was characterized by thin layer chromatography and its aerobic metabolism.

Anaerobic Metabolism of PG

The breakdown of PG is not restricted to aerobic environments. The microflora of the bovine rumen under anaerobic conditions rapidly degrade bioflavonoids such as rutin, quercitrin and naringin. It was observed by Simpson, et al. (1969), that PG was detected as a transitory intermediate. Hesperidin and naringin were also rapidly degraded anaerobically.

Anaerobic degradation of rutin to yield PG and other phenolic compounds by Butyrivibrio sp. C_3 was studied by Krishnamurty et al. (1970). PG and 3,4-dihydroxyphenylacetic acid are not further metabolized by this organism even in the presence of succinate or other such carbon sources.

Isolation of PG degrading bacteria from anaerobic enrichment cultures of the rumen microflora by Tasi and Jones (1976), yielded five strains identified as Streptococcus bovis and three as Coprococcus sp. $Pe_1 3$; $Pe_1 5$; $Pe_1 12$. This was

the first report to describe the isolation of gram-positive cocci capable of metabolizing an aromatic substrate anaerobically. A detailed study on one of the Coprococcus strains, Coprococcus sp. $Pe_1 5$, showed that the microorganism grew on PG as a sole source of carbon and energy but failed to grow on thirty-nine other aromatic compounds.

Patel et al. (1981), examined the initial steps in the anaerobic degradation of PG by Coprococcus sp. $Pe_1 5$ (Fig. 3). The authors also showed the direct spectral evidence for the chemical and the enzymatic reduction of PG to dihydro-PG. The cell-free extracts prepared from the bacteria grown on PG required NADPH in the initial reaction.

In the case of Coprococcus sp. $Pe_1 5$, the optimum pH for maximum enzyme activity was 7.4 and the Km for PG was 3.0×10^{-5}M. Although the organism was a strict anaerobe, the PGR from anaerobically grown cells was insensitive to air.

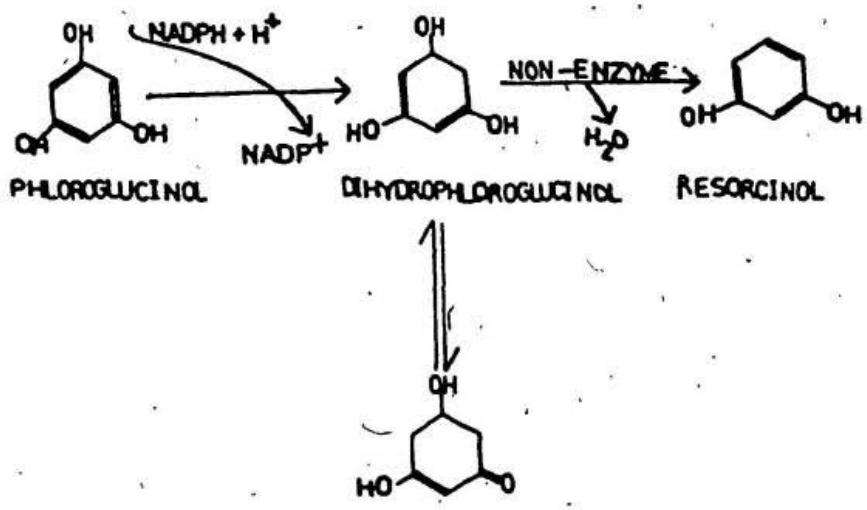
In the more recent past, Bernhard et al (1982) found a new strictly anaerobic, non-spore forming, sulfate-reducing bacterium, Pelobacter acidigallici, which utilized gallic acid, pyrogallol, 2,4,6-trihydroxybenzoic acid and PG. Their work complemented the findings of the earlier authors. (Whittel, 1976; Patel et al., 1981; Hang 1967; Mathur 1971).

Photometabolism of Phloroglucinol

The anaerobic photometabolism of PG by Rhodopseudomonas gelatinosa has been reported by Whittel et al., (1976). The cell-free extracts prepared from

FIGURE 3 INITIAL STEPS IN THE ANAEROBIC METABOLISM OF PHLOROGLUCINOL BY COPROCOCCUS SP.

PE 5 PATEL (1981)



the bacterium required NADPH in the decomposition of PG and the first product identified was dihydro PG.

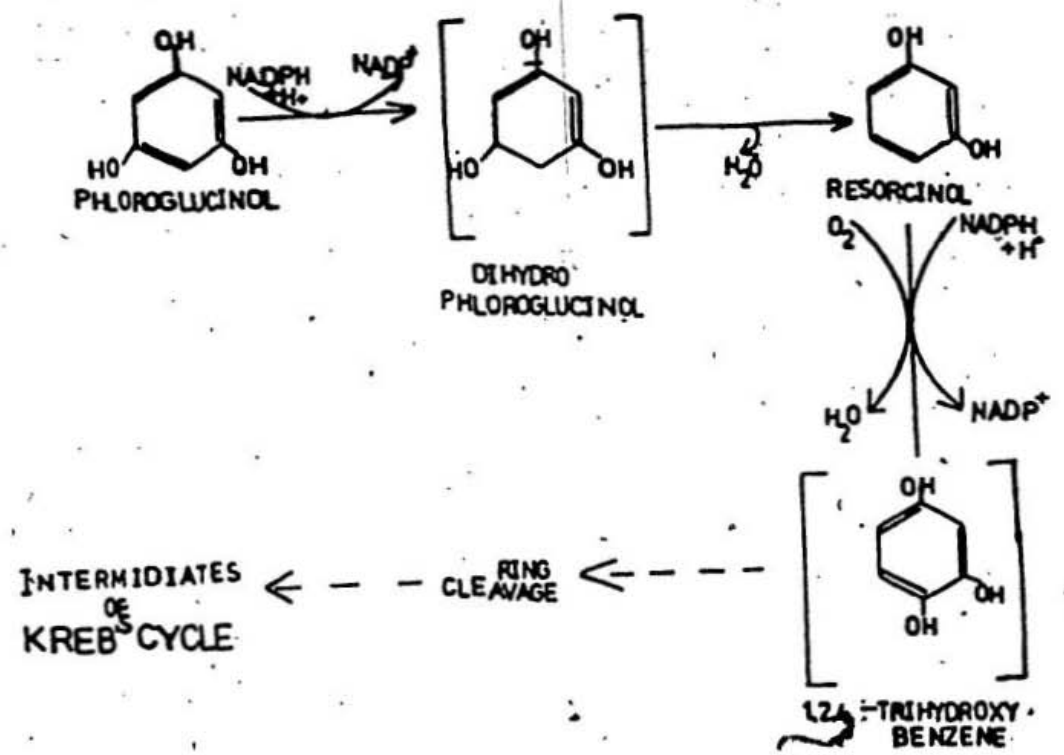
Carigie et al. (1965) have reported PG degradation by marine algae.

Fungal degradation of Phloroglucinol

Mathur (1971) studeid the utilization of PG in growing cell and resting cell fermentations with Penicillium sp. Mac 47 which was originally isolated and described by Robern (1965). Mathur proposed the pathway illustrated in Fig. 4. The authors proposed an enzyme complex which carried out the transformation of PG to resorcinol. They postulated that the product, resorcinol was held tightly by the enzyme complex which carried two activities, namely phloroglucinol reductase (PGR) and resorcinol hydroxylase (RH). A hypothetical intermediate 1,2,4-trihydroxybenzene was supposedly the target of the ring fission enzyme which formed intermediates of the TCA cycle.

Mathur (1971) also studied the physiological conditions necessary for optimal production of the enzyme(s) involved in the degradation as well as the purification and characterization of the enzyme(s). The PG and resorcinol enzyme activities were postulated to be closely related and to form a part of an enzyme complex involved in the degradation of PG by Penicillium sp. Mac M-47. The cell free extracts prepared from the fungus required NADPH as an electron donor for both PGR and RH activities. The optimum Ph for both enzyme activities was pH 7.3. The Km values for PGR and RH were 2×10^{-5}M and 1.43

FIGURE 4 PROPOSED PATHWAY FOR THE DEGRADATION OF PHLOROGLUCINOL BY PENICILLIUM SP. MAC M-47 MATHUR (1971)



x 10^{-3}M respectively. Attempts by the authors to detect any reaction product(s) were unsuccessful.

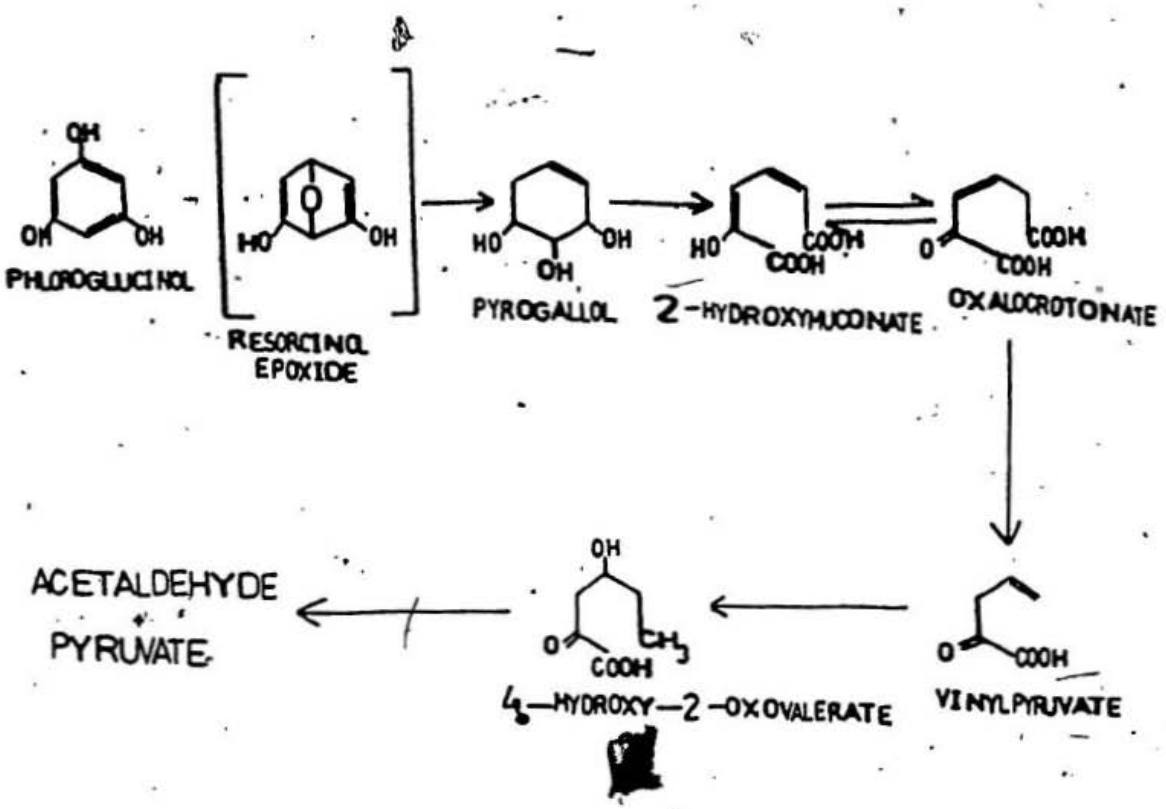
Jayasankar et al. (1969) studied the hydrolysis of phloridzin and phloretin to PG and phloretic acid by Aspergillus niger which were identified by chromatography and spectrophotometry.

Walker and Taylor (1983) have recently isolated a strain of Fusarium solani from soil which has been found to convert PG to pyrogallol which is further metabolized via the meta-fission pathway to yield pyruvate and acetate. They proposed a new pathway different from those proposed by earlier workers (Fig. 5).

Resorcinol Metabolism

The metabolism of resorcinol has received very little attention. Chapman and Ribbons (1976) showed some evidence that resorcinol is metabolized via the β-ketoadipate pathway. They elucidated the meta and ortho pathways of resorcinol metabolism in a strain of Pseudomonas putida. The first intermediate formed was hydroxyquinol which undergoes both ortho and meta cleavage reactions with the subsequent formation of both pyruvate and maleylacetate. The pyruvate formed is then channeled into the TCA cycle. Maleylacetate is reduced to β-ketoadipate by NADPH. Shailubhai et al. (1983) found a strain of Aspergillus niger which converted resorcinol to hydroxyquinol, follwed by ortho ring fission to form maleylacetate, which in turn is converted to β-ketoadipate.

FIGURE 5. PROPOSED PATHWAY FOR CATABOLISM OF PHLOROGLUCINOL BY FUSARIUM SOLANI, WALKER AND TAYLOR (1983)



A similar pathway for degradation of resorcinol in Trishosporon cataneum has been proposed by Gall and Nejahr (1979).

OBJECTIVES: The objectives of the present work were:

1. To isolate soil bacteria capable of degrading PG, a product of wood decomposition.

2. To determine the optimal condition for the degradation of PG by a selected bacterial strain. Bacillus sp. BPG-8 was chosen for this purpose.

3. To examine the initial steps in the degradation of PG by Bacillus sp. BPG-8.

4. To isolate and characterize the enzyme system involved in the initial reactions of PG-metabolism.

METHODS AND MATERIALS

Materials

All chemicals were of analytical grade and following acetic acid were purchased from Sigma Chemical Company. (St. Louis, Mo.). p-Dimethylaminonbenzaldehyde (PDAB), glacial acetic acid copper sulfate were purchased from J.T. Baker Chemical Company (Phillipsburg, N.J.). Sephadex gels were purchased fromPharmacia Fine Chemicals (St. Louis, Mo.). The jon exchange chromatography material, DEAE-Sephadex A-50-120 was product of Sigma Chemical Company, (St. Louis, Mo.).

Organisms, growth and maintenance

A gram positive rod was isolated from soil samples obtained from the South Side Hills area of St. John's Harbour. This location has large storage tanks owned by oil companies and the soil is contaminated with oil and petroleum hydrocarbons. The organism was isolated using the soil enrichment technique.

The bacterium was grown in a mineral salt medium containing 0.1% PG at 25°C for 18 hours. Cultures were maintained on trypticase soy agar (TSA) plates at 5°C and subcultured periodically.

Media preparation

A mineral salts medium (MSM) was used to culture the organism. The stock solutions were prepared as follows. 1 M potassium phosphate (Dibasic) solution was added to a solution of potassium phosphate (monobasic) until the pH was 5.5 The other stock solutions include magnesium sulphate (10 g/100 ml),

ammonium sulphate (50 g/100 ml) and yeast extract (YE, 1 g/100 ml). These four stock solutions were individually sterilized. The mineral salt medium was made as follows: To 900 ml of sterile distilled water were added stock solutions as follows: 10 ml of magnesium sulphate 1 ml of yeast extract, 100 ml of potassium phosphate and 1 ml of ammonium sulphate.

The pH was adjusted to 5.5 using 1 N Hydrochloric acid. To this medium PG was added aseptically to give a concentration of 0.1%.

Biochemical Tests

Characterization tests listed in Table 1 were performed for the identification of the organisms. A test for sporulation by the organism was carried out, with control tests simultaneously run on Bacillus subtilis and Pseudomonus sp. The cultures were inoculated on TSA agar plates and incubated at 25°C for three days. The cells were suspended in normal saline and subjected to heating at 70°C in a waterbath for 10 minutes. Heated samples were streaked on TSA plates which were incubated at 25°C. Only spore bearing organisms are expected to grow under these conditions so this is considered a spore confirmation test.

Large Scale Growth

Bacillus sp. BPG-8 was grown on TSA plates with PG on the lid to induce the cells. After good growth was obtained, the cells were inoculated in to 500 ml MSM medium containing 0.1 % PG. The cultures were incubated at 25°C and agitated at 150 rpm. After 18 hours incubation, 100 mls of the cell suspension was transferred into each of 4 flasks containing 400 ml of the same medium. The

flasks were incubated as before. At the end of this incubation time, the cells were harvested by centrifugation (10,000 rpm, 10 mins.). The pellet was then washed in 20 mM phosphate buffer. The pellet was stored in ice or frozen at -4°C until required.

Preparation of Cell Free Extracts

The pellet was suspended in a 20 mM phosphate buffer containing 1 mM EDTA, 1 mM 2ME and 15% glycerol. This buffer is henceforth referred to as phosphate buffer containing 15% glycerol. Approximately 2 grams of wet packed cells were suspended in about 3 ml of the buffer. The suspension was cooled in ice and sonicated for 3 minutes with inermittent gap of 30 seconds, sample and probe cooled in between (Braunsonic 2000, Canlab).

The disrupted cell suspension was centrifuged at 10,000 rpm for 10 minutes. This supernatant formed the source of the 'crude enzyme'.

Analytical Methods

Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

PG was determined by the method of Jayasankar and Bhat (1966). A standard curve was drawn and used to determine the PG concentration in the samples. To 1 ml aliquots (10-50 mg protein/ml) were added 2 ml 25% TCA and 2 ml 25% PDAB (in 99-100% glacial acetic acid) to estimate PG. The optical density of the coloured solution was measured at 534 nm.

All enzymes were assayed at room temperature using a Gilford Spectrophotometer (Oberlin, Ohio, U.S.A.). The reaction mixture contained in a total volume of 3 ml: 2.7 ml of phosphate buffer (pH 7.4), 0.05 ml NADPH (10 μmoles/ml), 0.1 ml PG (10 μmoles/ml), 0.2 ml enzyme. The reaction is initiated by the addition of the substrate PG. The disappearance of NADPH ws monitored on a Gilford Spectrophotometer (Oberlin, Ohio, U.S.A.) at 340 nm. From the plots obtained the initial velocity of the reaction was determined from the tangents to these plots. One enzyme unit was defined as the amount of enzyme that produces 1 μmole of product per min. per ml under standard assay conditions. Since the spectrophotometer used had a single beam, the endogenous activity was first measured without the substrate in the reaction mixture. Specific activity was defined as enzyme units per mg of protein. All the assays were run at room temperature.

Purification of the Enzyme

a) Ammonium Sulphate Fractionation

A saturated solution of ammonium sulphate was prepared by the addition of 72 g of enzyme grade ammonium sulphate in 100 ml water and adjusted with 1 N sodium chloride to pH 8. To the crude extract (25 ml) was added 10.8 ml of the ammonium sulphate saturated solution to give 30% saturation, stirred for 15 minutes and then centrifuged at 8,000 rpm for 10 min. To the supernatant solution (35 ml), 14 ml of saturated ammonium sulphate solution was added to obtain 45% saturation. The mixture was stirred for 15 minutes followed by centrifugation as before. To the 47 ml of supernatant solution was added 20 ml of

ammonium sulphate to give 60% saturation. Afteer equilibriating the suspension for 10 min. it was centrifuged. The supernatant solution 67 ml was decanted and 50 ml of ammonium sulphate was added to give 80% solution. The supernatant obtained was discarded. The precipitated proteins in various fractions were separately redissolved in minimum amounts of 20 mM phosphate buffer containing 15% glycerol. These samples were then dialyzed against two liters of the same buffer overnight. The enzyme activities in these samples were tested and recorded.

Concentration of Crude Extract by Ultra Filtration

Crude extract (15 ml) was filtered using a Diaflo ultra-filtration membrane (YM 10); purchased from Amicon Corporation Company, Danvers. The enzyme extract 15 ml was concentrated to 5 ml. The enzyme activity was determined before and after concentration as described before.

Gel Filtration and Molecular Weight Determination

Five grams of Sephadex G-150 was suspended in about 1 L of phosphate buffer containing 15% glycerol and allowed to swell for 48 hours at room temperature.

The column (2.5 cm x 26 cm) was initially coated with photoflo and dried for 2 hours at 37°C. The gel slurry was poured into the column by letting it slide along the side to avoid bubble formation. The packed column was set up in a cold room and equilibriated with buffer approximately 5 times the bed volume. Fractions (about 3 ml) were collected using an automatic fraction collector (LKB

2070 Ultrorac II, Fisher Scientific Company). To apply a sample, the excess buffer on top was removed with a Pasteur pipette. The column was standardized using proteins of known molecular weights. The standards were made at a concentration of 5 mg/ml and 2 ml samples of standards were run separately on the column. The protein concentration in the fractions was monitored by measuring absorbance at 280 nm. Also, enzyme activity in the fractions was carried out as previously described.

The proteins and their molecular weight used for the calibration of the column were: Cytochrome C (11,700), Soybean trypsin (20,000), Ovalbumin (44,000), Hemoglobin (64,000), Bovin serum albumin (68,000), Alcohol dehydrogenase (150,000), Catalase (230,000). The Kav values of the samples were determined using the following formula (Gel Filtration Theory and Practice, Pharmacia Fine Chemicals).

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

ve = elution volume
vt = bed volume
vo = void volume

<u>Protein Fractionation by Column Chromatography on a DEAE-Sephadex Column</u>

Ten grams DEAE-Sephadex A 50 were gently stirred into one liter of deionized water, and allowed to swell for 24 hours at room temperature. The suspension was filtered through Whatman No. 4 filter paper using a vacuum pump. The swollen gel was resuspended in 0.5 N hydrochloric acid and allowed to stand for 10 mins. for equilibration. After 3 changes in deionized water the exchanger was resuspended in 0.5 N sodium hydroxide for 10 mins. and again followed by washing with deionized water three times. The treated DEAE-Sephadex was resuspended in a minimum amount of deionized water followed by adjustment of the pH to 7 using 1 M potassium phosphate. Finally the gel was filtered and suspended in phosphate buffer containing 15% glycerol and stored at 4°C. This material was used for packing all columns required in the different purification experiments.

The column (2.5 cm x 9 cm) was initially coated with photoflo and dried for 2 hours at 37°C. The column was mounted on a stand and filled with the phosphate buffer containing 15% glycerol. The gel slurry was poured in along the side of the column reservoir to avoid any air bubbles. The packed column was set up in a cold room and washed with approximately 5 times the bed volume. To apply a sample, the excess buffer above the column material was removed using a Pasteur pipette. Fractions (3 ml) were collected using an automatic fraction collector. (LKB 2070 Ultrosac II, Fisher Scientific Company). The protein concentrations in the fractions were measured at 280 nm. The enzyme activity was completely eluted from column with (0.02 M to 0.8 M) potassium phosphate buffer (pH 7.4). Enzyme activities PGR and RH were measured as described previously.

Batch Purification of PG-reductase

DEAE-Sephadex A-50 was prepared as before. To 30 ml of the crude extract 5.4 g (wet weight) DEAE-Sephadex was added and stirred for 3 hours at 0-5°C in a cold room. The suspension was centrifuged and the pellet was resuspended in 0.8 M phosphate buffer (10 ml) containing 15% glycerol. To release the bound proteins, the solution was stirred for one hour and centrifuged. The supernatant (9 ml) was saved for enzyme activity and pellet was once again resuspended in 10 ml of 0.8 M phosphate buffer containing 15% glycerol. The suspension was centrifuged as before. The supernatant solution (8 ml) was saved and the pellet was resuspended in another 10 ml of 0.8 M phosphate buffer. After stirring for an hour the suspension was centrifuged. The pellet obtained was discarded and the supernatant solution (11 ml) was pooled together to give a final volume of 28 ml. The enzyme activity in the pooled extract was determined using the standard assay procedure.

RESULTS

Identification of Microorganisms

Electron and light microscopic examination of cells revealed non-flagellated rods. The spore staining of very old cultures showed very few spores and these were barely discernable under a light microscope. In the spore confirmation test which was described in the Methods and Materials Section, (Biochemical tests) the positive control and the unknown grew after heat treatment while the negative control failed to grow which confirmed that the unknown was a sporeformer. Table 1 shows the various characteristics that tentatively identify the unknown bacterium with the genus Bacillus.

Several biochemical tests (Table 2) were carried out to identify unknown isolates. These tests failed to clearly identify the bacterium to species level. Based on these observations and according to Bergey's manual of Determination of Bacteriology (Eighth Edition, 1974) the organism was tentatively identified as Bacillus sp. BPG-8.

Growth Requirements of BPG-8

The effect of pH, temperature and substrate concentration on growth were studied by modifying the physical and chemical environment of MSM medium containing PG. The aim of this experiment was to determine the optimal conditions required for the growth of BPG-8 as well as the stability of the substrate under these conditions.

Table 1 Characteristics of the Isolate

Test	Isolate	Bacillus sp.[a]	E. Coli[b]
Motility	-	-	+
Gram Stain	+	+	-
Acid-Fast Stain	-	-	-
Spore Stain	+	+	-
Oxygen (Anaerobicar)	Aerobic	Aerobic Facultative	Aerobic Facultative

+ = positive

- = negative

a - positive control

b - negative control

Table 2 Biochemical Tests of Bacillus sp. BPG-8

Characteristic	BPG-8	E. Coli
Colony Descriptions	Circular, raised, entire off white, opague.	
Cell Size	Length 2 to 2.5 M Width 1.0 to 1.2 M	
Motility	-	+
Temperatures:		
For Growth		
Maximum	37°C	
Minimum	20°C	
Optimum	25°C	
NO_3 - - NO_2	-	-
Indole	-	-
MR	-	-
VP	-	-
Simmon's Citrate	+	-
OF Glucose	Acid	-
OF	Acid	-
Decomposition of Casein	-	-
Gelatin	+	-
Growth in		
TSB + 5% - NACl	-	-
TSB + 0.02% Azide	-	-
TSB + 0.001% Lysozyme	-	-
Growth on		
Arabinose	-	-
Xylose	-	-
Mannitol	-	-
Glucose	+	-
Sabouarod dextrose broth	+	-

MR Methyl red test
VP Voges Proskayer

Growth of BPG-8 on PG

BPG-8 was grown on TSA plates with PG placed on the lid to induce the enzyme systems. Good growth of the cells was obtained on the plates after 48 hours at 25°C. Gultures grown on TSA plates were used to inoculate 100 ml MSM medium containing 0.001% yeast extract and 0.1% PG. Dark Brown Erlenmeyer fflasks (250 ml) were used to prevent photochemical decomposition of the substrate, PG. The cultures were incubated at 25°C and agitated at 150 rpm. After 18 hours, 10 ml of the inoculum was inoculated into 70 ml of fresh MSM. The flasks were incubated as before. The growth was measured hourly by determining the turbidity at 600 nm in a spectrophotometer. The samples were withdrawn at one hour intervals.

Figure 6 illustrates the growth cycle of the organism at 25°C in MSM medium containing 0.1% PG. The stationary phase was reached in about 14 to 16 hours. This experiment was used to obtain rough idea about the time of incubation needed for large scale growth of BPG-8 under similar conditions.

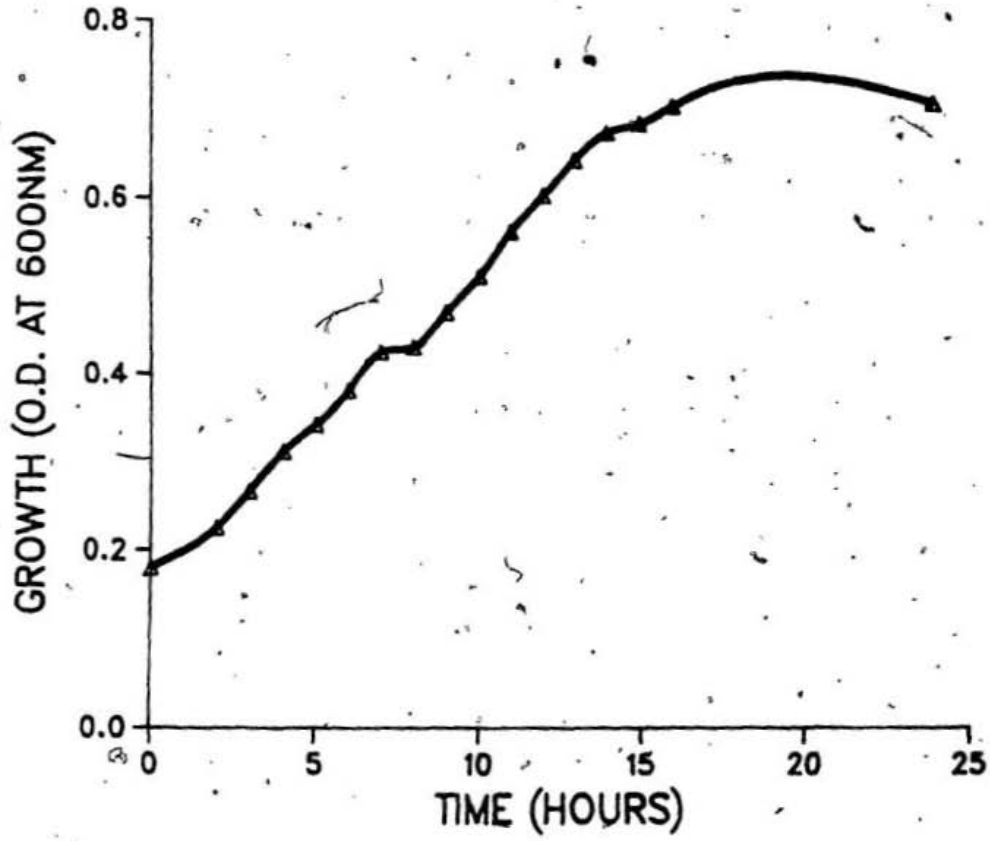
Phloroglucinol Utilization by Resting Cells of Bacillus sp. BPG-8

Two flasks containing 500 ml MSM medium were inoculated with cells grown on plates containing PG. They were incubated for 18 hours at 25°C as before. They were harvested by centrifugation, washed three times with sterile 20 mM phosphate buffer containing 1 mM ethylenediaminetraacetic acid (EDTA), and 1 mM 2-Mercaptoethanol (2ME) pH 7.4. This buffer is henceforth referred to

Figure 6 - Growth of Bacillus sp. BPG-8 on PG

Erlenmeyer flasks (250 ml) containing 70 ml MSM with 0.1% PG and 0.001% Y.E. (pH 5.5) were inoculated with 10 ml of freshly grown (18 hrs old) cells and incubated at 25°C on a shaker (Psychrotherm, New Brunswick) and agitated 150 rpm. Samples (1ml) were withdrawn at one hour intervals and growth measured by optical density readings measured in a Gilford Spectrophotometer. Flask without PG showed no growth.

FIGURE 6



as phosphate buffer. The washed cells were resuspended in 5 ml of the same buffer. Flasks containing 120 ml fresh MSM medium were inoculated with the 5 ml washed cell suspension. The suspension gave an optical density reading of 0.6 at 600 nm. (132 mg total protein per flask). The flask was incubated at 25°C at 150 rpm in a psychrotherm and 1 ml aliquots were removed at hourly intevals. The cells were removed by centrifugation and the supernatent was retained and used for estimating PG concentration according to the method of Jayasankar and Bhat (1965).

The rate of PG utilization was studied using this resting cell suspension. The results of the PG concentration analysis in the medium and the time sequence revealed that the substrate was utilized without any lag (Figure 7). The complete disappearance of 0.1% PG required 5 hours.

Growth of BPG-8 on Various Aromatic Substrates

Bacillus sp. BPG-8 was grown on TSA plates. Duplicate flasks each containing 40 ml MSM medium were inoculated with 1 ml cell suspensions prepared from the growth on plates and various aromatic substrates were added to the flasks. The flasks were incubated and change in absorbance at 600 nm was noted. Control flasks without added cells were also set up to determine chemical changes. The flasks were incubated at 25°C with shaking at 150 rpm. The concentrations of the substrates used was 0.1%.

Table 3 shows results from an experiment to determine if Bacillus sp. BPG-8 could grow on other aromatic substrates other than PG. It is clear that BPG-8 does have the ability to grow on these substrates.

Table 3 Growth on Various Aromatic Substrates

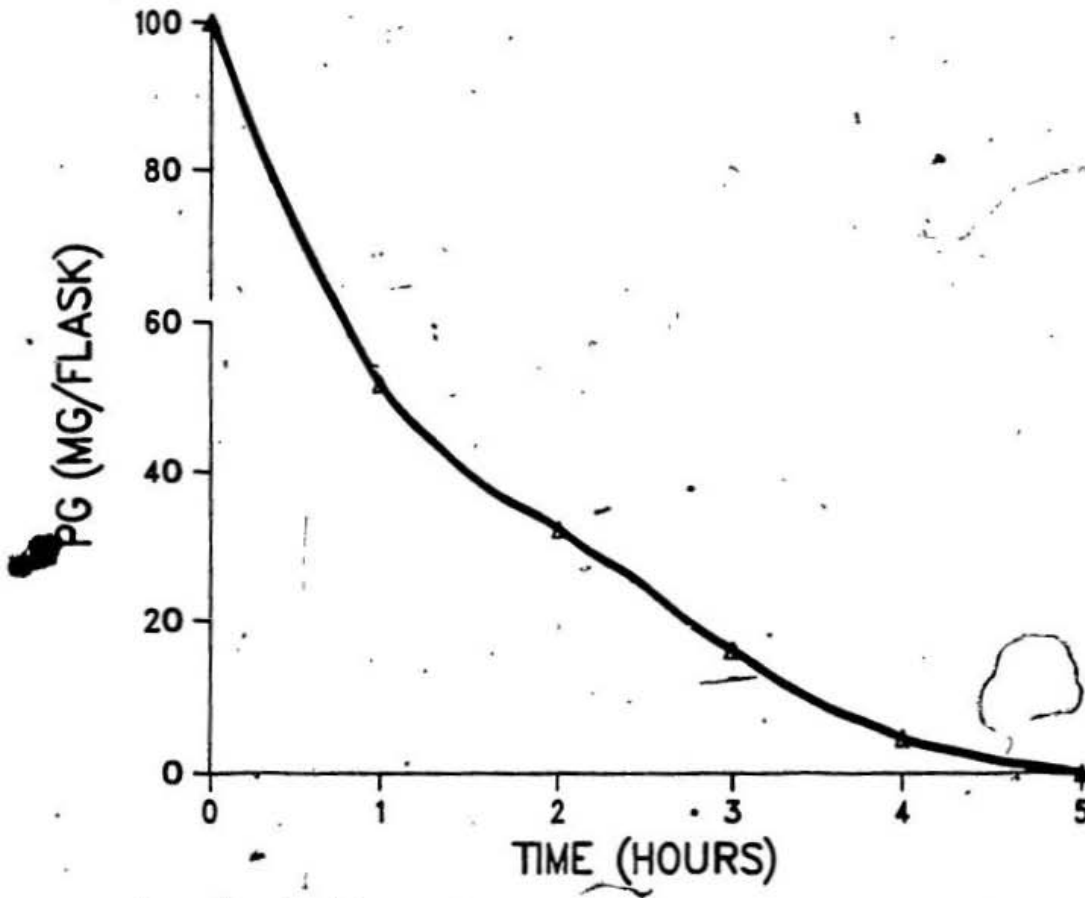
Substrate	Growth
Phloroglucinol	+
Resorcinol	-
Catechol	-
Phenol	-
Pyrogallol	+
Orcinol	+
Gallic Acid	-
1, 2, 4 Benzenetriol	-
2, 4 - Dihydroxy Benzoic Acid	-
2, 5 - Dihydroxy Benzoic Acid	-
2, 6 - Dihydroxy Benzoic Acid	-

The cultures were grown as described in Materials and Methods. The substrate concentrations used were 0.1%.

Figure 7 - Phloroglucinol Utilization by Resting Cells Suspensions

Erlenmeyer flask (250 ml) containing 120 ml MSM with 0.001% Y.E. 0.1% PG (pH 5.5) were inoculated with 5 ml washed cell suspension. This flask containing 125 ml cell suspension (5.3 mg/ml protein) were incubated at 25°C on a shaker agitated at 150 rpm. Samples (1 ml) were withdrawn at one hour interval and centrifuged (1000 rpm, 10 mins). The clear supernatant solution was used to determine the residual PG using the method of Jayasankar and Bhat (1966).

FIGURE 7



Effect of Substrate Concentration on the Growth of BPG-8

The optimal PG concentration was determined by inoculating 1 ml cell suspensions into flasks containing MSM (pH 5.5) with 0.001% YE. PG concentrations in the flasks varied between 0.0 to 0.4%. For each concentration of PG, duplicate flasks were inoculated and incubated at 25°C in a Psychrotherm, with shaking at 150 rpm. The organism's growth was determined by measuring turbidity at 600 nm in a Spectrophotometer.

The optimal substrate concentration was determined to be 0.1% as seen in Figure 8. PG concentrations in excess of 1% appear to be inhibitory to the growth. This is not surprising since PG has been known to be antibacterial agent.

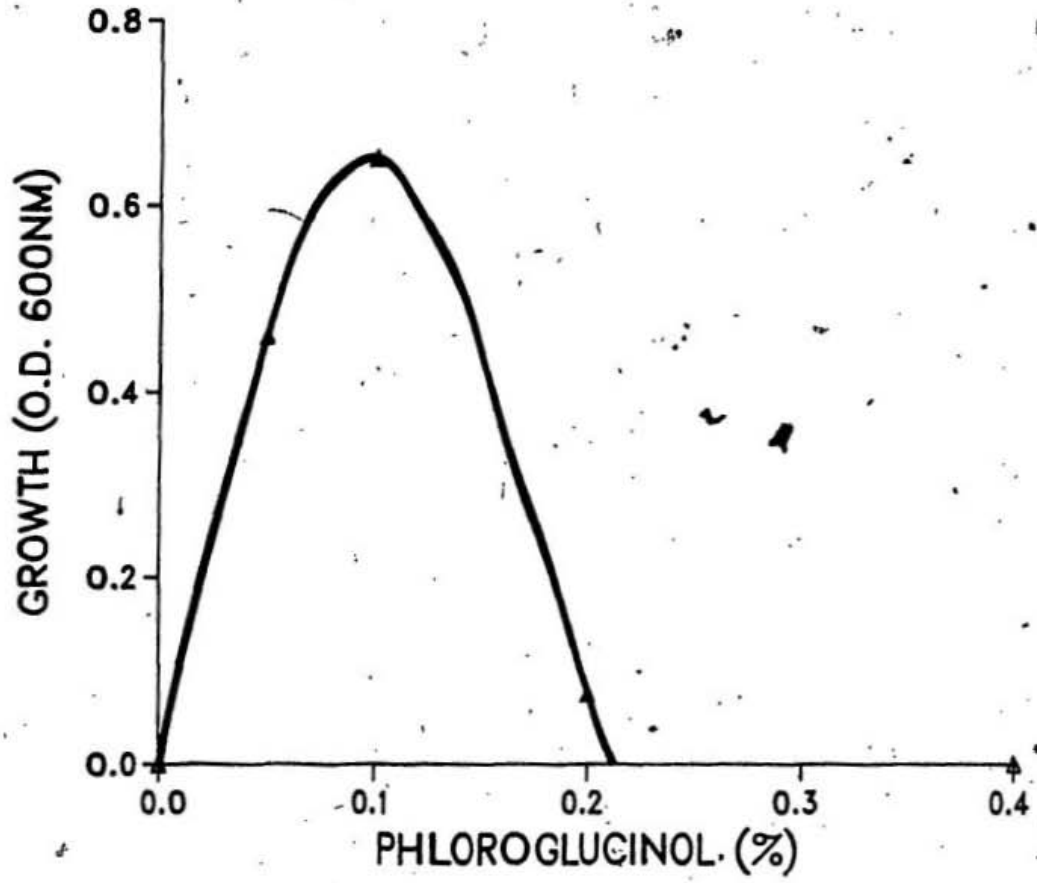
Optimal Temperature for the Growth of Bacillus sp. BPG-8

The incubation temperatures ranged from 0° to 40°C. For this experiment cells were induced on TSA plates with PG on the lid. Cell suspensions were prepared from these induced cells in a normal saline solution. Duplicate flasks containing 40 ml MSM medium with 0.1% PG and 0.001% YE (pH 5.5) were inoculated with 1 ml of the cell suspension. The flasks were incubated at various temperatures for 18 hours and the growth was recorded by measuring the turbidity (absorbance at 600 nm). The flasks were incubated in a Dubenoff metabolic shaking incubator (GCA/Precision Scientific) at 10° to 40 °C at 40 rpm. For 0° and 5°C temperatures, a refrigerated circulating water bath (Masterline Forma Scientific Company) was used.

Figure 8 - Effect of Substrate Concentration on the Growth of BPG-8

Erlenmeyer flasks (250 ml) contained 40 ml MSM with 0.001% YE (pH 5.5). The concentration of PG varied from 0 - 0.4%. The flasks were inoculated with 1 ml suspension of freshly grown cells and incubated at 25°C in a shaker (Psychrotherm) agitated at 150 rpm. The growth in each flask was determined by optical density method after 18 hours.

FIGURE 8



The optimal temperature for growth was found to be 25°C while the maximum was 37°C. The minimal temperature ws 20°C as shown in Figure 9. This means that the growth in soils in Newfoundland is expected to slow due to relatively low temperatures in this region.

Optimal pH for Growth

Replicate Erlenmeyer flasks containing 40 ml MSM with 0.1% PG were used for this experiment. The pH of the medium in the flasks varied between 5 and 8. For pH adjustment either 1 N hydrochloric acid or 1 N sodium hydroxide was employed. The flasks were inoculated with 1 ml MSM bacterial suspension prepared from freshly grown cells on TSA plates. The flasks were incubated at 25°C in a psychrotherm and agitated at 150 rpm. The growth was measured spectrophotometrically by absorption at 600 nm at 24 and 48 hours.

Figure 10 shows that the optimal pH for the growth is 5.5. Brown[redacted]e medium was observed at pH 6.5 to 8.0 indicating the breakdown of PG into quinoes and their polymers.

Purification of phloroglucinol reductase

Ammonium Sulfate Fractionation of the Crude Extract

Table 4 shows the results of ammonium sulfate fractionation of curde extract solution. As is evident from the table, the PGR activity precipitated between 65% and 80% ammonium sulphate. However the ammonium sulfate appeared to inhibit the enzyme activity. About 87% of PGR and 93% of RH activity was lost during ammonium sulfate treatment.

Figure 9 - The Effect of Temperature of Incubation on the Growth of BPG-8

Erlenmeyer flasks (250 ml) contained 40 ml MSM with 0.1% PG and 0.001% YE (pH 5.5). They were inoculated with 1 ml cell suspension (in physiological saline) and incubated at various temperatures indicated. For 10-40°C temperature range shaker. Water-baths were used and for 0° and 5 °C temperatures, a refrigerated circulating water bath was used. The growth was determined by measuring the optical density at 600 nm using a Spectrophotometer after 18 hrs.

FIGURE 9

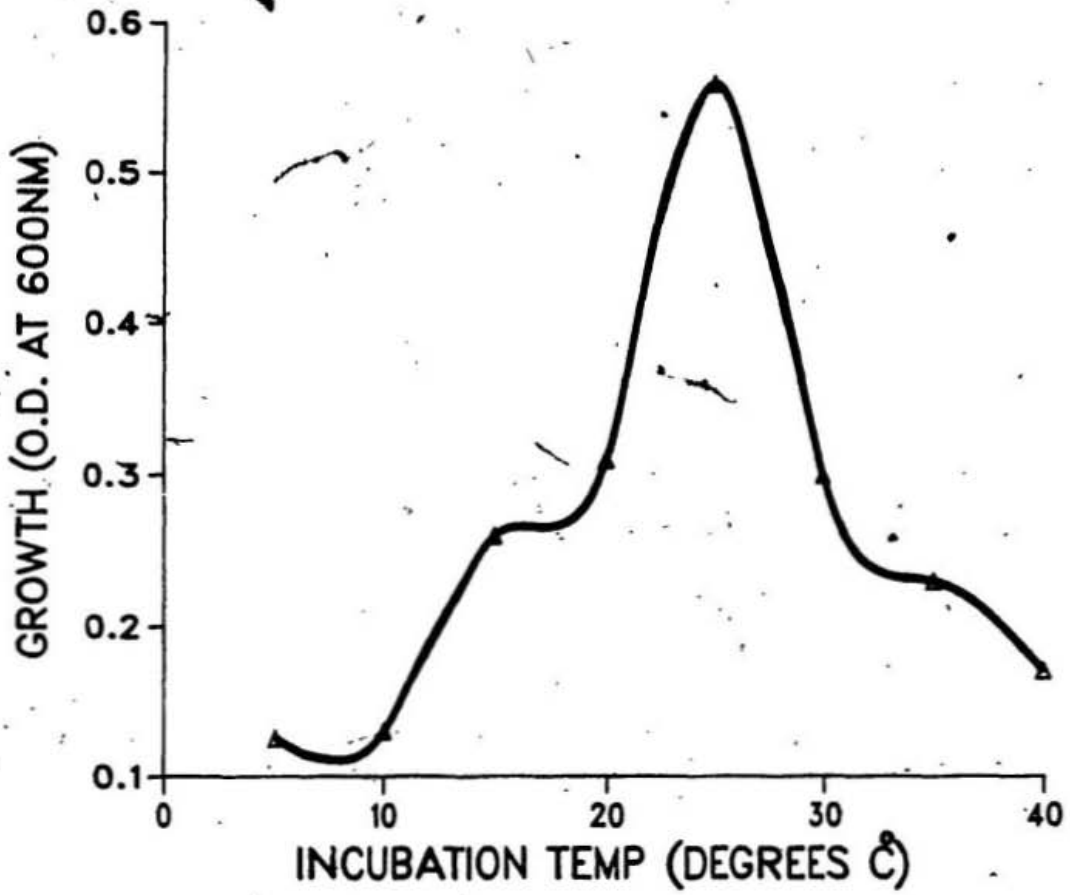

0.6
0.5
0.4
0.3
0.2
0.1
GROWTH (O.D. AT 600NM)
0
10
20
30
40
INCUBATION TEMP (DEGREES C)

Figure 10 - Effect of pH on the Growth of BPG-8

Erlenmeyer flasks (250 ml) contained 40 ml MSM with 0.1% PG and 0.001% Y.E. The pH of the medium in each flask was adjusted to a given value using either 1 N HCl or 1 N NaOH. The flasks were inoculated with 1 ml suspension of freshly grown cells and incubated at 25°C in a shaker (Psychrotherm, New Brunswick) and agitated at 150 rpm. The growth in each flask was determined by measuring the optical density at 600 nm using a Gilford Spectrophotometer after about 18 hours.

FIGURE 10

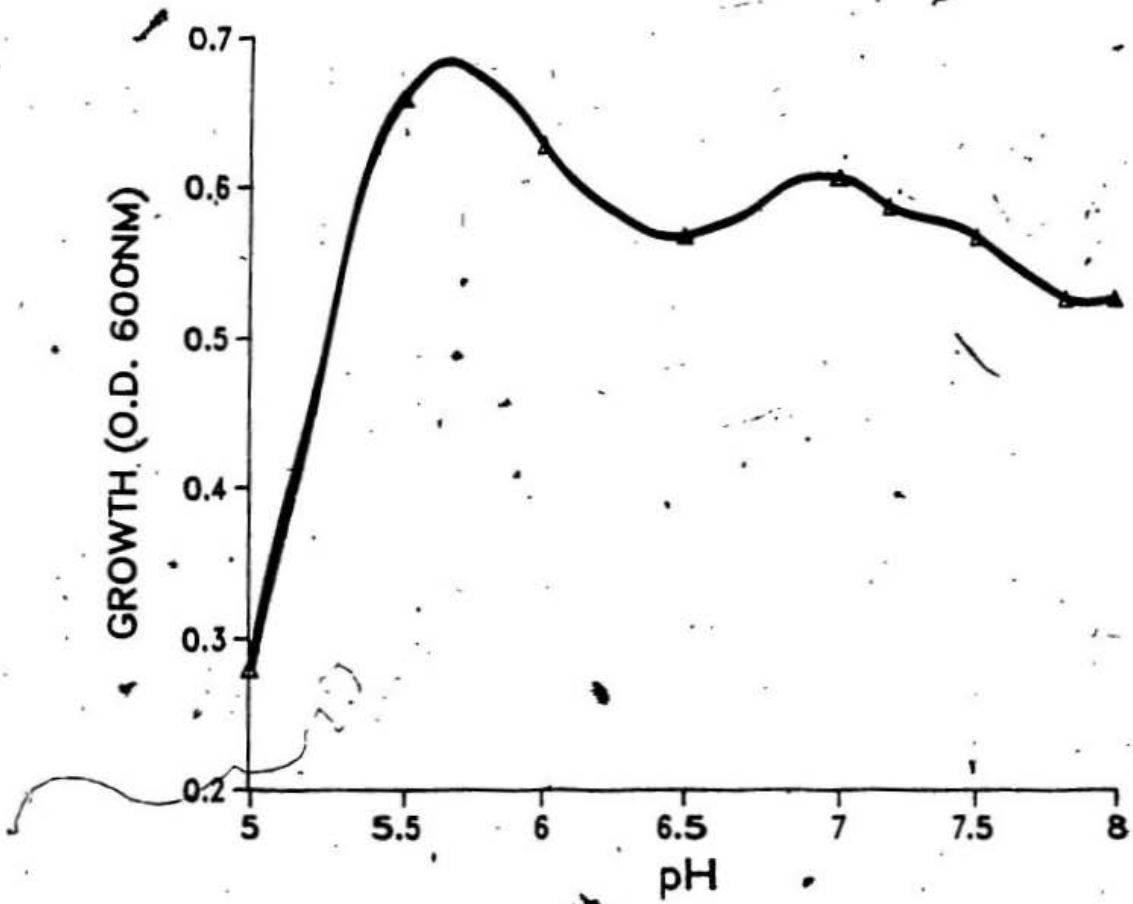


Table 4 - Ammonium Sulfate Fractionation of the Crude Extract

Ammonium Sulphate Fraction	Volume (ml)	Protein (mg)	PG-reductase			R-hydroxylase		
			Total Units	Specific Activity	% Recovery	Total Units	Specific Activity	% Recovery
Crude Extract	25	120	3.3	0.03	100	0.91	0.01	100
30%	1.3	2.3	0.0	0.0	0.0	0.0	0.0	0.0
50%	1.4	3.2	0.03	0.01	1	0.0	0.0	0.0
65%	2.2	16.5	0.24	0.01	7.1	0.03	0.0	3.3
80%	2.1	14.9	0.15	0.01	4.6	0.03	0.0	3.3

Enzyme unit is defined as the amount of extract required to produce 1 μmole of product per min. per ml. Specific activity is defined as a number of enzyme units per mg protein.

Enzyme Concentration by Ultra Filtration

The results obtained after ultra filtrtion through amicon filter showed that PGR activity was increased three-fold and RH activity was increased four fold after the reduction of the volume from 15 ml to 5 ml. The results are summarized in Table 5.

Gel Filtration on Sephadex G-150 Column and Molecular Weight Determination

Figure 11 shows the protein profile and the distribution of the enzyme activities in different fractions obtained on a Sephadex G-150 column as described earlier. Two large protein peaks were observable. The PGR and RH activities appeared in the same fractions. However the Peak-tubes for these enzyme activities were different. Table 6 summarizes the results obtained.

The molecular weight of the PGR was determined by gel filtration on a calibrated Sephadex G-150 column. Several proteins of known molecular weights were used to obtain a standard plot (Figure 12). The molecular weight of PGR was found to be 155,000 by this method.

Column Chromatography on a DEAE-Sephadex Column

Figure 13 shows the protein profile and the enzyme activities obtained on a DEAE Sephadex A-50 Column as described earlier. The PGR activity appeared in two large peaks. In contrast no RH activity was associated in these eluted peaks. Table 7 summarizes the results obtained.

Batch Purification of PGR

Table 5 - The Concentration of Crude Extract by UIltrafiltration

Treatment	Volume	Total Protein (mg)	PG-reductase			R-hydroxylase		
			Total Units	Specific Activity	Recovery (%)	Total Units	Specific Activity	Recovery (%)
Crude Extract	15 ml	90	0.36	0.00	33%	0.18	0.00	25%
Membrane Filtration	5 ml	39	0.36	0.01	100%	0.24	0.01	100%

Crude extract (15 ml) was concentrated to (5 ml) using 10 µm Amicon membrane filter. PG-reductase and R-hydroxylase activity was determined as described in Materials and Methods.

Figure 11 - Gel Filtration on a Sephadex G-150 Column

A concentrated sample (55 mg of protein) was applied on a column (2.5 cm x 26 cm) containing Sephadex G-150. The column was washed with 20 M phosphate buffer containing 15% glycerol (pH 7.4). The protein concentration in the fractions was determined spectrophotometrically at 280 nm. The PG-reductase (12 mg) and R-hydroxylase (9 mg) activity in the fractions were determined as described in the Materials and Methods.

(△-△) Protein

(●-●) PGR, enzyme units/ml

(■-■) RH, enzyme units/ml

FIGURE 11

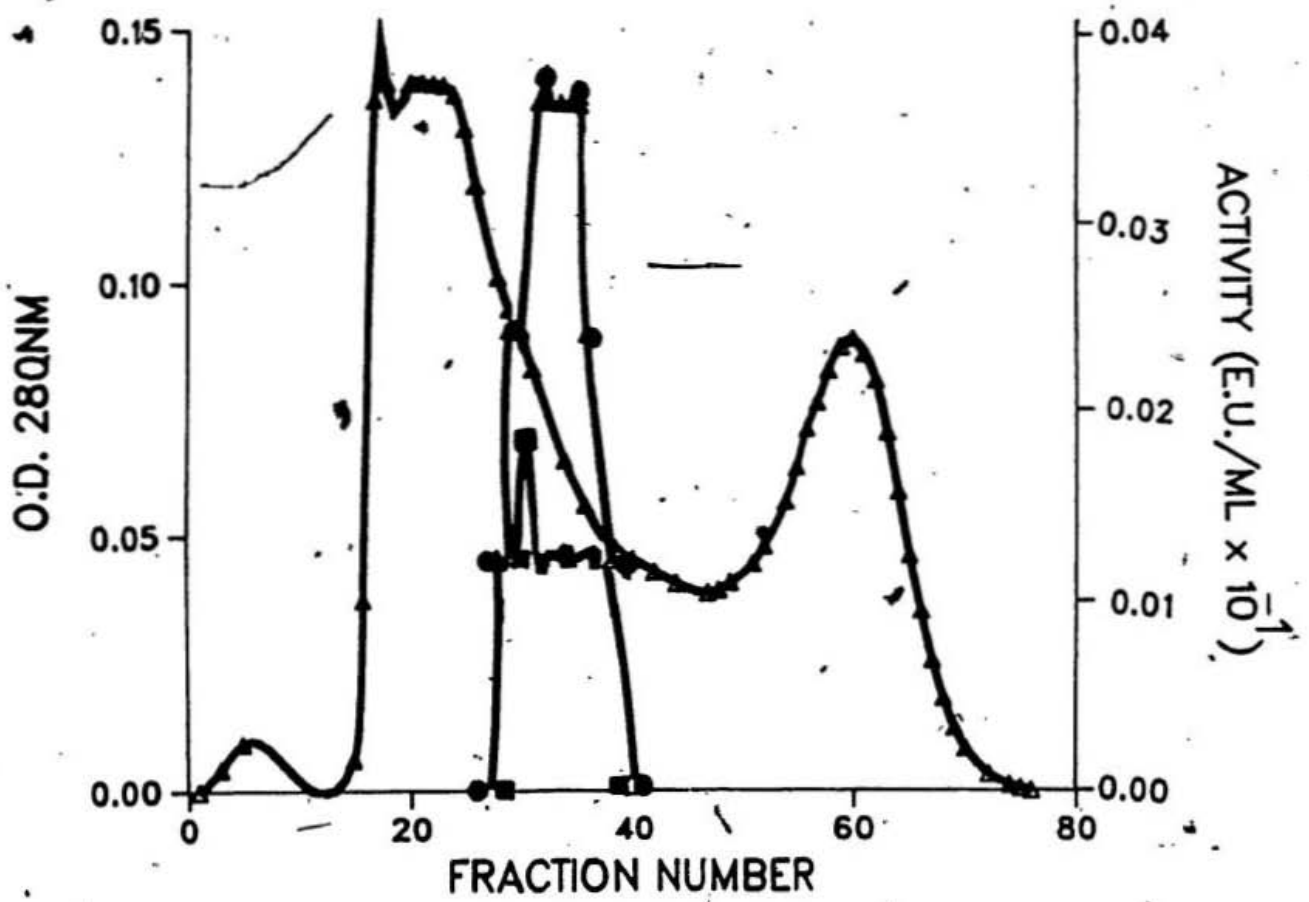

0.15
0.10
0.05
0.00
O.D. 280NM
0.04
0.03
0.02
0.01
0.00
ACTIVITY (E.U./ML x 10⁻¹)
0
20
40
60
80
FRACTION NUMBER

Table 6 - Purification of PG-reductasé on Sephadex G-150 Columns

Steps	Total Volume (ml)	Total Protein (mg)	Total Units		SP Activity		% Recovery		Purification Fold	
			PGR[a]	RH[b]	PGR[a]	RH[b]	PGR[a]	RH[b]	PGR[a]	RH[b]
Crude Extract	16	96	1.34	0.96	0.02	0.01	100	100	1	1
Ultra Filtration	7	55	2.52	1.68	0.05	0.03	188	175	2.5	3
Sephadex G-150	14	10	0.3	0.12	0.03	0.02	22	13	1.5	2

PGR[a] = Phloroglucinol reductase.

RH[b] = Resorcinol hydroxylase.

Figure 12 - Standard Plot for Molecular Weight Determination Using Sephadex G-150 Column

A column (2.5 cm x 26 cm) packed with Sephadex G-150 was used for molecular weight determination. The standard proteins used for the calibration of the column were Cytochrome C (11,700), Soybean trypsin (20,100), Ovalbumin (45,000), Hemoglobin (64,000), Bovine serum albumin (68,000), Alcohol dehydrogenase (150,000) and Catalase (230,000). Linear regression analysis was performed to obtain the plot.

A = Cytochrome C

B = Soybean trypsin

C = Ovalbumin

D = Hemoglobin

E = Bovin serum albumin

F = Alcohol dehydrogenase

G = Catalase

FIGURE 12

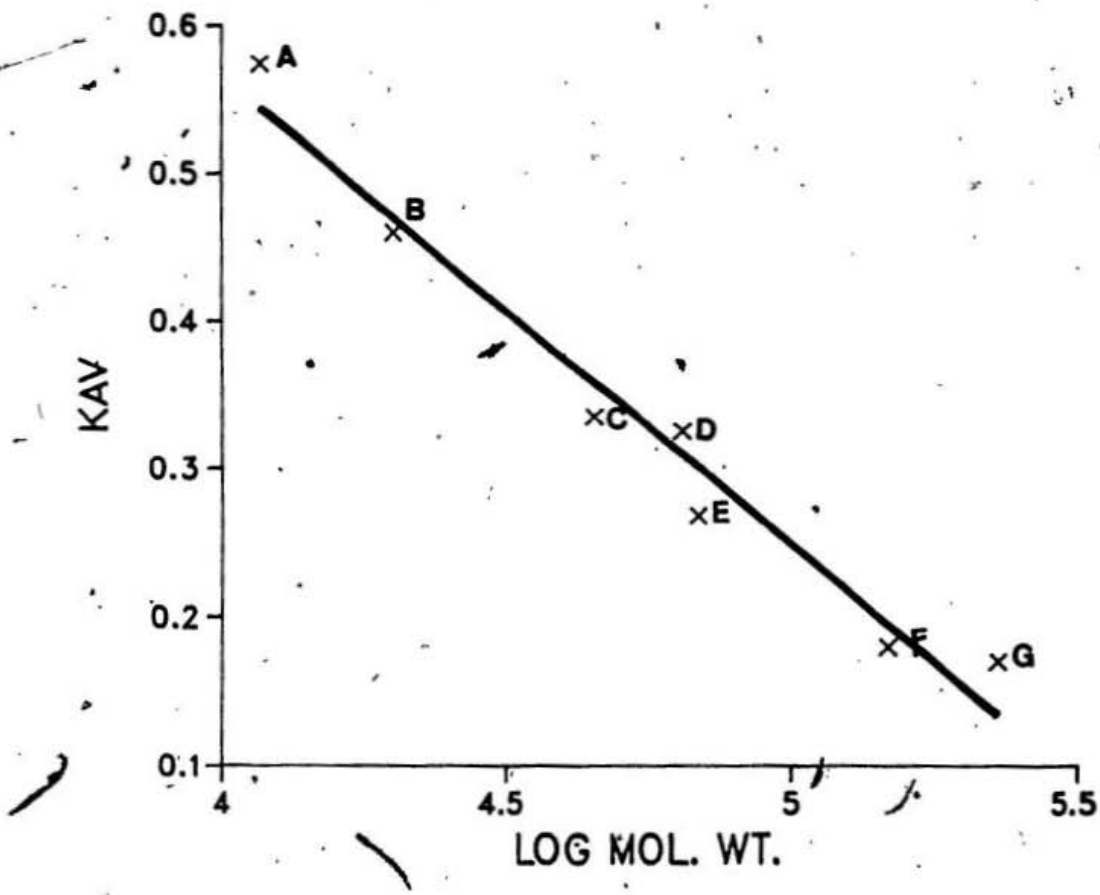


Figure 13 - Column Chromatography on a DEAE-Sphadex A-50 Column

A DEAE-Sephadex A-50 column (2.5 cm x 9 cm) was washed with 20 mM phosphate buffer containing 15% glycerol (pH 5.5). A crude extract (163 mg) was applied on a column. The column was washed with 20 mM phosphate buffer containing 15% glycerol pH (7.4). The protein concentration in the fractions was determined spectrophotometrically at 280 nm. The enzyme activity was completely eluted from the column with (0.02 M to 0.8 M) potassium phosphate buffer containing 15% glycerol pH (7.4). The PG-reductase activity in the fractions was determined as described in the Materials and Methods.

▲-▲ - Protein

●-● - PGR, enzyme units/ml

FIGURE 13

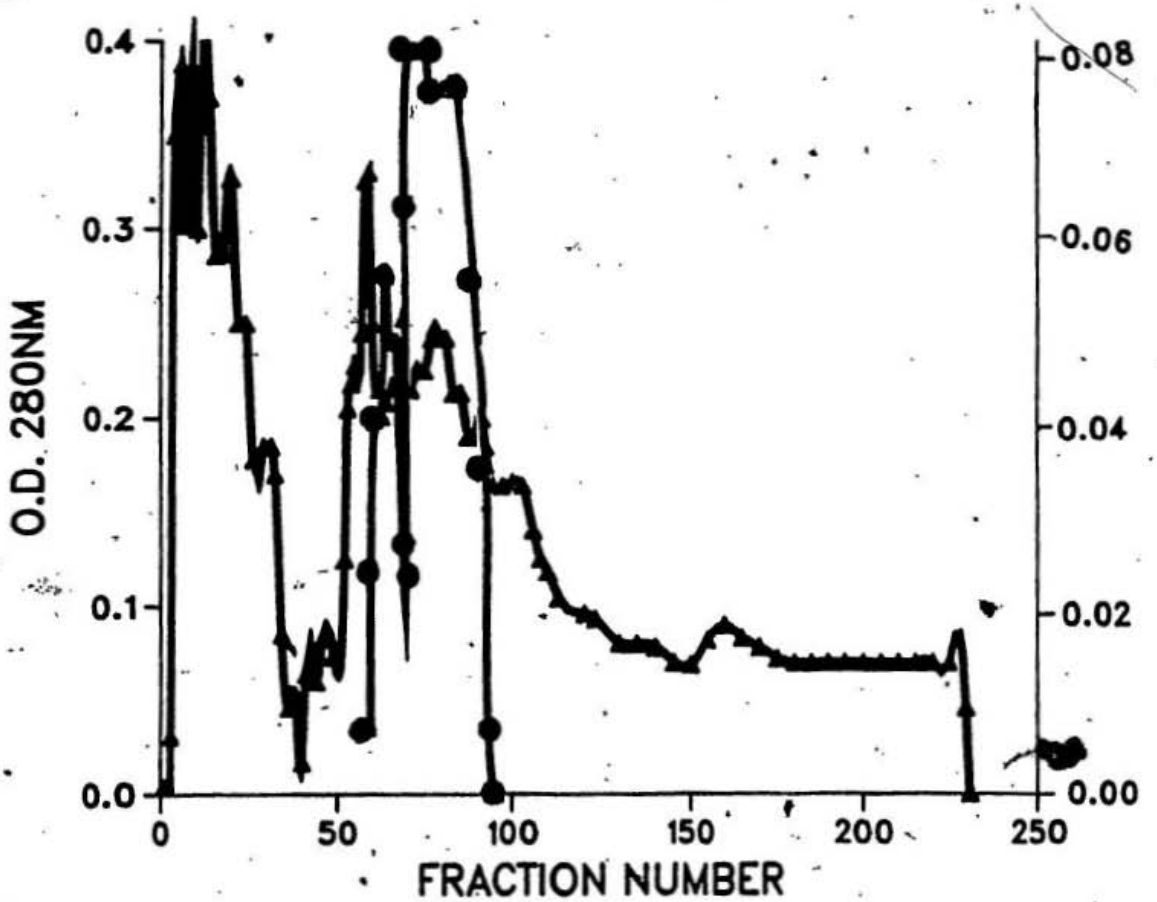

0.4
0.3
0.2
0.1
0.0
O.D. 280NM
0.08
0.06
0.04
0.02
0.00
ACTIVITY (E.U./ML)
0
50
100
150
200
250
FRACTION NUMBER

Table 7 - Purification of PG-reductase on DEAE Sephadex A-50 Column

Steps	Total Volume (ml)	Total Protein (mg)	Total Units		Sp Activity		% Recovery		Purification Fold	
			PGR[a]	RH[b]	PGR[a]	RH[b]	PGR[a]	RH[b]	PGR[a]	RH[b]
Crude Extract	34	163	18.5	4.5	0.12	0.1	100	100	1	1
DEAE Sephadex Column										
PK. I	31	19	1.1	0	0.05	0	6	0	2.4	0
PK. II	95	67	4.6	0	0.06	0	24	0	2.0	0

Enzyme unit is defined as the amount of extract required to produce 1 µmole of product per min. per ml.

Specific activity is defined as a number of enzyme units per mg protein.

PGR[a] = phloroglucinol reductase.

RH[b] = resorcinol hydroxylase

Attempts were made to separate the PGR and RH activity by batch purification using DEAE-Sephadex A-50 anion exchanger. The bound protein was eluted with 0.8 M phosphate buffer (pH 7.4) as described in Materials and Methods.

Table 8 summarizes the results obtained. About 50% of the PGR activity was bound to the ion exchangers and was recoverable by elution with the higher concentration of the phosphate buffer. In contrast no RH activity was associated in this eluted fraction.

Properties of PG-reductase and R-hydroxylase

Effect of enzyme and substrate concentration

Varying concentrations of the crude enzyme were tested to determine the rate of PGR using the standard assay system.

Figure 14 depicts the effect of increasing volume of extract on the PGR activity. The PGR activity increased with increasing volume of crude extract up to 1 ml per assay mixture. Further increases in the amount of crude extract did not increase the PGR activity. The linear portion of the plot was used to determine the appropriate concentration of the extract to be used in the subsequent experiment.

Keeping the other conditions constant, PGR activity was determined at various concentrations of the substrate, PG. The Km was determined by Lineweaver Burke plot and straight line was obtained by regression analysis.

Table 8 - Batch Purification of PG-reductase

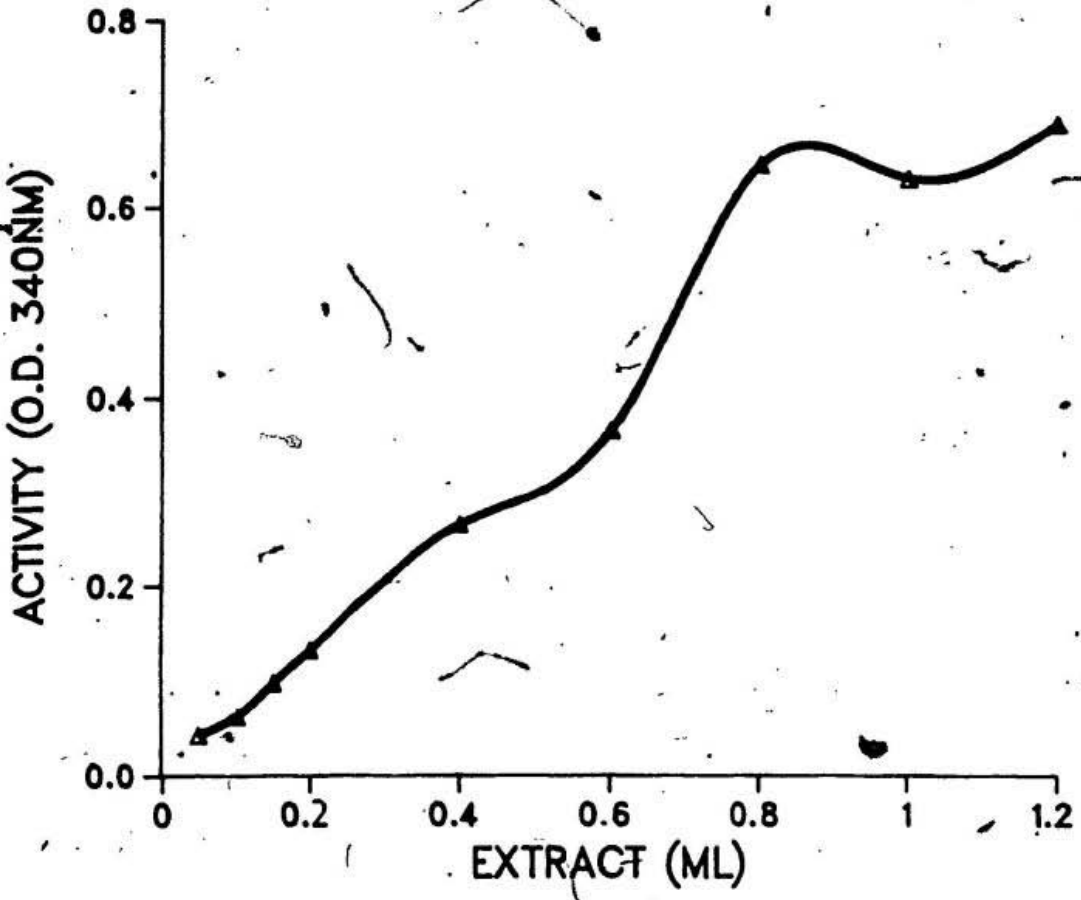
Step	Volume	Total Protein (mg)	PG-reductase Total Units	PG-reductase Specific Activity	PG-reductase Recovery (%)	R-hydroxylase Total Units	R-hydroxylase Specific Activity	R-hydroxylase Recovery (%)
Crude Extract	30 ml	108	1.98	0.018	100	0.72	0.01	100
Unbound Activity	24 ml	10.08	0.06	0.01	3.0	0	0	0
Bound Activity	24 ml	25.3	1.01	0.04	51	0	0	0

For details see Materials and Methods.

Figure 14 - Effect of Enzyme Concentration on the PG-reductase Activity

The reaction mixture in a total volume of 3.0 contained reduced $NADP^+$(0.5 μmole), PG(1 μmole) and varying concentrations of enzyme as indicated. The oxidation of reduced $NADP^+$ was monitored at 340 nm using a Gilford Spectrophotometer. The reaction was initiated by adding PG to the reaction mixture. The reaction was usually allowed to run between 3-10 min. Initial velocity of the reaction was determined by drawing tangents to the lines obtained.

FIGURE 14



The effect of PG concentrations on the rate of NADPH oxidation is shown in Figure 15 from the Lineweaver-Burk Plot (1934). The Km for PG was calculated to be 2×10^{-4}M. Substrate inhibition was observed at concentration higher than 4×10^{-3} M PG.

Effect of metal ions, NaCl, KCl

The following metal salts were used for this experiment. Zinc sulfate, nickel sulfate, ferrous chloride, ferrous sulfate, magnanese chloride, magnesium sulfate, calcium chloride and ferrous ammonium sulphate. Enzyme samples (1 ml) were incubated separately with different metal ions (3 μmoles/ml conc.) at 0°C for 10 minutes. To determine the PGR activity, 0.2 ml aliquots of the extract which were incubated with metal ions were used in the standard enzyme assay. An enzyme sample without added meltal ions was used as a control.

The effect of various metal ions on PGR activities is shown in Table 9. Zinc sulphate, ferrous chloride, manganese chloride, calcium chloride and ferrous ammonium sulphate inhibited more than 70% of the PGR activity at a concentration of 3 μM. Nickel sulfate and magnesium sulfate had a slightly lower inhibitory effect on PGR activity. Calcium chloride was found to be a potent inhibitor and the inhibition was about 95% of the original activity of PGR.

Freshly prepared crude extract of BPG-8 grown on PG was tested for the PGR and RH activities in the presence and absence of varying concentrations of sodium chloride and potassium chloride. Standard assay conditions were used except for the presence of the salts added to the reaction mixtures.

Figure 15 - Double Reciprocal Plots Showing the Effect of Substrate (PG) Concentration on the Reaction Rate

Reaction mixture (3 ml) contained 2.7 ml of 0.1 M phosphate buffer (pH 7.4), 0.05 ml NADPH (10 μmoles/ml), 0.2 ml enzyme (5 mg/ml) and phloroglucinol as indicated. The oxidation of NADPH was monitored at 340 nm.

FIGURE 15

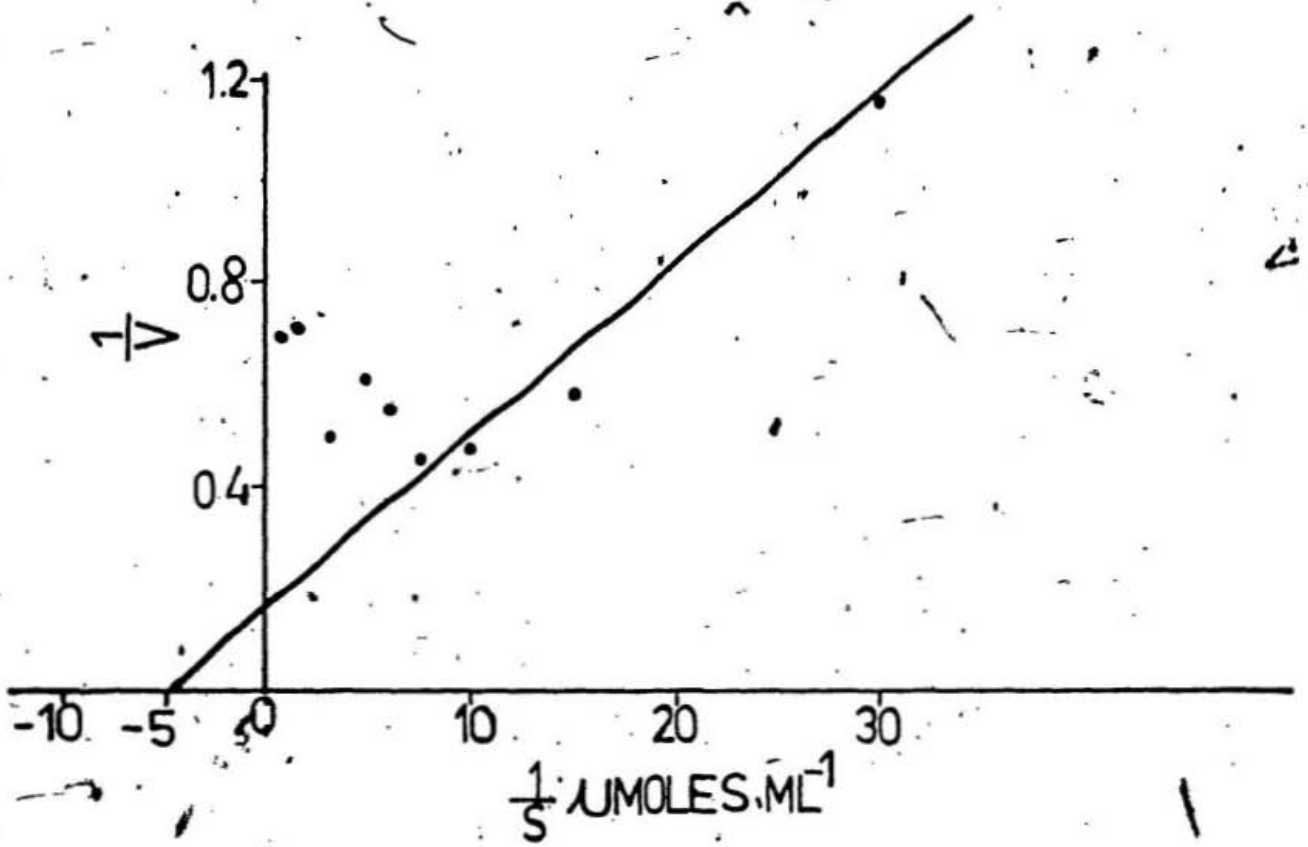


Table 9 - The Effect of Metal Ions on the PG-reductase Activity

Additions	% Inhibition
None	100
Zinc sulphate	70
Nickel sulphate	55
Ferrous chloride	78
Ferrous sulphate	66
Manganese chloride	78
Magnesium sulfate	55
Calcium chloride	95
Ferrous ammonium sulphate	78

The crude dialysed enzyme (1 ml) and metal ions (3 µmoles) were incubated in ice (0-4°C) for 10 minutes, and then assayed for PG-reductase activity according to the standard assay. The PG-reductase activity in the absence of added metal ion was taken as 100%.

Table 10 shows the effect of varying concentrations of sodium chloride on PGR and RH activites. The PGR activity decreased with increasing concentration of sodium chloride (between 50 - 400 μmoles per 3 ml assay mixture). The inhibition ranged between 12 and 87 percent. There was a rapid loss of RH. For example, only 25% of RH activity was retained at 50 μ moles/assay. With further increases in concentration, no RH activity was observed.

Table 11 shows the effect of increasing concentrations of potassium chloride (50 to 400 μmoles/assay) on PGR and RH activities. The loss of PGR acticity increased with increasing concentration of potassium chloride. The loss of RH was more dramatic. For example only 11 percent of the original activity remained in the presence of 50 μmoles of potassium chloride with further increases in potassium chloride concentrations complete disappearance of RH activity observed.

Effect of different buffers

Crude extract prepared from freshly grown cells was divided into 6 aliquots. Each was then dialysed against different buffers for 3.5 hours. The buffer consisted of 20 mM potassium phosphate alone or with one of the following: dithiothreitol, (DTT 1 mM), 2 mercaptoethanol (5 mM), phenylmethyl sulfonyl fluoride (PMSF 1 mM), Cystein HCl and 15% glycerol. After dialysis, the extracts were stored at 0-4°C on ice and samples were withdrawn at 0, 20 and 42 hours intervals to determine the residual activity.

Table 10 Effects of Sodium Chloride on the PGR and RH Activities

Sodium Chloride µmoles/Assay	PG-reductase E.u/ml	% Activity	R-hydroxylase E.u/ml	% Activity
Control	0.530	100	0.192	100
50	0.480	89	0.048	25
100	0.300	56	0.036	18
200	0.141	27	0	0
400	0.072	13	0	0

PG-reductase and R-hydroxylase activity were determined as described in Materials and Methods in the presence of increasing concentrations of sodium chloride, ranging from 50 - 400 µmoles/3 ml, assay mixture.

Table 11 Effects of KCl Concentrations on the PG-reductase and the R-hydroxylase Activities

Potassium Chloride µmoles/assay	PG-reductase E.u/ml	PG-reductase Activity %	R-hydroxylase E.u/ml	R-hydroxylase Activity %
Control	0.576	100	0.228	100
50	0.312	89	0.024	11
100	0.156	27	0.012	5
200	0.048	8	0	0
400	0	0	0	0

PG-reductase and R-hydroxylase activities were determined as described in Materials and Methods in the presence of increasing concentrations of potassium chloride, ranging from 50 to 400 µmoles/assay.

The effects of different buffers on the PGR activities are presented in Table 12. The loss of PGR activity was much more when fresh extract was dialysed separately in buffers containing DTT (1 mM), PMSF (1 mM) and 2ME (5 mM). Only 17% of the original activity was retained after 20 hours of dialysis. Also inhibition of 67% PGR activity was detected in the presence of cycsteine-HCl (1 mM). After dialysis and storage for 42 hours in 15% glycerol buffer PGR activity was found to be stable (66.7%).

Inhibition of PGR activity was detected after 42 hours storage in all other buffers as indicated in Table 12.

The Effect of Sodium Phosphate buffer on the PGR and RH Acitivies

Sodium phosphate (20mM) buffers with pH range between 5.7 and 8 were prepared. Freshly prepared crude extract was divided into 5 ml portions and dialysed separatedly against buffers adjusted to different pH's for 16 hours. The dialysed samples were kept on ice and the PGR and RH activities were determined.

In order to determine the effect of sodium phosphate buffer (pH 5.7 to 8) on the stability of PGR and RH activities the fresh crude extract prepared in a 20 mM phosphate buffer containing 15% glycerol was dialysed in various buffers adjusted to different pH for 16 hours at 4°C. The loss of PGR activity was observed in buffers between pH 5.7 to 7.4 while between pH 7.6 to 8 the enzyme activity retained was calculated to be 50% of the original. Table 13. Inhibition of RH activity was detected in buffers with pH 5.7 to 8.

Table 12 - Effects of Different Buffers on the Stability of the PG-reductase

Buffers	Activity after Dialysis					
	0 Hours E.u/ml	% Residual Activity	20 Hours E.u/ml	% Residual Activity	42 Hours E.u/ml	% Residual Activity
20 mM Potassium Phosphate	0.012	17	0	0	0	0
+ DTT	0.012	17	0	0	0	0
+ PMSF	0.012	17	0	0	0	0
+ 2ME	0.012	17	0	0	0	0
+ 15% Glycerol	0.048	66.7	0.048	66.7	0.048	66.7
+CYS.HCl	0.024	33.3	0	0	0	0

A freshly prepared crude extract containing PG-reductase activity (0.072 E.U./ml) was dialysed in various buffers indicated above and stored on ice at 0, 20 and 42 hrs. Samples were removed and the residual PGR activity was determined using the standard assay method.

Table 13 The Effect of Sodium Phosphate Buffer on PG-reductase and R-hydroxylase Activities

pH	PG-reductase E.u/ml	o/o Recovery	R-hydroxylase E.u/ml	o/o Recovery
Control	0.036	100	0.012	100
5.7	0	0	0	0
6.0	0	0	0	0
6.5	0	0	0	0
7.0	0	0	0	0
7.4	0	0	0	0
7.6	0.018	50	0	0
8.0	0.018	50	0	0

Freshly prepared crude extract in 20 mM KH_2PO_4 (pH 7.4) containing 1 mM EDTA, 1 mM 2ME and 15% glycerol was dialyzed for 16 hours against 20 mM sodium phosphate buffers adjusted to pH 5.7 to 8 and also containing EDTA, 2 ME and 15% glycerol as above.

The control sample represents extracts in the potassium phosphate buffer.

The Effect of Potassium Phosphate buffer on the PGR and RH Activities

Potassium phosphate (20 mM to 0.8 mM) containing 1 mM EDTA, 1 mM 2ME and 15% glycerol (V/V) was prepared. Freshly prepared crude extract was divided into 3 ml portions and dialysed separately against phosphate buffers adjusted to different molarities for 20 hours. The dialysed samples were then kept on ice and the PGR and RH activities were determined.

The effects of varying the concentrations of potassium phosphate on PGR and RH activities are presented in Table 14. This activity was found to be stable over a concentration range between 0.4 M to 0.8 potasssium phosphate buffer. The loss of PGR and RH activity was much more at lower concentrations (0.02 M to 0.3 M) and only 66 percent of PGR and 40 percent of the original activity was retained after dialysis at these low concentrations of potassium phosphate buffer.

The Effect of imidazole buffer on the PGR and RH Activities

Imidazole (0.025 M) containing 1 mM EDTA, 1 mM 2ME and 15% glycerol (V/V) was adjusted to pH (7.4) using 0.1N HCl. Freshly prepared crude extract was dialysed against the imidazole buffer for 10 hours. The PGR and RH activity was determined.

Table 15 shows the effect of an imidazole buffer (pH 7.4) on enzyme activity. Only 4.5 percent of the activity of PGR was retained after dialysis at 4°C for 10 hours. The RH was undetectable in the same extract.

Table 14 Effect of Potassium Phosphate Buffers on PGR and RH Activities

Potassium Phosphate (mM)	PGR E.U/ml	Percent of Activity	RH E.U/ml	Percent of Activity
Control	0.09	100	0.03	100
0.02	0.06	66	0.012	40
0.1	0.06	66	0.012	40
0.3	0.06	66	0.012	40
0.4	0.09	100	0.03	100
0.5	0.09	100	0.03	100
0.6	0.09	100	0.03	100
0.8	0.09	100	0.03	100

Different portions (about 1 ml) were dialysed against different concentrations ranging from 20 mM to 800 mM of potassium phosphate adjusted to the same pH for 20 hours. The dialysed extracts were then tested for the residual activity using the standard assay. The crude enzyme preparation contained 4.5 mg;ml protein.

Table 15 Effects of Imidazole Buffer on the Enzyme Stability

	PG-reductase E.u/ml	Percent Activity	R-hydroxlyase E.u/ml	Percent Activity
Control	0.528	100	0.132	100
After 10 hrs. of Dialysis	0.024	4.5	0	0

The PG-reductase and the R-hydroxylase activity were determined as described in Materials and Methods after dialysing the fresh crude extract against 25 mM Imidazole buffer (pH 7) for 10 hours. The control consisted of extract prepared in 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM 2ME.

The Effect of Poly buffer-74 on the PGR and RH Activities

Effect of Poly buffer-74 (pH 4 to 7.4) containing 1 mM EDTA, 1 mM 2ME and 15% glycerol was determined spectrophotometrically (Gilford instrument Oberlin, Ohio, U.S.A.) by performing enzyme assay. The reaction mixture contained the following: 0.1 M phosphate buffer 2.5 ml; NADPH 0.5 μmoles; enzyme 0.2 ml (5 mg/ml protein); and poly buffer (0.2 ml). A reaction mixture without poly buffer was used as a control. The change in absorbance at 340 nm was observed.

Table 16 shows the effect of poly buffer 74 on the stability of PGR and RH activities. More than 55 and 70 percent inhibition of PGR and RH respectively was observed.

Effect of pH on Enzyme Activity

In this experiment, three buffers were adjusted to different pH's varying from 6 to 8.4. These included 0.1 M morpholinopropane.sulfonic acid (MOPS) pH 6.5 to 7.9, 0.1 M Tris-HCl pH 7.4 to 8.4 and 0.1 M potassium phosphate pH 6 to 8. These buffers each contained 1 mM EDTA, 1 mM 2ME and 15% glycerol. The reaction mixture contained in a final volume of 3 ml: 2.7 ml, 0.1 mM phosphate buffer, 0.5 μmoles NADPH, 1 μmole of PG and 0.2 ml enzyme (4.8 mg/ml protein). The change in absorbance at 340 nm was measured using a Gilford spectrophotometer.

The PGR activity in a crude enzyme sample was determined using different

Table 16 Effects of Poly Buffer-74 on the Enzyme Activities

PH	PG-reductase E.u/ml	Percent Activity	R-hydroxylase	Percent Activity
Control	0.528	100	0.132	100
4	0.120	23	0.012	9
5	0.120	23	0.012	9
6	0.168	32	0.024	18
7.4	0.228	43	0.036	27

A total volume of 3 ml contained 2.5 ml of phosphate buffer (0.1 M), NADPH (0.5 umoles), crude extract (0.2 ml). The oxidation of reduced $NADP^+$ was monitored at 340 nm using a Gilford Spectrophotometer.

buffers with pH ranging between 6 and 8.4. Table 17 shows that Tris HCl (0.1 M) was a poor buffer giving least PGR activity while potassium phosphate (0.1 M) gave the highest activity. The activity obtained in the presence of Mops was similar to that obtained in the presence of Tris-HCl.

Enzyme Stability:

Effect of Storage Temperatures on the Stability of the PG-reductase and R-hydroxylase

The effect of storage temperature on the crude extract was studied at 4°C and -20°C. The fresh extract was assayed for enzyme activity and kept on ice (0-4°C) in a cold room. After 48 hour interval, a sample was removed and the residual activity was determined.

In another sample of curde extract, activity was determined before it was frozen and stored at -20°C. After keeping it frozen for 48 hours, it was thawed and assayed for the level of PGR and RH.

The PGR and RH activities at 0-4°C decayed very rapidly (Table 18). In 48 hours the PGR activity decreased by almost 95% whereas the RH activity disappeared completely.

In the case of where the crude extract was frozen and stored at -20°C for 48 hours and then thawed, there was no loss in the PGR and RH activities. Table 19 summarizes the results.

The Effect of Glycerol on the Stability of PG-reductase and R- hydroxylase

Table 17 - Effect of pH on PG-reductase

pH	Potassium Phosphate E.u/ml	MOPS E.u/ml	Tris-HCl E.u/ml
6	0.018	-	-
6.5	-	0.012	-
7	0.066	0.024	-
7.4	0.072	0.012	0
7.8	-	-	0
8	0.036	0.006	0.006
8.2	-	-	0.012
8.4	-	-	0.018

A freshly prepared extract in 20 mM potassium phosphate buffer containing 1 mM EDTA and 1 mM 2 ME, containing 0.11 E.U./ml of PG-reductase was used in this experiment. It contained 4.2 mg/ml protein.

The above buffers (0.1 M) containing 1mM EDTA, and 1 mM 2-ME were used to determine the activity of PG-reductase.

Table 18 - The Effect of Storage at (4°C) on the PG-reductase and R-hydroxylase Activities

Time (Hours)	PG-reductase		R-hydroxylase	
	NADPH	NADH	NADPH	NADH
0	0.225 (100%)	0.266 (100%)	0.145 (100%)	0.097 (100%)
48	0.012 (5%)	0.024 (9%)	0.0 (0%)	0.0 (0%)

The fresh enzyme solution in the 20mM phosphate buffer was kept on ice at (0-4°C) for 48 hours.

Table 19 - The Effect of Storage at (-20°C) on the PG-reductase and R-hydroxylase Activities

Time (Hours)	PG-reductase		R-hydroxylase	
	NADPH	NADH	NADPH	NADPH
0	0.225 (100%)	0.314 (100%)	0.145 (100%)	0.132 (100%)
48	0.217 (96%)	0.312 (100%)	0.132 (91%)	0.132 (100%)

The fresh enzyme solution in the 20 mM phosphate buffer was kept on ice at (0-4°C) for 48 hours. before enzyme assays were performed. The oxidation of reduced $NADP^+$ and NAD^+ was examined using the stored sample.

In order to determine the effect of glycerol on the enzyme activity, six tubes each containing 7 ml phosphate buffer (20 mM) with 1 mM EDTA and 1 mM 2ME were prepared. Glycerol was then added to each tube to give a concentration range between 2% to 15% (V/V). To each tube was added approximately 2 g wet packed, freshly grown and washed cells. The cell suspensions were sonicated separately as described before. The cell debris was removed by centrifugation (10,000 rpm, 10 min.) and the clear supernatant solutions obtained were transferred into six clean tubes, stored on ice. The enzyme activity in the crude extracts was measured at different time intervals.

The effects of increasing concentrations of glycerol on the PGR and RH are illustrated in Figures 16 and 17 respectively. Increases in PGR and RH activities were observed with increasing concentrations of glycerol. Glycerol (15%) in the buffer offered protection to the PGR and RH activity.

Chemical and Enzymatic Reduction of Phloroglucinol

A reaction mixture containing 2.7 ml of 0.1 M phosphate buffer pH (7.4), 0.05 ml NADPH (10 μmoles/ml), 0.2 ml enzyme (0.7 mg/ml) in both the cuvettes. 0.04 ml (10 μmoles/ml) PG was added to sample cuvettes and spectra were recorded using a double beam Shimudzu UV-260 Spectrophotometer (Kyoto, Japan). The spectra were recorded at different time intervals.

A reaction mixture containing 2.7 ml potassium phosphate buffer 0.1M, pH (7.4) and 1 μmole of PG was prepared in a cuvette. The reference cuvette contained 2.8 ml of the same buffer. Using a double beam Shimadzu UV-260

Figure 16 - Effect of Glycerol on the Stability of PG-reductase

Crude extracts of freshly grown cells of BPG were prepared in 20 mM potassium phosphate buffer containing increasing concentrations of glycerol. These extracts were then stored on ice and the PGR activity determined at various time intervals indicated.

Extract A = 15% glycerol

Extract B = 10% glycerol

Extract C = 8% glycerol

Extract D = 2% and 4% glycerol

Extract E = Control

FIGURE 16

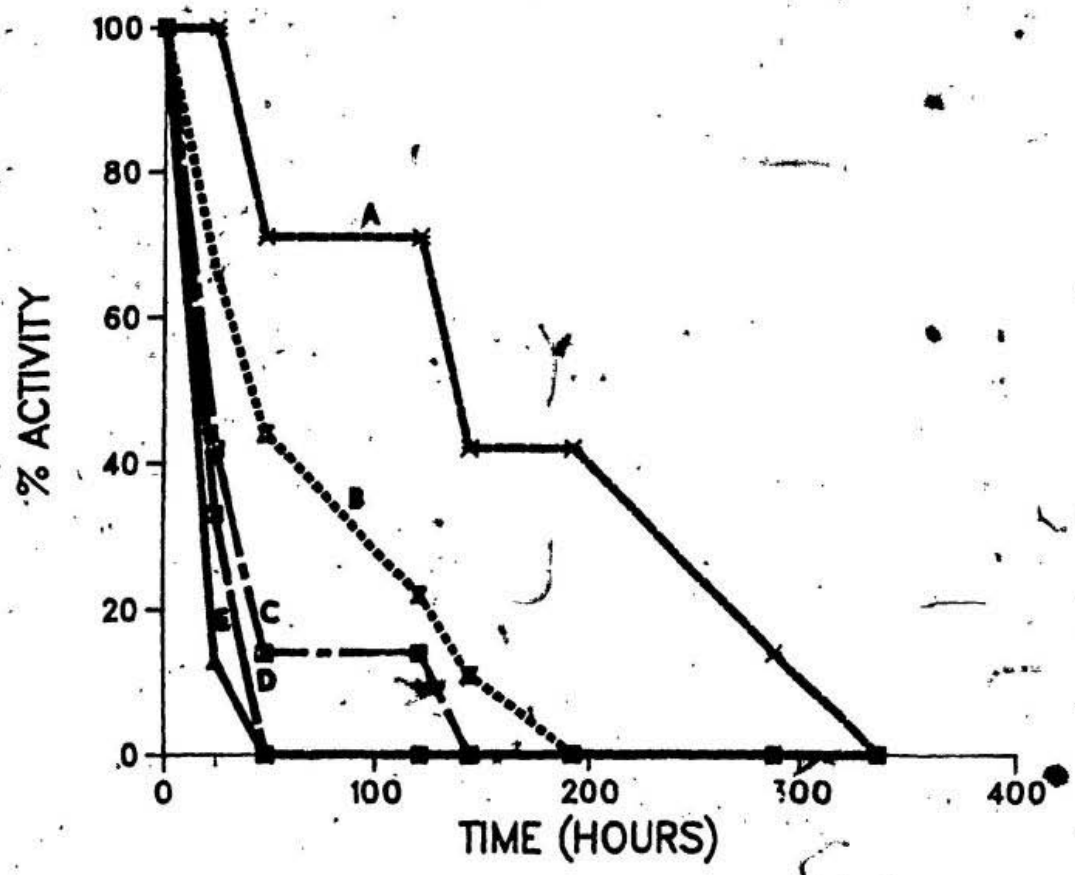


Figure 17 - Effect of Glycerol on the Stability of R-hydroxylase

Crude extracts of freshly grown cells of BRG-8 were prepared in 20 mM potassium phosphate buffer containing increasing concentrations of glycerol. These extracts were then stored on ice and the PGR activity determined at various time intervals indicated.

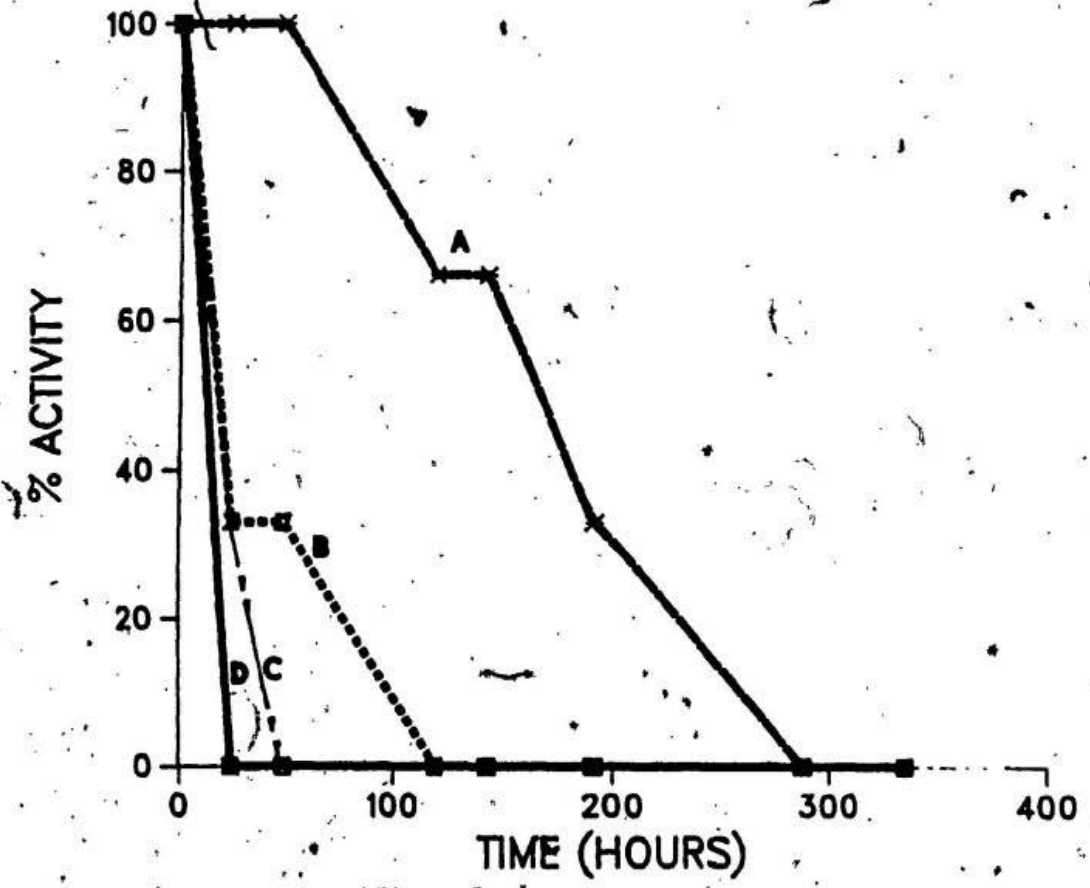
Extract A = 15% glycerol

Extract B = 10% glycerol

Extract C = 8% glycerol

Extract D = 2%; 4% and Control

FIGURE 17



Spectrophotometer, the spectrum of PG was obtained. To chemically reduce PG 2 mg of sodium borohydride in 0.1 ml was added into both the cuvettes and the spectra were recorded at different time intervals.

When PG and NADPH were incubated with the enzyme, a rapid oxidation of NADPH was observed. Figure 18 shows the spectral shift observed in the enzymic reduction of PG to dihydro PG. An identical spectrum was given by the chemical reduction of PG to dihydro PG. Figure 19. By the addition of appropriate quantities of enzyme to a reaction mixture containing the coenzyme and PG it was possible to show stoichiometric conversion of NADPH to NADP.

Induction of PG-Reductase

For this experiment six Erlenmeyer flasks were prepared as follows: Flask a, 0.1% PG, Flask B, 0.1% PG plus 1% sodium succinate, flask C, 0.1% PG plus 1% glucose, flask D, 1% succinate, flask E 1% sodium pyruvate and flask F 1% glucose. The flasks were inoculated with 25 ml of BPG-8 cell suspension, freshly grown and incubated at 25°C in a psychrotherm for 18 hours. Cells from each flask were harvested separately by centrifugation and washed three times with 20 mM potassium buffer. The washed cells were resuspended in 2 ml of the same buffer and sonicated as described before. The clear supernatant solutions obtained after centrifugation were decanted into clean tubes. The PGR activity in these crude extracts was determined using the standard assay procedure.

Table 20 shows that PGR activity appears in cells grown in the presence of either PG alone or in the presence of PG plus many other carbon substrates such

Figure 18 - Enzymatic Reduction of Phloroglucinol to Dihydrophloroglucinol

Reaction mixture (3 ml) contained 2.7 ml of 0.1 ml phosphate buffer pH (7.4), 0.05 ml NADPH (10 μmoles/ml), 0.2 ml enzyme (0.7 mg/ml) in both cuvettes. 0.04 ml (10 μmole/ml) PG was added to sample cuvettes and the spectra were recorded using Shimadzu UV-260 model Spectrophotometer.

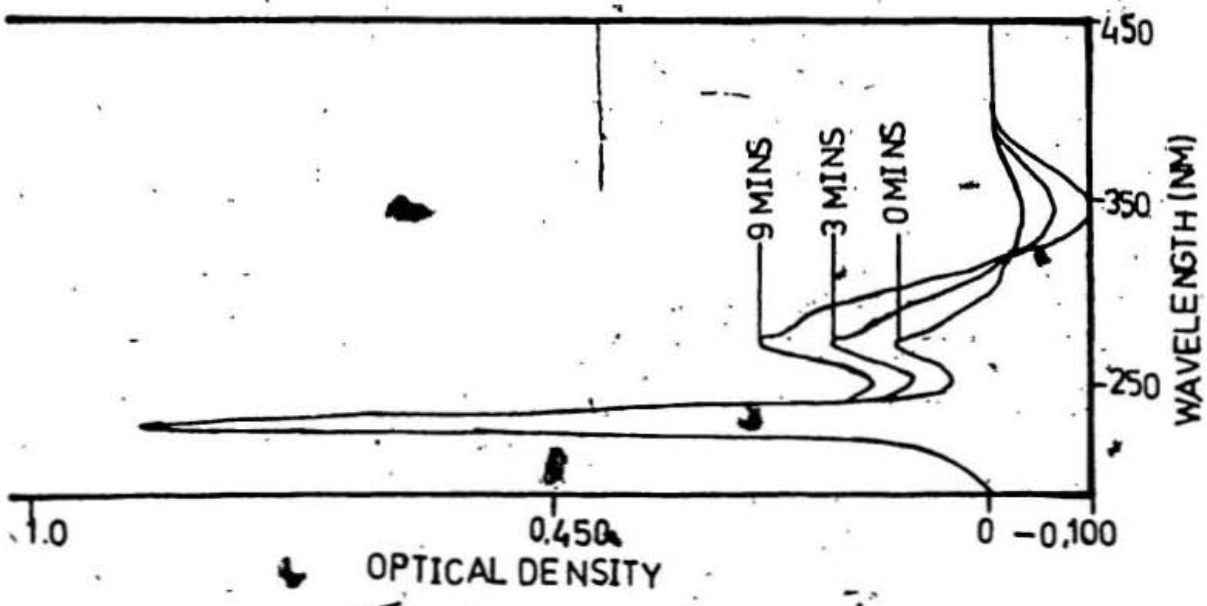

9 MINS
3 MINS
0 MINS
450
350
250
WAVELENGTH (NM)
1.0
0.450
0
-0.100
OPTICAL DENSITY

FIGURE 18

Figure 19 - Chemical Reduction of Phloroglucinol

The reaction mixture (3 ml) contained 2.7 ml of 0.1 M phosphate buffer (pH 7.4) and 1 μmole PG (in 0.1 ml). The reference cuvette contained 2.8 ml of the same buffer. The reduction of PG was initiated by adding 1 mg sodium borohydride (0.1 ml of the same buffer) and the spectra of the reaction mixture were recorded at the times indicated.

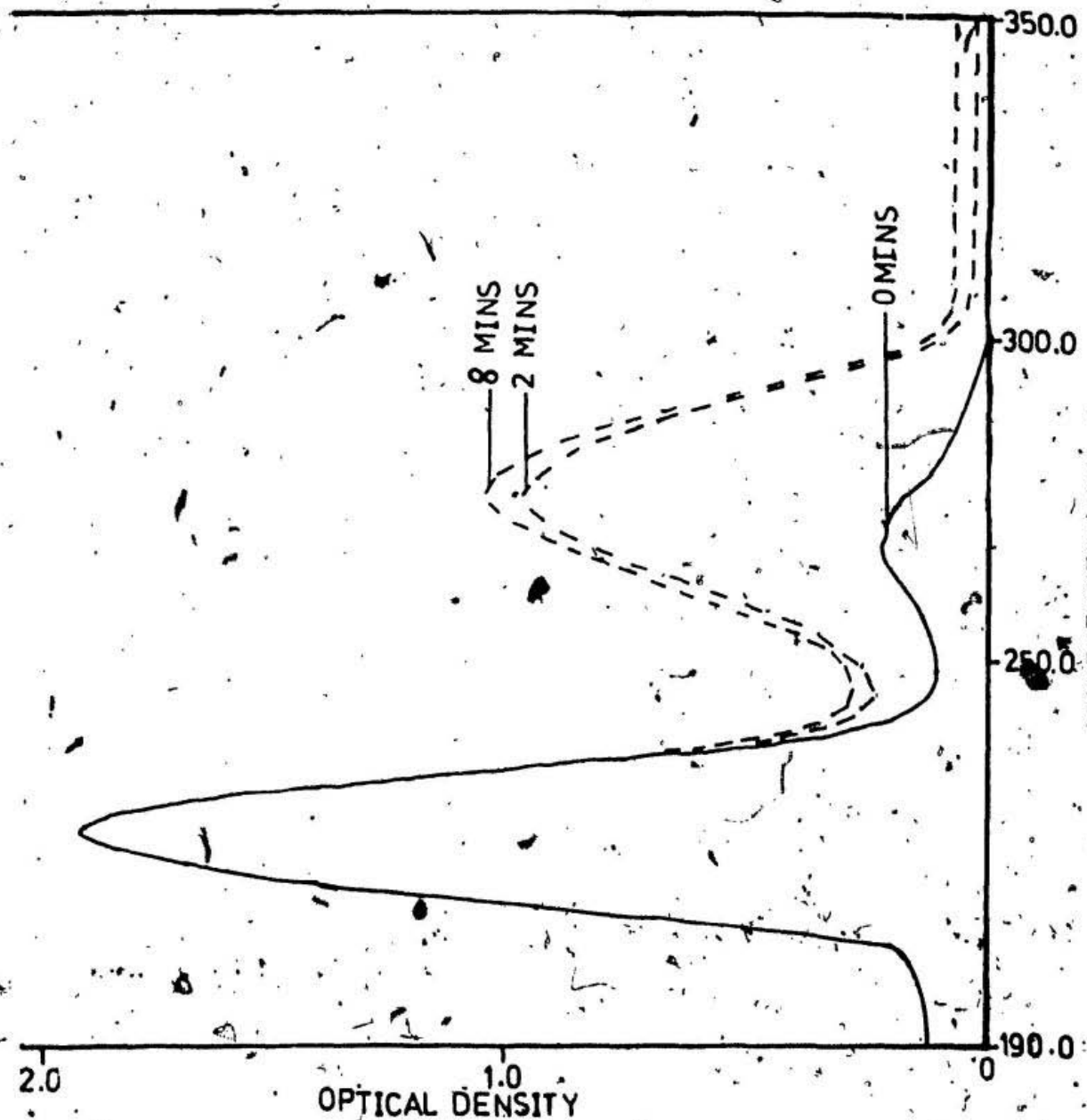

350.0
300.0
250.0
190.0
WAVELENGTH (NM)
0 MINS
2 MINS
8 MINS
2.0
1.0
0
OPTICAL DENSITY

FIGURE 19

Table 20 - Inducibility of PG-Reductase in the Presence of Various Carbon Sources

Growth Substrates	Activity (E.U/ml)	Protein (mg/ml)	Specific Activity
PG	0.048	3.5	0.013
PG+succinate	0.048	5.75	0.01
PG+pruvate	0.036	2.5	0.014
PG+glucose	0.024	3.5	0.01
Succinate	0	4.2	0
Pyruvate	0	2.5	0
Glucose	0	1.6	0

Cells were grown in the presence of the above substrates described in Materials and Methods. Crude extracts of cells grown on these substrates were tested for the PG-reductase activity using standard assay procedures.

E.U = enzyme units

Specific activity was defined as E.U. per mg protein.

as succinate, pyruvate and glucose. No PGR activity was detected in cells grown in the absence of PG.

Effect of Incubation Time on PG-Reductase Activity

Five flasks containing each 500 ml of MSM medium were inoculated with freshly grown cells of BPG-8 on a shaker ina psychrotherm and agitated at 150 rpm at 25°C. Flasks were removed at different time intervals and the cells were harvested and washed as before. Washed cells were resuspended in 20 mM phosphate buffer (pH 7.4) and sonicated as described above. The cell debris was removed by centrifugation and the clear supernatant solution formed the source of the enzyme. Table 21 shows the results obtained.

In order to assess the time at which the enzyme was maximally produced, the BPG-8 was grown for various time intervals as described in Materials and Methods. As depicted in Table 21, PGR and RH activities reach a peak in about 18 hours and decline thereafter. A rapid decline of PGR and RH activity was observed after 24 hours. RH activity was not detected after 36 hours.

Table 21 - The Effect of Time Incubation on the Enzyme Activities

Substrate	Activity after (E.u/ml) 12 hrs.	18 hrs.	24 hrs.	36 hrs.	65 hrs.
PG +NADPH	0.048	0.06	0.048	0.036	0.012
PG+NADH	0.048	0.036	0.036	0.024	0.0
R+NADPH	0.012	0.024	0.012	0.006	0.0
R+NADH	0.024	0.024	0.012	0.0	0.0

The cells of BPG-8 were grown for different time intervals indicatedabove in separate flasks and used for preparing crude extracts. PG-reductase and the R-hydroxylase activities in the crude extracts were determined using NADH and NADPH as electron donors according to the standard assay procedure.

DISCUSSION

A gram positive Bacillus isolated from soil by enrichment technique was identified using biochemical tests listed in Table 2. Based on these results the organism was assigned to the genus Bacillus. Bergey's Manual of Determinative Bacteriology (Eighth edition, 1974) lists several additional diagnostic tests that failed to identify the species of the organism.

Under electron microscopy and light microscopy, cells appeared as rods. The size of the cell was very small 2 - 2.5 m length and 1.0 - 1.2 m width. The spore stain was carried out on the bacterium but even old cultures showed few spores. So to indicate that the unknown is a sporeformer, the spore confirmation test was carried out. Tables 1 and 2 review results which permitted the unknown bacterium to be tentatively identified as Bacillus sp. BPG-8. To my knowledge, this is the first report on the aerobic utilization of PG by a gram positive, sporulating bacterium. There are few, reports available in the literature about gram positive and negative bacterium utilizing PG. Gram positive cells included: Mycobacterium sp. (Bernhein, 1965); Brevibacterium fuscum (Nakugwa and Takeda, 1962); Streptococcus bovis and Coprococcus sp. (Tusi and Jones, 1976), Pelobacter acidigallici (Bernhard et al., 1982).

Gram negative bacteria included: Pseudomonas sp. Mac 451, (Robern, 1965), Butyrivibrio sp. C_3 (Krishnmurty et al., 1970), Rhodopseudomonas gelatinosa (Whittel et al., 1976).

Physical and chemical factors play an important role in the metabolism of

aromatic ompounds by microorganisms. Temperature, pH, aeration and a favourable supply of inorganic ions are of prime importance for growth and metabolism. The medium used to grow the Bacillus sp. BPG-8 resembles the medium described for Pseudomonas sp. Mac 451 (Robern, 1965). The optimum pH of the medium for the growth of the latter was 7.2 - 7.4, but for BPG-8 it was 5.5. This may be due to higher stability of PG under acidic environment.

The optimum temperature for the growth of Pseudomonas sp. and Penicillium sp. Mac 47 was 30°C. Coprococcus sp. Pe_15 (Patel et al., 1981) grew best at 37°C while Bacillus sp. BPG-8 grew optimally at 25°C. The temperaturs above 25°C and pH above 5.5 caused browing of the medium due to the decomposition of the substrate. In the case of Pseudomonas sp. and Penicillium s[. the organisms were grown at pH 7.5 and the incubation temperature of 30°C. It is difficult to interpret the results obtained by these authors in the light of our observation.

Pseudomonas sp. and Bacillus sp. gave best growth when 0.1% PG was incorporated into the medium, higher concentrations resulted in decreased growth. Concentrations higher than 0.2% inhibited the growth of the organism completely. In contrast under optimum conditions 0.25% PG was utilized completely in 11 hours by a growing culture of Penicillium sp.

The generation time for BPG-8 in MSM containing 0.1% PG and 0.001% YE was estimated to previously be 3.75 hours (Worthman, 1985). This is much higher than that of E. coli which has a generation time of about 25 to 30 mins.

(Brock, 1979). The stationary phase began after 12 to 14 hours of growth. The time of harvest was important for obtaining good enzyme activity. In the case of Coprococcus sp. the optimal PGR activity occurred between 36 to 48 hours, while in the case of BPG-8 it was between 12 to 14 hours. In the case of Pseudomonas sp. the cells were harvested after 18 hours of growth and those of Penicillium sp. were harvested after 36 hours, showed optimal activity (Robern, 1965; Mathur, 1971).

Thornton (1928) was able to isolate microorganisms from the soil capable of growing in pure culture which utilized PG, resorcinol, cresol and resorcyclic acid as the sole sources of carbon. In contrast Coprococcus sp. $Pe_1 5$ failed to grow at the expense of any of 39 different aromatic or flavonoid compounds tested. Bacillus sp. BPG-8 grew on pyrogallol, orcinol and PG but not on any of the other compounds tested. Gallic acid, pyrogallol, 2,4,6-trihydroxy benzoic acid and PG were the only substrates utilized by Pelobacter acidigallici (Bernhard et al., 1982).

The enzyme from Bacillus sp. BPG-8 and Penicillium sp. MAC M-47 carried two kinds of activities, one specific for PGR and another for RH. In contrast, enzymes from Pseudomonas sp. MAC 451 and from Coprococcus sp. $Pe_1 5$ showed only the PGR activity. In case of BPG-8 PGR and RH activities were partially purified by the ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. However the partially purified protein retained RH activity.

Similarly the enzyme complex from Penicillium sp. was purified by a

combination of ultrafiltration, protamine sulfate treatment ammonium sulfate precipitation, DEAE Sephadex column chromatography and gel filtration using Sephadex G-200 (Mathur, 1971). The enzyme complex was purified 20-fold and showed PGR and RH activities.

The PGR from Pseudomonas sp. was purified by a combination of various techniques including streptomycin treatment, ammonium sulfate precipitation, column chromatography on a DEAE-cellulose column and a Bio-gel P-300 column. (Hang, 1967). This enzyme was purified twenty three-folds and showed only PGR activity. The crude extract of Pseudomonas sp. also showed on PGR activity.

The PGR of Coprococcus sp. was purified by protamine sulfate treatment, ammonium sulfate precipitation and gel filtration using Sephadex G-200 (Patel, 1981). In this also the crude extract as well as the purified enzyme carried only PGR activity.

In the case of BPG-8 the PGR activity was inhibited by ammonium sulfate treatment. Only 13% of the PGR and 7% of RH activities were recorded in the 60-80% ammonium sulfate fraction. In contrast the enzyme of Penicillium sp. was not affected by ammonium sulfate. About 84% of the PGR and 83% of the Rh activities were recoverably in the 45%-65% ammonium sulfate fraction.

The PGR and RH activities were not separated when a sample of crude extract was run through a Sephadex G-150 column (Figure 6). Similar observations were also made for the enzyme from Penicillium Mac 451 (Mathur, 1971).

Attempts to separate the two enzyme activities on the ion-exchange column surprisingly yielded two activity peaks (Figure 8) both carrying PGR activity but no RH activity. This experiment was repeated twice and the results obtained were similar. Perhaps the high dilution during the column chromatography makes it difficult to detect the RH activity. It is to be noted that in the crude extract as well the ratio of PGR activity to that of RH activity is very high.

When a batch purification using the same ion-exchange material was performed, the bound enzyme released also had a hgher ratio of PGR activity over th RH activity. The purpose of using this technique was to purify the enzyme in a lesser time compared to the column chromatography. About 50% of the PGR activity was recoverable by this method.

Purification of the enzyme from Bacillus sp. by chromatofocusing was unsuccessful because the eluting buffers, imidazole and poly buffer were found to be inhibitory to PGR and RH activities. Other workers have not reported the use of this method for the purification of a similar enzyme.

PGR from the Bacillus sp. BPG-8 resembles the reductase described in Pseudomonas sp. MAC 451. (Hang, PhD. Thesis), Penicillium sp. MAC M-47 (Mathur, 1971) and anaerobe Coprococcus sp. $Pe_1 5$ (Patel et al., 1981). All four enzymes carry out the reduction of Phloroglucinol with NADPH as an electron donor, and in all cases the reductase activity was stimulated by 2-mercaptoethanol. However, the fungal enzyme complex used NADH at a 50% efficiency, whereas the enzyme from Coprococcus sp. $Pe_1 5$ exhibited only 3 to 4%

activity under similar conditions. In the present studies, NADH may be substituted for NADPH as an alternate electron donor but because of the high endogenous activity with NADH, NADPH was used as an electron donor. RH from the Bacillus sp. carry out the hydroxylation of resorcinol with NADPH as an electron donor. However NADH can also abe used in this reaction.

The Km values for PG was 2×10^{-4} M and that for resorcinol was 0.25×10^{-4} M in the case of BPG-8 (Worthman, 1985). In the case of enzyme complex from Penicillium sp. (Mathur, 1971) the Km for PG was 2×10^{-5} M and that for resorcinol was 1.43×10^{-3} M. Thus the enzymes from both the organisms show higher affinity for PG compared to that for resorcinol.

The crude enzyme from Bacillus sp. when stored at 4°C fro two days, showed a 90% loss of PGR and a complete loss of the RH activity. While in the case of Coprococcus sp. (Patel, 1981) only 9% of the initial activity was lost within five days in the presence of 2-mercaptoethanol.

No differences were observed in the PGR and RH activities in crude extract stored at 4°C for a week in the case of Penicillium sp. Thus the enzymes from Bacillus sp. appears to be more unstable compared to other enzymes described in the literature. Freezing and thawing had little or no effect on the PGR or RH from BPG-8. Similar observations have been reported for the enzymes from Coprococcus sp., Penicillium sp. and Pseudomonas sp. (patel, 1981; Mathur, 1971; Robern, 1965).

The stability of the enzyme (PGR) is affected by different buffer systems (Table 12). The loss of PGR activity was much higher when fresh extract was dialysed separately in buffers containing DTT, PMSF, 2ME and Cysteine HCl. The increasing concentrations of glycerol (15%) offered protection to the PGR and RH activities. The earlier workers did not report the use of glycerol for stabilizing the enzyme. (Patel,1981; Mathur, 1971; Robern, 1965).

The PGR and RH activities from BPG-8 was inhibited by potassium chloride used as a gradient on DEAE-Sephadex A-50 column while in the case of Penicillium sp. enzyme complex was not inhibited when potassium chloride was used as a gradient on the same column. In the case of BPG-8 sodium chloride also inhibited the enzyme (Table 18). There was a more rapied loss of RH than PGR activity in the same extract. This suggested that there may be two different enzymes.

The results so far obtained in this work indicates that the PGR and RH activities may form two active sites of a single enzyme containing more than one polypeptide. Unless two separate enzymes with either PGR orRH activities can be obtained it is difficult to draw any definite conclusions with respect to the proposed enzyme complex.

Sodium phosphate buffers with pH range between 5.7 to 8 also inhibited the PGR and RH activities. However, potassium phosphate did not seem to affect these acticities. A higher concentration of potassium phosphate appeared to stabilize the enzyme activities (Table 14). Optimum activities were found over a

concentration range between 0.4 M to 0.8 M potassium phosphate (monobasic) buffer. Such enzyme stabilization by potassium phosphate has not been reported by other workers (Robern, 1965; Mathur, 1981; Patel, 1981).

The pH optimum for the Bacillus sp. was found to be 7.4 (Table 17) in the presence of 0.1 M potassium phosphate. Similar results were obtained for the enzymes Penicillium sp., Pseudomonas sp. and Coprococcus sp. (Mathur, 1971; Robern, 1965; Patel, 1981).

The molecular weight of PGR from Bacillus sp. was found to be 155,000. The molecular weight of the Coprococcus enzyme was reported to be 130,000 (Patel, 1981), while the fungal enzyme (Mathur, 1971) ws found to be 76,000. Thus PGR from Bacillus sp. appears to be larger than enzymes from other sources.

Both BPG-8 and Coprococcus sp. have PGR which is inducible by PG. However, in the case of Coprococcus sp. Pe$_1$5 low levels of PGR activity was detected in cells grown on other substrates. This perhaps represented the low levels of constitutive synthesis of the enzyme.

The PGR and RH activities from a Bacillus sp. reached a peak in about 18 hours while PGR activity from Penicillium sp. reached a peak in about 24 hours. The enzyme activity was observed in the crude extract prepared from 24 hours and 36 hours old cultures of Penicillium sp. In contrast PGR activity was detected in cells of BPG-8 grown for about 65 hours. The RH activity, however, declined rapidly after about 24 hours in the case BPG-8.

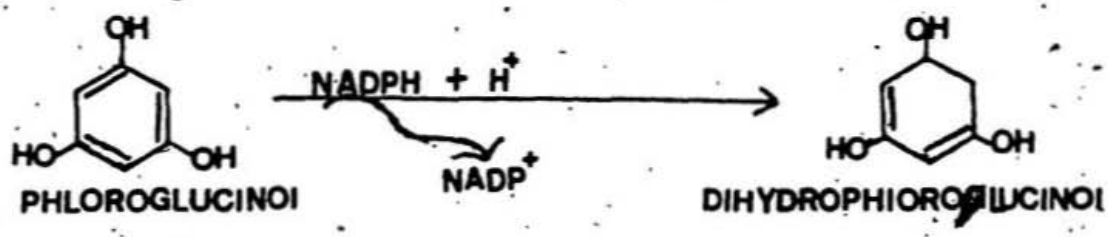
Robern (1965) in the case of Penicillium sp. showed that resting cells of this fungus degraded PG and that in a culture medium a compound that absorbed light at 278 nm was detected. This compound was not further metabolized by the fungus. Hang (1967) showed that Pseudomonas sp. grown on PG produced a similar compound with similar absorption peak at 278 nm. This intermediate was detected in the culture medium by its spectral characteristics as well as by colour reactions.

Jamieson (1970) has shown that dihydro PG is an intermediate product formed during PG metabolism by Pseudomonas sp. They claim to have isolated dihydro PG from this culture medium in an organic solvent system. However, their nuclear magnetic resonance spectral analysis of the isolate product suggest that the dihydro PG decomposed to give resorcinol.

Whittle. (1976) detected 2-OXO-4-hydroxyadipate and dihydro PG in photosynthetic cultures of Rhodopseudomonas gelatinosa growing on PG as the sole carbon source. A soluble extract of these cells reduced PG to dihydro PG in the dark in the presence of NADPH as hydrogen donor. The authors proposed a pathway of PG degradation of Rhodopseudomonas gelatinosa which included initial reduction of PG to dihydro PG and subsequent hydration of the benzene nucleus.

Coprococcus sp. $Pe_1 5$ (Patel, 1981) carried out a similar NADPH dependent initial step in PG degradation to yield dihydro PG.

The proposed initial step in the metabolism of PG by Bacillus sp. BPG-8 is,



PHLOROGLUCINOL

DIHYDROPHLOROGLUCINOL

CONCLUSIONS

The following conclusions were drawn from these observations:

1. The organism isolated was tentatively identified as Bacillus species BPG-8. It was a gram + rod, that produced endospores.

2. The organism was shown to utilize PG, orcinol and pyrogallol as sole sources of carbon and energy.

3. The organism degraded PG most readily at 25°C. Aeration was essential for better growth and PG utilization.

4. The optimum pH was 5.5. The pH above 5.5 and temperatures above 25°C caused browning of the medium.

5. The optimum PG concentration was 0.1%. Higher concentrations inhibited the growth.

6. The resting cell suspension required five hours for complete utilization of 0.1% without any lag.

7. Bacillus sp. BPG-8 carried an inducible PGR.

8. The enzyme requires electron donors such as reduced $NADP^+$ and NAD^+.

9. The PGR activity was inhibited by potassium chloride, sodium chloride, sodium phosphate, imidazole buffer and poly buffers.

10. Metal ions such as Zn^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+} did not stimulate the PGR activity.

11. The effect of the metal ions was more pronounced with respect to RH activity than PGR activity.

12. The PGR and RH activities were unseparable by gel filtration and ion exchange column chromatography.

13. The ratio of PGR to RH activity was always higher in all enzyme preparations.

14. The spectral changes observed during chemical reduction of PG by sodium borohydride indicate the formation of dihydro-PG.

15. Evidence is presented to show that enzymatic reduction of PG in the presence of NADPH forms dihydro PG.

16. The data presented suggest that BPG-8 may carry an enzyme complex with two separate activities, namely PGR and RH.

Future Work

- Affinity column chromatography.

- Rechromatography of the peaks obtained on Sephadex G-150 and ion exchange (DEAE-Sephadex A-50 Column).

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