SOME ASPECTS OF INDUCTION MECHANISMS OF 2,3-DIHYDROXYBENZOATE OXYGENASE BY *PSEUDOMONAS FLUORESCENS*

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Robert Timothy Powell

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SOME ASPECTS OF INDUCTION MECHANISMS OF 2,3-DIHYDROXYBENZOATE OXYGENASE BY PSEUDOMONAS FLUORESCENS

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ABSTRACT

Robert Timothy Powell. SOME ASPECTS OF INDUCTION MECHANISMS OF 2,3-DIHYDROXYBENZOATE OXYGENASE BY PSEUDOMONAS FLUORESCENS. (Under the direction of Takeru Ito) Department of Biology, July 1979.

The soil bacterium islolated by Ayers (1978) on 2,3-DOB selective medium was identified as Pseudomonas fluorescens. This strain grew best at room temperature (24-25°C) under aerated conditions.

Glutamate was chosen as an alternate carbon source in the growth medium due to its lesser interference with 2,3-DOB oxygenase production by P. fluorescens. Glucose and fructose completely inhibited 2,3-DOB oxygenase induction. High enzyme induction was observed using 0.2 g 2,3-DOB per liter of glutamate growth medium. This finding resulted in a reduced 2,3-DOB expenditure. No alternate, less expensive inducers were found to elicit production of 2,3-DOB oxygenase. Among the compounds tested in this study, 2,3-DOB was the only substrate of the enzyme.

The temperature optimum for 2,3-DOB oxygenase was established at 37°C and complete enzyme denaturation occurred between 50°C and 55°C. The pH optimum was near pH 8.0 with abrupt reduction in activity above that pH.

A permease-mediated transport system for 2,3-DOB in this organism was indicated. The rate of transport was extremely rapid in starved cells. (Approximately 85% of 2,3-DOB exposed to the cells was taken up within 30 minutes.) The rate of transport was probably inhibited in growing cells by glutamate or accumulated metabolites. Production of

2,3-DOB oxygenase began immediately after addition of 2,3-DOB to growing cultures, but in starved cells a two-hour lag period was observed between addition of 2,3-DOB and production of 2,3-DOB oxygenase. The lag period was attributed to limited availability and utilization of the "free amino acid pool" within the starved cells.

The presence of phospholipids in the ultracentrifuged extract appeared to protect 2,3-DOB oxygenase activity against the effects of sonification. However, no direct relationship between enzyme stability and membrane phospholipids could be established in this study.

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INTRODUCTION

A strain of Pseudomonas fluorescens produced 2,3-dihydroxybenzoate oxygenase when grown in a medium containing 2,3-dihydroxybenzoate (2,3-DOB) as a sole carbon source. This enzyme catalyzed incorporation of one molecule of oxygen into 2,3-DOB with simultaneous release of carbon dioxide and α-hydroxymuconic semialdehyde (Fig. 1, Appendix: Ribbons and Senior, 1970). Occurrence of the substrate of this enzyme, 2,3-DOB, and of its derivatives in nature has been well documented (Rosenberg and Young, 1974; Byers, 1974). Such documentation will appear in the section of REVIEW OF LITERATURE.

Ayers (1978) isolated a similar soil bacterium, and purified a 2,3-DOB oxygenase from the organism in order to investigate a possibility of involvement of two separate enzymes in this oxygenase reaction. He concluded that there was no evidence to indicate the presence of two enzymes.

The following problems were considered before undertaking the studies presented in this thesis. First, the soil organism isolated by Ayers was yet unidentified. Secondly, the induction mechanism of this enzyme was entirely unknown, and the known inducer of this enzyme, 2,3-DOB, was relatively expensive. The third problem was whether 2,3-DOB induced its transport carrier or not. Fourthly, it was necessary to determine the pH and the temperature optima and other characteristics of the enzyme in order to assay the enzyme activity properly. Consequently this investigation was concerned with a) identification of the organism isolated by Ayers (1978) as Pseudomonas fluorescens, b) examina-

tion of a number of compounds to find optimal and inexpensive induction conditions, c) studies of 2,3-DOB transport across the cell wall/cell membrane of the organism, and d) determination of the pH and temperature optima, and other characteristics of the enzyme.

REVIEW OF LITERATURE

In relation to the planned studies listed in the INTRODUCTION, in this section the following items were discussed: a) natural occurrence of 2,3-DOB, the substrate of 2,3-DOB oxygenase, b) 2,3-DOB oxygenase, c) metabolism, especially catabolism of 2,3-DOB by Pseudomonas species, d) some aspects of regulatory mechanisms of 2,3-DOB catabolism.

Production of 2,3-DOB and its derivatives by several organisms has been reported frequently. Goris and Canal (1936) identified it as a constituent of the buds of Populus balsamifera. This compound was isolated from Claviceps paspali and shown to be a metabolite of major importance in its submerged fermentation of lysergic acid d-hydroxy-ethylamide (Arcamone et al., 1961). Aerobacter aerogenes and Escherichia coli tryptophan auxotrophs able to utilize anthranilic acid for growth accumulated 2,3-DOB in the growth medium (Pittard et al., 1961). The compound was also isolated and characterized by Dyer et al. (1964) and considered to be an intermediate in the biosynthesis of streptomycin by Streptomyces griseus. Catlin et al. (1968) identified 2,3-DOB as an intermediate in the shikimic acid pathway of biosynthesis of aromatic compounds from mutant strains of Streptomyces rimosus unable to produce oxytetracycline.

Among the 2,3-DOB derivatives, itoic acid (2,3-dihydroxybenzoyl-glycine) was the first one described in literature as a natural product. Itoic acid, secreted by the gram-positive spore-forming species of bacteria, Bacillus subtilis, when grown in an iron-deficient growth medium, was isolated and identified (Ito and Neilands, 1958). This was

the first report of a 2,3-DOB derivative to function as a possible iron transporting ionophore. Earlier, Garibaldi and Neilands (1956) observed that the production of this Iron Transporting Orthophenolic Acid (itoic acid), then still unidentified, was inversely proportional to the iron present in the culture medium. Peters and Warren (1968) found that under conditions of iron deficiency three strains of Bacillus subtilis produced 2,3-DOB, itoic acid, or both of these compounds. Enterochelin, a cyclic trimer of 2,3-dihydroxybenzoyl-L-serine, was produced by E. coli and A. aerogenes (O'Brien and Gibson, 1970). The same compound, named enterobactin by Pollack and Neilands (1970), was a product of Salmonella typhymurium. All these compounds, 2,3-DOB and its derivatives, seemed to have one common function, that was to transport ferric ions into a microbial cell from the exterior after chelating with the extremely insoluble ions, thus solubilizing them.

A precursor of the biosynthetic pathway leading to 2,3-DOB was chorismate, which was successively converted to isochorismate and 2,3-dihydro-2,3-dihydroxybenzoate (Young et al., 1967; Young et al., 1969; Young and Gibson, 1969a,b). Luke and Gibson (1971) found cell extracts of E. coli converted 2,3-DOB and L-serine to enterochelin in the presence of magnesium ions and ATP.

Enzymatic cleavage of 2,3-DOB by Pseudomonas fluorescens was accomplished by 2,3-DOB 3,4-oxygenase (Ribbons and Senior, 1970). Incorporation of both atoms of oxygen into the organic substrate and release of carbon dioxide and α-hydroxymuconic semialdehyde were the result of this cleavage. The reaction product formed by the meta-cleavage of the aromatic ring, α-hydroxymuconic semialdehyde, was reported by Dagley

et al. (1964) in other pseudomonads to be further metabolized by sequentially induced enzymes.

Regulating factors of biosynthesis of 2,3-DOB 3,4-oxygenase have been sparsely studied. According to Ribbons and Senior (1970), induction of the dioxygenase responsible for 2,3-DOB oxidation by P. fluorescens was elicited by the substrate 2,3-DOB in mineral media, but not by 2,3-dihydroxy-p-toluate (Fig. 1, Appendix), a non-inducing substrate for the enzyme. Benzenoid cleavage of 2,3-dihydroxy-p-toluate gave the ring-fission product of 3-methylcatechol studied by Catelani et al. (1968). However, 3-methylcatechol itself was not oxidized by extracts of P. fluorescens grown with 2,3-DOB, nor was it formed anaerobically from 2,3-dihydroxy-p-cumate (Ribbons and Senior, 1970). 2,3-Dihydroxy-p-cumate was also found to be a substrate for extracts of P. fluorescens cultured in 2,3-DOB mineral media, and to accumulate a compound that maximally absorbed at 393 nm (DeFrank and Ribbons, 1976). 2,3-Dihydroxy-p-cumate, however, was not an inducer of the enzyme necessary for the ring cleavage.

Enzymes of paraffin and aromatic compound degradation were thought to show low specificity for their substrates (Dagley et al., 1963). The 'meta' catechol dioxygenase from fluorescent pseudomonads oxidized several ring-substituted alkyl derivatives of catechol (Ribbons, 1970; Sala-Trepat et al., 1972; Bayly and Whigmore, 1973). Evans et al. (1972) reported that a 3,4-oxygenase from a Pseudomonas species grown on 2,4-dichlorophenoxyacetate attacked 3-chlorocatechol, 5-chlorocatechol and 3,5-dichlorocatechol. The broad specificity of some of these enzymes seemed to allow aromatic compounds with various ring substituents to be

metabolized by the same enzymes, thus to contribute a great deal to the versatility of Pseudomonas species.

Certain substances structurally and metabolically related to the substrate of the induced enzyme were also the inducers of the enzyme, although some of them did not serve as substrates of the enzyme and others did. For example, Halvorson (1960) found induction of yeast β-glucosidase by the expected substrate cellobiose to be very poor. Methyl-β-glucoside exhibited greater induction and was hydrolyzed much faster than cellobiose, whereas thiomethyl-β-glucoside was not hydrolyzed while being an excellent inducer. Induction by the latter which was not a substrate of the induced enzyme was an example of gratuitous induction.

Growth substrates often induced both the uptake systems that transported them and the enzymes that initiated their catabolism (Lin, 1970). Growth of a mutant of P. putida on adipate resulted in induction of not only the β-ketoadipate uptake system but also the enzymes of both the catechol and protocatechuate degradative pathways. It was thus suggested that these enzymes, which were the subject of "coordinate induction" by adipate, shared a common regulatory gene with the β-ketoadipate uptake system for the induction (Parke and Ornston, 1976). On the other hand a compound inherent to a catabolic pathway induced an enzyme catalyzing the reaction that produced that compound. Work with P. putida by Ornston (1971) showed cis, cis-muconate to be the inducer of the enzyme that produced it from catechol. Cross-induction of enzymes of one pathway by metabolites of the other was reported for benzoic acid and para-hydroxybenzoic acid pathways in P. putida (Hosokawa, 1970).

Mechanisms used by P. fluorescens to govern the synthesis of the inducible enzyme involved in 2,3-DOB oxidation were not known, and were chosen as a subject of the studies undertaken in this thesis.

MATERIALS AND METHODS

Microorganism

A previously isolated strain of *Pseudomonas* capable of metabolizing 2,3-dihydroxybenzoic acid as a sole carbon source was used (Ayers, 1978). This organism was identified as *Pseudomonas fluorescens* in this study by the nutritional and biochemical methods described by *Stanier et al.* (1966) as described in RESULTS AND DISCUSSION. It was maintained on Difco nutrient agar slants and subcultured once a month.

Growth media

The standard growth medium was prepared by dissolving 0.25 g NH_4NO_3, 0.375 g K_2HPO_4, 0.125 g NaH_2PO_4, 0.025 g yeast extract, and 1.0 g carbon source in 50 ml tap water. To this solution, 172.5 ml de-ionized water and 2.5 ml bacterial salt solution (containing 20 g $MgSO_4 \cdot 7H_2O$, 0.2 g $FeSO_4 \cdot 7H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.02 g $MnCl_2 \cdot 4H_2O$, 0.002 g $CuSO_4 \cdot 5H_2O$ in one liter 0.1N HCl) was added. The pH of the solution was adjusted to 7.0 with 1N potassium hydroxide before the total volume was brought to 225 ml. The growth medium was transferred to a one liter flask, sealed with a gauze and cotton stopper, and autoclaved for 20 minutes. The carbon sources added for testing for optimum growth and optimum 2,3-DOB oxygenase production were galactose, glucose, fructose, sodium acetate, sodium glutamate, sodium citrate, sodium succinate, sodium lactate, and DL-malic acid. The standard growth medium containing glutamate will be referred to as the glutamate growth medium. A known inducer of the enzyme, 2,3-DOB, was also added in variable concentrations for testing optimum enzyme production.

Various other phenolic compounds were added singly in the place of 2,3-DOB in some experiments. Since most of these phenolic compounds studied were labile at autoclave temperatures, their various amounts were singly dissolved in a potassium phosphate buffer to make the final buffer concentration 10 mM, the final pH 7.0, and the final volume 25 ml, and the solutions were filtered through a Metricel filter with pore size of 0.02 μm and aseptically added to the autoclaved standard growth medium. This brought the total volume of the complete growth medium to 250 ml. The phenolic compounds studied were benzoate, para-hydroxy-benzoate, catechol, protocatechuate, ortho-vanillin, ortho-vanillate, 3-methylcatechol, and 4-methylcatechol (Research Organic/Inorganic Chemical Corporation). Some phenolic compounds tested for inducers proved inhibitory to bacterial growth. In such cases, 25 ml of a 5 mM solution of these compounds was prepared and aseptically added to the growth medium 10-12 hours after inoculation.

Growth and harvest of the bacteria

Flasks containing the growth media were inoculated, and incubated for 20-24 hours at room temperature with continuous shaking at 150 revolutions per minute on a gyratory shaker (New Brunswick Scientific Co.) to provide adequate aeration. Cells were then collected by centrifugation at 10,000 g for 15 minutes. The supernatant was discarded and the cells were washed in 20 ml 0.02 M potassium phosphate (pH 7.8) twice, and suspended in 7-10 ml 0.02 M potassium phosphate buffer (pH 7.8). The washed cell suspension was stored in ice.

Substrate solutions

The concentration of the substrate solution for the induced enzyme 2,3-DOB oxygenase was 10 mM 2,3-DOB in 20 mM potassium phosphate buffer (pH 7.8).

Oxygen consumption determination

Oxygen consumption was measured polaragraphically by a Clark oxygen electrode (Yellow Springs Instrument Co.). Oxygen content of the reaction medium was assumed equal to that in air-equilibrated pure water (e.g., 273, 268, 263 mM at 22°, 23°, and 24° respectively).

Dry weight estimation of the bacterial cells

The dry weight of the bacterial cells was estimated by the method of Ito (unpublished). The method was as follows. Cell suspensions were diluted appropriately with distilled water, usually 1:100, and the optical density at 540 nm was determined by a Coleman 124 Linear Absorbance Spectrophotometer. If the optical density was more than 0.5, the cell suspension was diluted further. The concentration of the cells in the diluted suspension in mg dry weight cells/ml was calculated by dividing the respective optical density by the extinction coefficient, 3.68 $(mg/ml)^{-1}cm^{-1}$. The dry weight of the cells in mg/ml of the original suspension was calculated by multiplying the dilution factor (100) by the concentration obtained from the diluted suspension.

Preparation of cell free extracts

Approximately 3-5 ml of washed whole cells suspended in 0.02 M potassium phosphate buffer (pH 7.8) were sonified for 6 minutes at an

operating power setting of 4 with a 130 watt, model W-140D sonifier (Bronson Co.). The microtip probe and solution were cooled in an ice bath during the treatment to prevent temperature increase and protein denaturation. The sonified suspension was centrifuged at 10,000 g for 15 minutes to remove any unbroken cells and large cell fragments. The supernatant portion (crude extract) was stored at 4°C. In some experiments, the crude extract was further centrifuged at 100,000 g for one hour by a Beckman L5-75 ultracentrifuge. The latter will be referred to as the ultracentrifuged (UC) extract.

Protein determinations

Protein concentrations in the crude and UC extracts were determined by the method of Layne (1957).

Determination of pH and temperature optima

One liter of the glutamate growth medium to which 0.4 g 2,3-DOB was added as the inducer was inoculated with *P. fluorescens*. The culture was incubated for 20 hours, harvested, and a crude extract obtained. The 2,3-DOB oxygenase activity was determined by the oxygen electrode in a 10 mM 2,3-DOB solution in various buffers (acetate, phosphate, and carbonate in 20 mM concentrations) whose pH values ranged 4.0 to 10.0. The pH of the substrate solutions following oxygen uptake determinations was monitored as a precautionary measure. The activity was also determined at temperatures ranging from 0°C to 55°C in the presence of 10 mM 2,3-DOB in potassium phosphate buffer (0.02 M, pH 7.8). This was accomplished by the use of a reaction vessel within a temperature-controlled water bath.

Examination of enzyme stability in crude, ultracentrifuged and phosphatide-treated extracts

Three liters of the glutamate growth medium to which 0.4 g 2,3-DOB per liter were added was inoculated with P. fluorescens and grown for 21 hours at room temperature under aerated conditions. The cells were centrifuged for 5 minutes at 15,000 g and washed with 0.02 M potassium phosphate buffer (pH 7.8). Washed whole cells were suspended in 15 ml 0.02 M potassium phosphate buffer (pH 7.8). A 2 ml aliquot of this cell suspension was saved on ice. The rest of the cells were sonified, centrifuged, and the supernatant saved as a crude extract. A 2 ml aliquot of the crude extract was saved on ice. The remaining crude extract was subjected to ultracentrifugation for 1 hour at 100,000 g and the supernatant was obtained as a UC extract. A 2 ml aliquot of the UC extract was saved on ice. To three 3 ml aliquots of the remaining UC extract, either 5 mg phosphatidylcholine (PC), or 5 mg phosphatidyl ethanolamine (PE), or 5 mg of both PC and PE (PC + PE) was added. These were then sonified in order to dissolve the phospholipids as miscelles and to form a PC extract, a PE extract, and a (PC + PE) extract. A 1 ml aliquot of the UC extract with no phospholipids added was sonified to see the effects of sonification alone.

Dry weight of the cell suspension and protein content of each of the extracts were determined. The 2,3-DOB oxygenase activity was determined by the oxygen electrode immediately, and at 1, 3, 7 and 10 days after the extracts were prepared. The extracts were refrigerated during storage.

Enzyme induction time and transport of 2,3-DOB by starved cells

One-half liter of the glutamate growth medium was inoculated with P. fluorescens and grown to 20 hours maturity under aerated conditions. The cells were centrifuged 5 minutes at 10,000 g and washed twice with 0.02 M potassium phosphate buffer (pH 7.8). The cell paste was finally suspended in 100 ml of the phosphate buffer and placed in a refrigerator (4°C) for 20 hours.

To each of six 50 ml centrifuge tubes was added 9 ml of the cell suspension and 1 ml bacterial salt solution, and 1 ml 10 mM 2,3-DOB in 0.02 M potassium phosphate buffer (pH 7.0). The time at which 2,3-DOB was added was the "zero" time (T_o). A control for each was prepared by substituting 1 ml 0.02 M potassium phosphate buffer (pH 7.0) for the 2,3-DOB. At 30 minutes, 1 hour, 2 hour, 3 hour, 4 hour, and 5 hour intervals following the 2,3-DOB addition, an experimental and a control centrifuge tube were centrifuged for 5 minutes at 10,000 g. The supernatant was saved and the cells washed once in 10 ml 0.02 M potassium phosphate buffer (pH 7.8), this supernatant was added to the previous one. The combined supernatants were brought to a total volume of 50 ml in a volumetric flask with 0.02 M potassium phosphate (pH 7.8) and absorbance was recorded at 305 nm. Likewise, 1 ml of the original 10 mM 2,3-DOB solution was diluted 1:50 with 0.02 M potassium phosphate (pH 7.8) and the absorbance at 305 nm was recorded in order to determine the millimolar extinction coefficient (approximately 3.7) for 2,3-DOB at 305 nm. The cell sediment was resuspended in 5 ml 0.02 M potassium phosphate buffer (pH 7.8). Each cell suspension was checked for dry weight as well as for 2,3-DOB oxygenase activity.

Enzyme induction time and transport of 2,3-DOB by growing cells

Six one-liter flasks containing 225 ml each of the glutamate growth medium were inoculated with P. fluorescens. These cultures were allowed to incubate at room temperature for 14 hours under aerated conditions. Twenty-five ml of 10 mM 2,3-DOB in 0.02 M potassium phosphate (pH 7.0) was added to each of the five flasks. The time was recorded and designated T_o. The cells from the flask to which no 2,3-DOB was added were harvested and washed once in 0.02 M potassium phosphate (pH 7.8) and suspended in 10 ml of that buffer. The contents of each of the other five flasks were centrifuged for 5 minutes at 10,000 g and washed at 1 hour, 2 hour, 3 hour, 4 hour, and 5 hour intervals. The supernatant and wash at each time were combined and diluted with 0.02 M potassium phosphate (pH 7.8), and the absorbance at 305 nm was determined. Dry weight and 2,3-DOB oxygenase activity were determined for the cell suspensions obtained from the six flasks.

Electron microscopy techniques

One liter of cells were grown, harvested, and uniformly suspended in distilled water. A 1:1 mixture of cells and 2% phosphotungstic acid (pH 6.8) was prepared for the purpose of negative staining (Kay, 1967). A small drop of the mixture was placed on collodion-coated, copper grids as whole mounts and observed with a Hitachi HS-8 transmission electron microscope. Shadow-casted whole mounts were effected when whole mounted grids were placed in a vacuum evaporator and casted with a carbon-platinum rod.

Bacterial cells were fixed in Kellenberger's standard fixative

(Kay, 1967), dehydrated by a series of ethanol treatments, and embedded in Arladite 6005. Sections (approximately 600 Å Thick) were double stained with lead and uranium salts to complete sectioned staining and observed with the transmission electron microscope.

RESULTS AND DISCUSSION

Results of biochemical and physiological tests (Table 1, Appendix) performed on the bacterial species isolated from Pitt County soil on a selective medium containing 2,3-DOB as the sole carbon source (Ayers, 1978) were identical with those expected of Pseudomonas fluorescens, whose characteristics were established by Stanier et al. (1966). Sectioned bacterial cells observed with a transmission electron microscope revealed electron dense material and a scalloped cell wall (Fig. 2, Appendix). Whole mount preparations of these cells indicated polar multitrichous flagellation (Fig. 3, Appendix). The sonicated cells were well disrupted as evidenced by the electron micrograph (Fig. 4, Appendix).

Rapid oxygen uptake was exhibited by cells grown on 2,3-DOB when placed in that substrate solution, but little oxygen consumption occurred in the presence of potassium phosphate buffer (0.02 M, pH 7.8) alone (Fig. 5, Appendix). Results from testing growth media containing various carbon sources with and without the inducer 2,3-DOB indicated maximal enzyme production in the glutamate growth medium (Table 2, Appendix). Glucose in the growth medium completely inhibited 2,3-DOB oxygenase induction due to catabolite repression. Repression of enzyme synthesis also occurred when fructose was used as a carbon source. Figure 6 (Appendix) showed dependence of enzyme induction on the inducer concentration. A 0.2 g 2,3-DOB per liter of the glutamate medium gave high enzyme induction. This amount represented an 80% saving of the 2,3-DOB expenditure made when 2,3-DOB was the only carbon source in

a growth medium (Ayers, 1978). The temperature optimum for 2,3-DOB oxygenase was 37°C. Complete enzyme denaturation occurred between 50°C and 55°C (Fig. 7, Appendix). The pH optimum for the enzyme was near pH 8.0 with abrupt reduction in activity above that pH (Fig. 8, Appendix).

An immediate rapid uptake of 2,3-DOB was observed by starved, non-growing bacterial cells, whereas a much slower uptake was seen by a growing culture in the glutamate growth medium (Fig. 9, Appendix). The slower uptake indicated that the 2,3-DOB transport was inhibited by either glutamate (an alternate carbon source) or other nutrients or possibly metabolic products of the growing cells. It was difficult to determine from the data whether the transport involved a permease system (facilitated diffusion) or a mere simple diffusion. However, two characteristics seemed to suggest that the transport was by a permease system rather than by mere simple diffusion. Firstly, the rate of the transport was extremely rapid in the starved cells. Secondly, the transport was inhibited by some substances. The observations that cells rapidly accumulated certain compounds were taken as a suggestive evidence that the entry of nutrients into bacterial cells were mediated by selective permeation systems (Gale, 1947). Rickenberg and Lester (1955) showed convincing evidence that such a system regulated the internal concentration of β-galactosides in E. coli. If the transport system of 2,3-DOB in this organism was indeed a permease-mediated type, the system appeared to be constitutive, for these cells were not adapted to 2,3-DOB at T_o. It was not known whether the strain used in this study was a mutant which lacked the ability to repress the synthesis of

a specific transport system for 2,3-DOB or the transport system was a non-specific carrier for carbon sources. Although 2,3-DOB appeared to accumulate in a high concentration in the starved, non-growing cells within 30 minutes, production of the enzyme began after a two-hour exposure to the inducer (Fig. 10, Appendix). It was possible that this lag period was attributed to the limited availability and utilization of the "free amino acid pool" within the starved cells, and that protein turnover could have supplied the necessary supply of intracellular amino acids which were then resynthesized into the enzyme. Since the total cell density remained constant in the starved cells, 2,3-DOB oxygenase was probably the major newly synthesized protein. Although addition of the inducer to the rapidly-growing culture elicited immediate switch-on of production of the induced enzyme, the enzyme formed a smaller proportion of the newly synthesized protein (Fig. 10, Appendix).

Ayers (1978) indicated that the purified enzyme lost almost its entire activity in about a week, but that the crude enzyme extract appeared to be relatively more stable. In order to find if the stability was related to possible association of the enzyme with membrane lipids derived from the cell fragments, effects of phospholipids on the stability of the enzyme were examined. The turbid crude extract evidently contained cell fragments but no whole cells, whereas the clear UC extract was practically free of particulate matter. The oxygenase activity was consistently higher in the UC extract than in the crude extract throughout the ten-day period except at the day 7 (Fig. 11, Appendix). It was possible that the crude extract contained inhibitors,

or proteases which were removed by ultracentrifugation. The phospholipid extracts showed a higher activity and a longer retention of activity than the sonified UC extract. This result appeared to indicate that phospholipids protected against the damage caused by sonification, which was evidenced by the lower activity of the sonified UC extract relative to that of the UC extract (unsonified).

Of the nine compounds studied, none were gratuitous inducers of 2,3-DOB oxygenase. None but 2,3-DOB served as the substrate for the enzyme, although possible presence of permeability barrier in this organism against some of the substrates tested might have prevented interaction of these substrates with the enzyme. The induced enzyme exhibited not only high substrate specificity but also high inducer specificity for 2,3-DOB.

Degradative pathways of benzoate, catechol, para-hydroxybenzoate, and protocatechuate by this P. fluorescens strain probably followed the well-established β-ketoadipate pathway for many Pseudomonas species. That is, benzoate was metabolized via catechol and p-hydroxybenzoate via protocatechuate with eventual conversion of both metabolites into β-ketoadipate (Ornston, 1971). Results presented in Table 3 (Appendix) were certainly in accord with the presence of the separate degradative pathways from benzoate and p-hydroxybenzoate. However, the enzyme induction pattern by catechol needed explanation. It appeared that catechol and/or its metabolic products were the inducers for the synthesis of enzymes necessary for not only the catechol degradative pathway but also the p-hydroxybenzoate degradative pathway. Since both pathways converge at β-ketoadipate, this compound might have been the

inducer of all of these enzymes. However, since cis, cis-muconate but not catechol was an inducer of catechol oxygenase in Pseudomonas described by Ornston (1971), it was possible that the catechol oxygenase of this organism under study was induced by a small amount of cis, cis-muconate produced from catechol by the basal level of catechol oxygenase possibly present in the unadapted cells. High oxygen uptake of the cells grown on catechol when exposed to protocatechuate occurred with the strain of P. fluorescens of the present study (Table 3) indicating induction of protocatechuate oxygenase by any one of the intermediates on the catechol degradative pathway namely catechol, cis, cis-muconate, β-ketoadipate enol-lactone and β-ketoadipate (Fig. 1, Appendix). Similar examples of cross-induction of certain enzymes of the protocatechuate and catechol pathways by metabolites of one or the other have been reported (Hosokawa, 1970; Kemp and Hegeman, 1968; Ornston, 1966). Poor growth was noted when ortho-vanillin, ortho-vanillic acid, 3-methylcatechol, and 4-methylcatechol were present in low concentrations in the growth medium. The results (Table 3, Appendix) indicated an inability of this P. fluorescens strain to metabolize any of these diphenols. It was also possible that some impurities of these diphenols employed were the growth inhibitors.

SUMMARY

The soil bacterium isolated by Ayers (1978) on 2,3-DOB selective medium was identified as Pseudomonas fluorescens. This strain grew best at room temperature (24-25°C) under aerated conditions.

Glutamate was chosen as an alternate carbon source in the growth medium due to its lesser interference with 2,3-DOB oxygenase production by P. fluorescens. Glucose and fructose completely inhibited 2,3-DOB oxygenase induction. High enzyme induction was observed using 0.2 g 2,3-DOB per liter of glutamate growth medium. This finding resulted in a reduced 2,3-DOB expenditure. No alternate, less expensive inducers were found to elicit production of 2,3-DOB oxygenase. Among the compounds tested in this study, 2,3-DOB was the only substrate of the enzyme.

The temperature optimum for 2,3-DOB oxygenase was established at 37°C and complete enzyme denaturation occurred between 50°C and 55°C. The pH optimum was near pH 8.0 with abrupt reduction in activity above that pH.

A permease-mediated transport system for 2,3-DOB in this organism was indicated. The rate of transport was extremely rapid in starved cells. (Approximately 85% of 2,3-DOB exposed to the cells was taken up within 30 minutes.) The rate of transport was probably inhibited in growing cells by glutamate or accumulated metabolites. Production of 2,3-DOB oxygenase began immediately after addition of 2,3-DOB to growing cultures, but in starved cells a two-hour lag period was observed between addition of 2,3-DOB and production of 2,3-DOB oxygenase. The

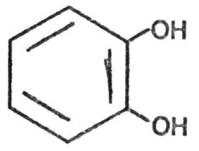
lag period was attributed to limited availability and utilization of the "free amino acid pool" within the starved cells.

The presence of phospholipids in the ultracentrifuged extract appeared to protect 2,3-DOB oxygenase activity against the effects of sonification. However, no direct relationship between enzyme stability and membrane phospholipids could be established in this study.

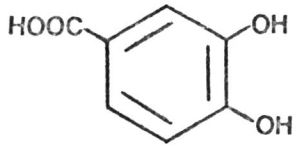
APPENDIX

APPENDIX (Continued)

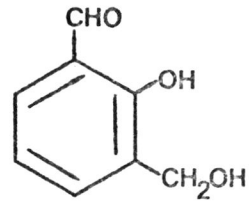
Fig. 1. Structures of some compounds discussed.



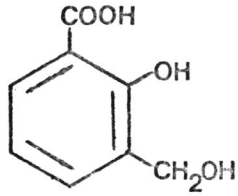
CATECHOL



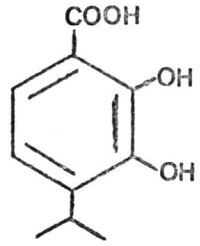
PROTOCATECHUIC ACID



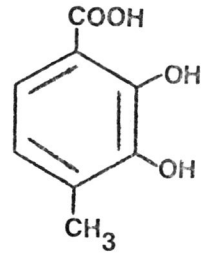
ORTHO-VANILLIN



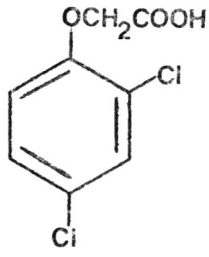
ORTHO-VANILLIC ACID



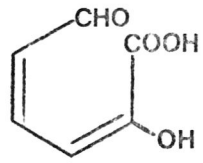
2,3-DIHYDROXY-P-CUMATE



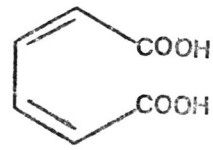
2,3-DIHYDROXY-P-TOLUATE



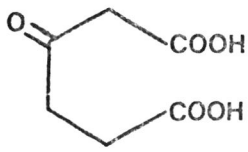
2,4-DICHLOROPHENOXY-ACETATE



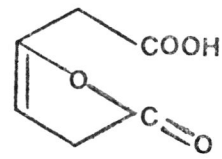
α-HYDROXY-MUCONIC SEMIALDEHYDE



CIS,CIS-MUCONATE



β-KETOADIPATE



β-KETOADIPATE ENOL-LACTONE

APPENDIX (Continued)

Table 1. Characters used in differentiation of Pseudomonas species within the fluorescent group (from Stanier et al., 1966). The third column represents results obtained with the Ayer's organism, and the characteristics established by Stanier et al.

Cyt-c : Cytochrome c

v : variable

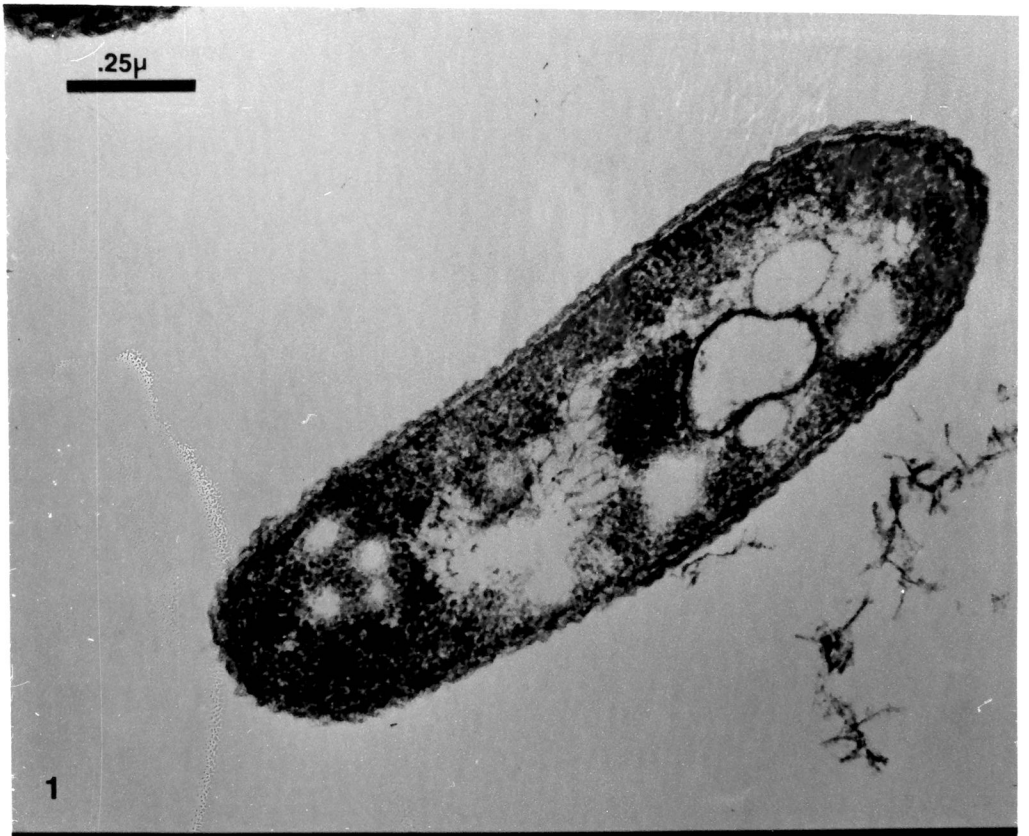
	P. aeruginosa	Ayer's organism and P. fluorescens	P. putida
Monotrichous flagellation	+	-	-
Growth at 4°C	-	+	v
Growth at 41°C	+	-	-
Gelatin liquification	+	+	-
Utilize meso-inositol	-	+	-
Cyt-c principal Soret band	428	427	425

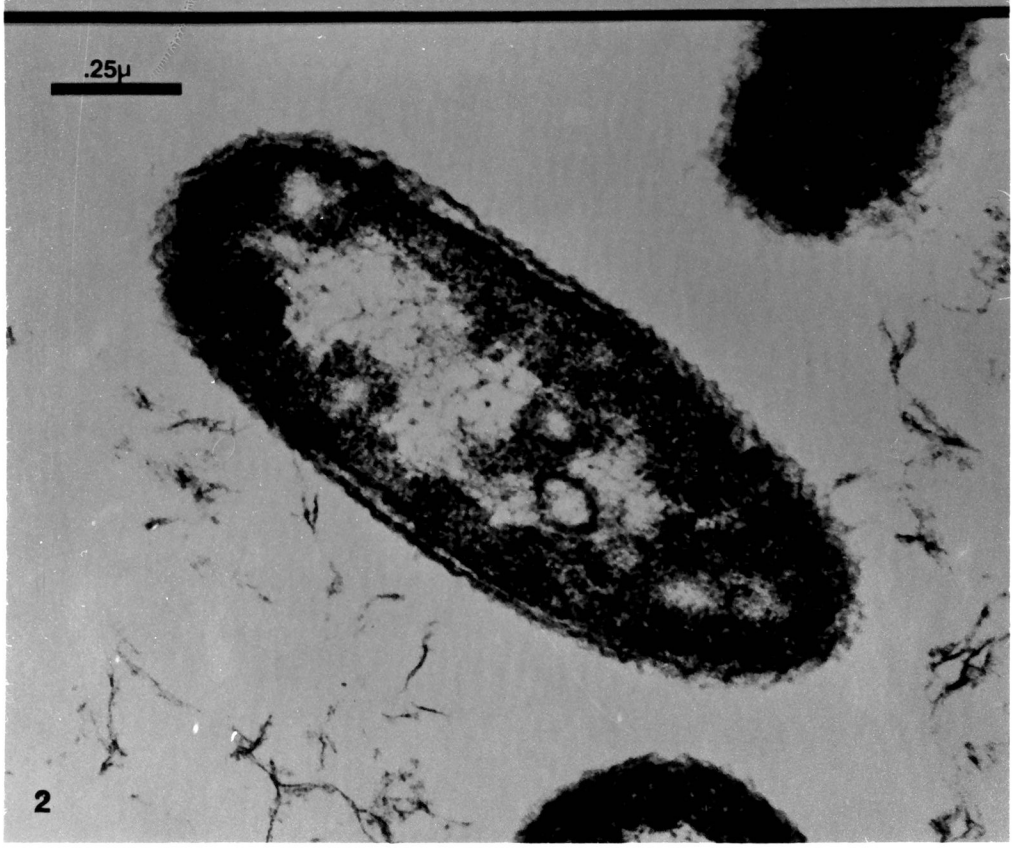
OTHER TESTS

Motility	+
Indol	-
Catalase	+
Oxidase	+
Lipase	+

APPENDIX (Continued)

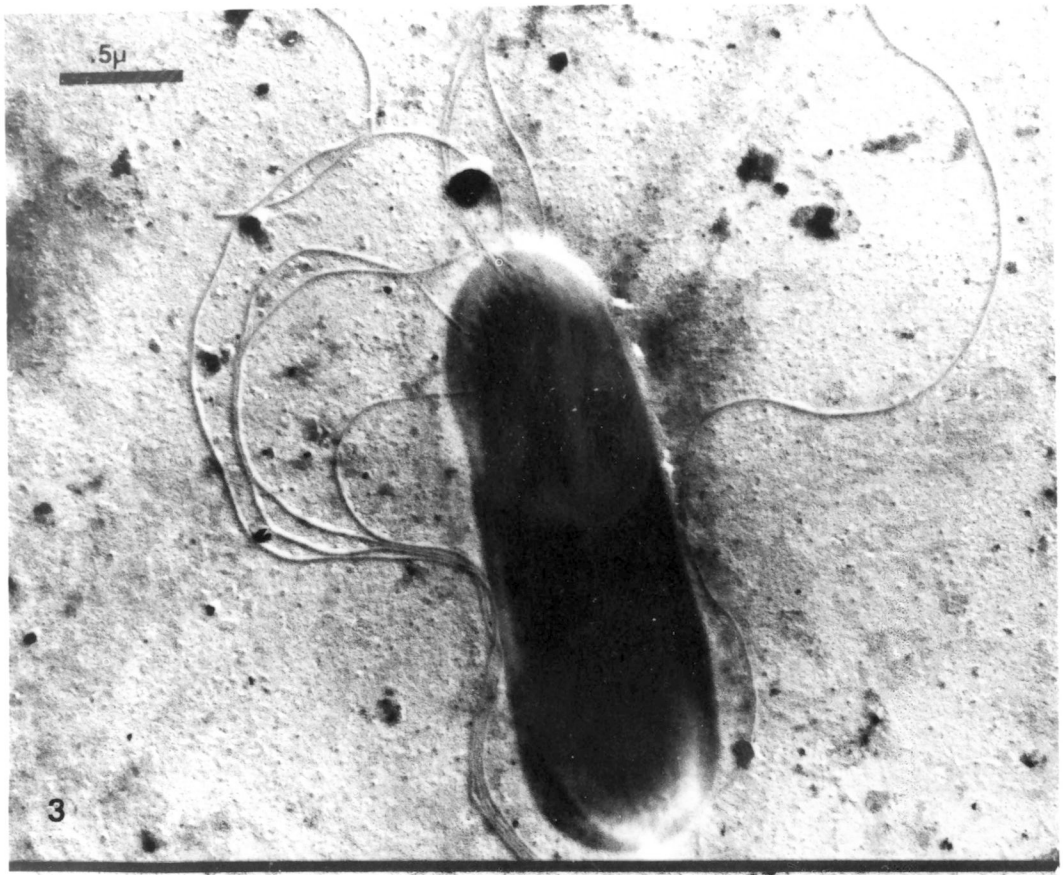
Fig. 2. Electron micrographs of sectioned Pseudomonas fluorescens, X68,400.

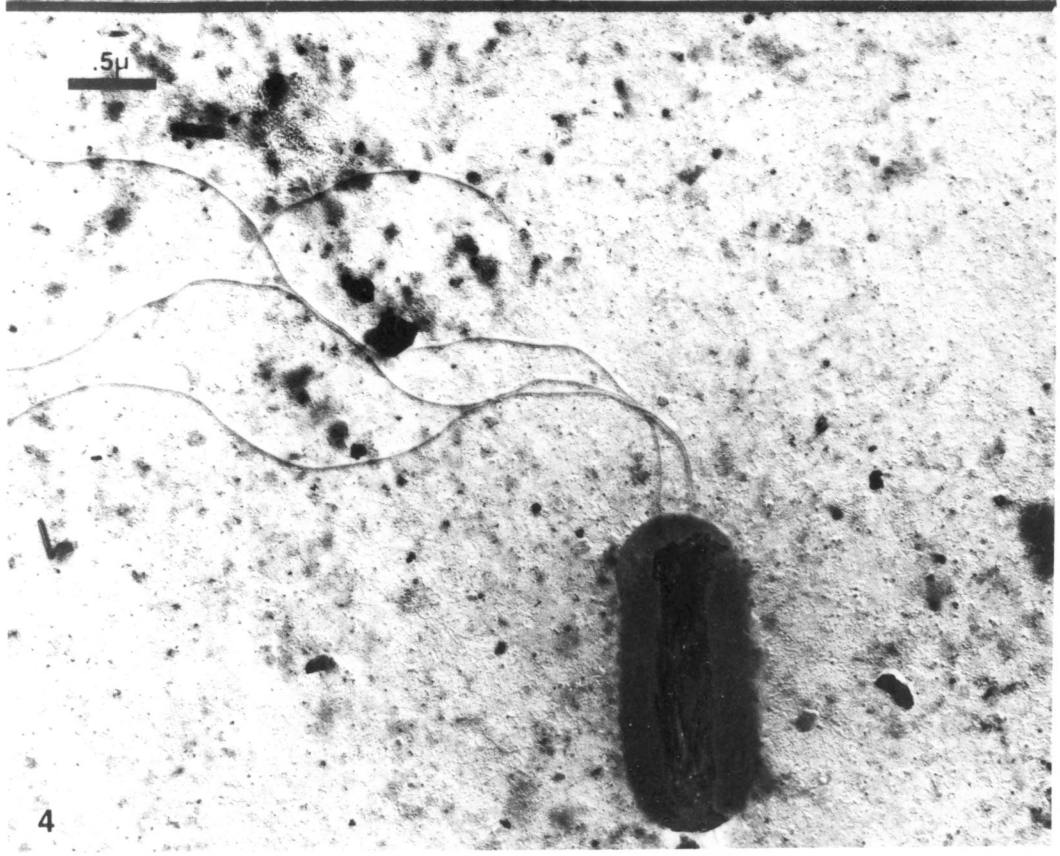

.25μ
1


.25μ
2

APPENDIX (Continued)

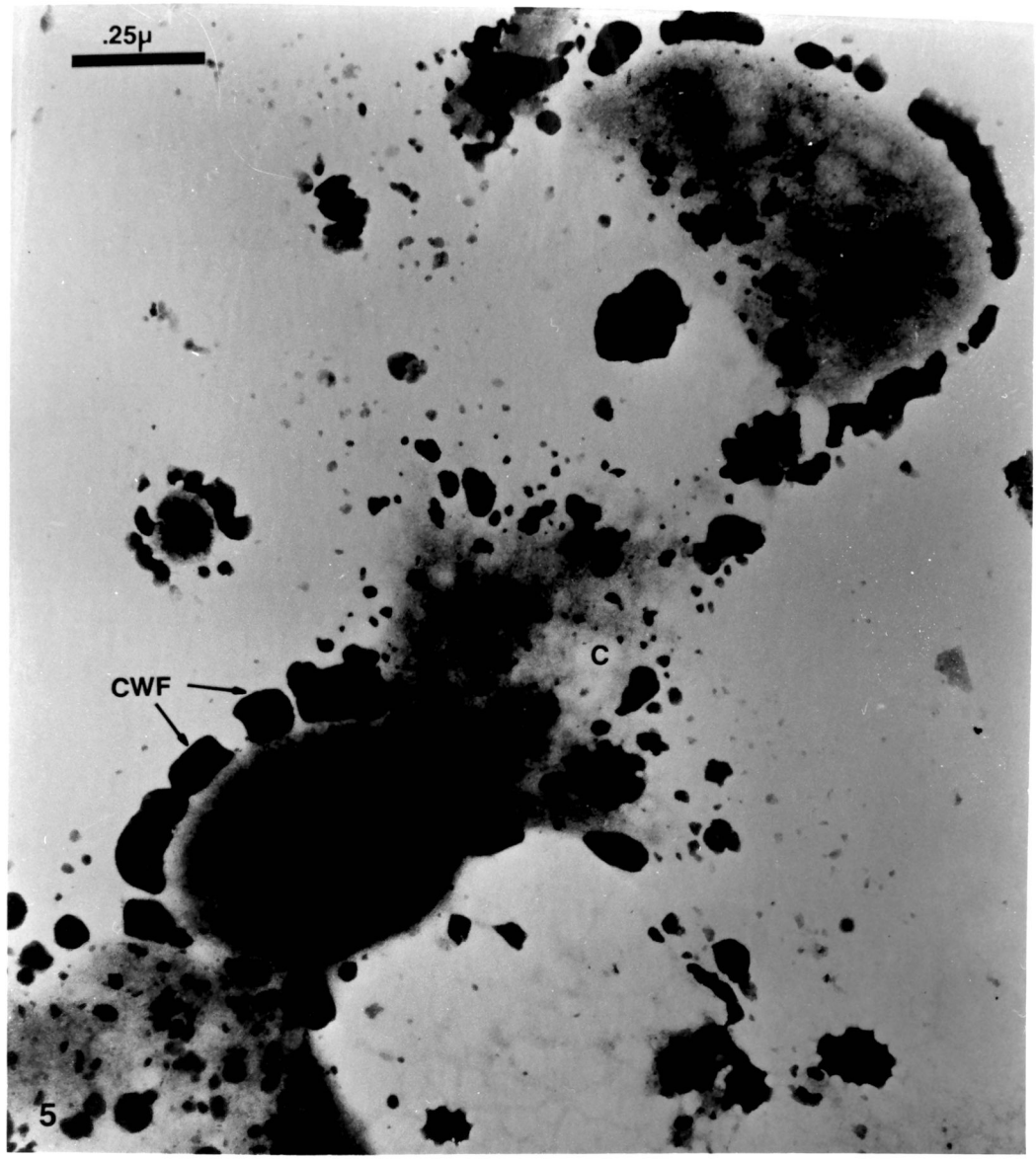
Fig. 3. Negatively stained, shadow-casted whole mount preparations of Pseudomonas fluorescens, X22,000 and X32,000 respectively.


.5μ
3


.5μ
4

APPENDIX (Continued)

Fig. 4. Negatively stained whole mount preparation of sonicated Pseudomonas fluorescens with cell wall fragments (CWF) and cytoplasm (C) labelled, X68,400.


.25μ
CWF
C
5

APPENDIX (Continued)

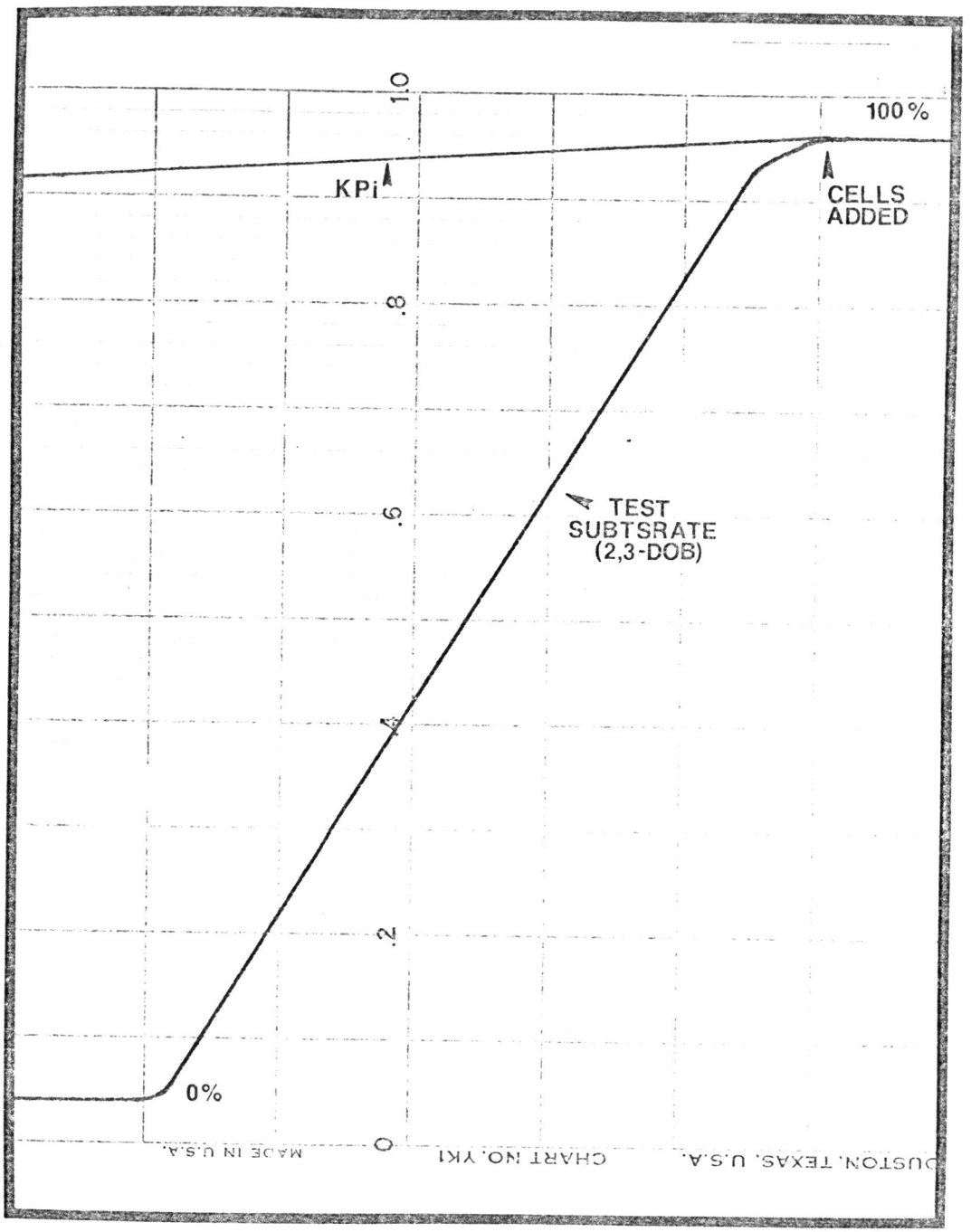
Table 2. Effect of various carbon sources on induction of 2,3-DOB oxygenase.

Enzyme activity is expressed in micro-moles oxygen uptake per minute per milligram cells dry weight. The amount of 2,3-DOB added to each growth medium was 1 g/liter where applicable.

CARBON SOURCE	SPECIFIC ACTIVITY WITHOUT 2,3-DOB	SPECIFIC ACTIVITY WITH 2,3-DOB
ACETATE	0.078	0.127
CITRATE	0	0.128
SUCCINATE	0.020	0.159
MALATE	0.018	0.142
LACTATE	0.040	0.195
GLUCOSE	0.002	0.002
FRUCTOSE	0.027	0.011
GALACTOSE	0.016	0.186
GLUTAMATE	0	0.220

APPENDIX (Continued)

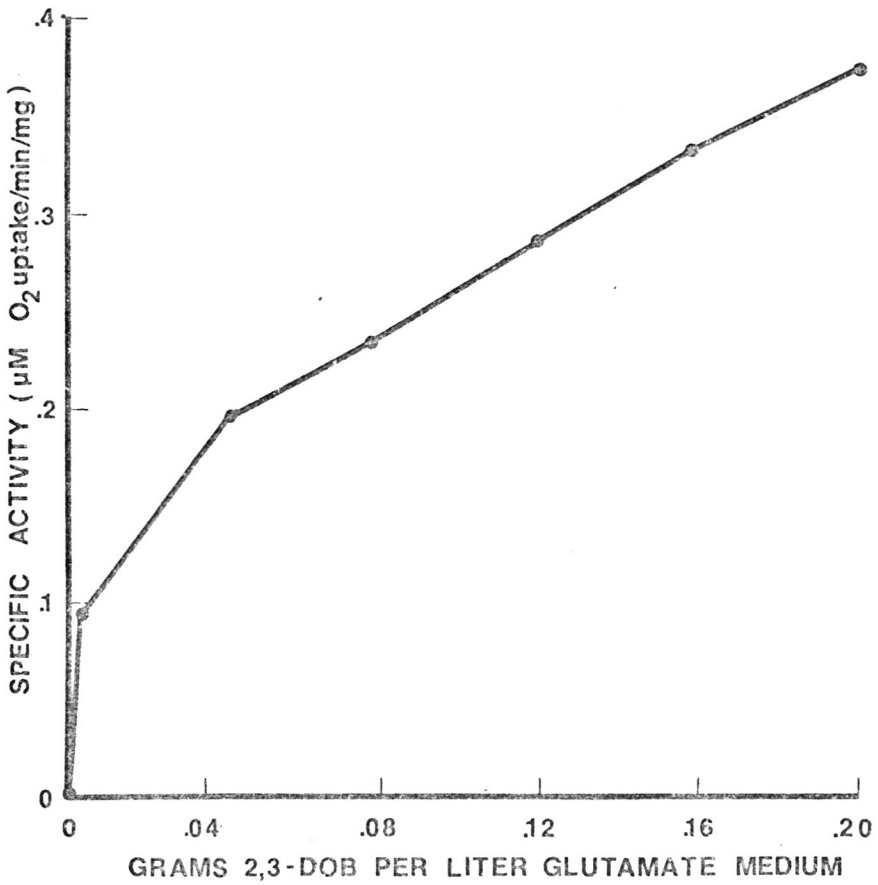
Fig. 5. Oxygen uptake by 2,3-DOB-grown cells when mixed with 2,3-DOB solution (10mM) and potassium phosphate buffer (20mM, pH 7.8) determined by the oxygen elctrode. The dry weight of the cells used was 8.97 mg/ml and the temperature was 24°C.


1.0
.8
.6
.4
.2
0
100%
KPi
CELLS
ADDED
TEST
SUBTSRATE
(2,3-DOB)
0%
MADE IN U.S.A.
CHART NO. YK1
HOUSTON, TEXAS, U.S.A.

APPENDIX (Continued)

Fig. 6. Effect of the inducer (2,3-DOB) concentration on induction of 2,3-DOB oxygenase.

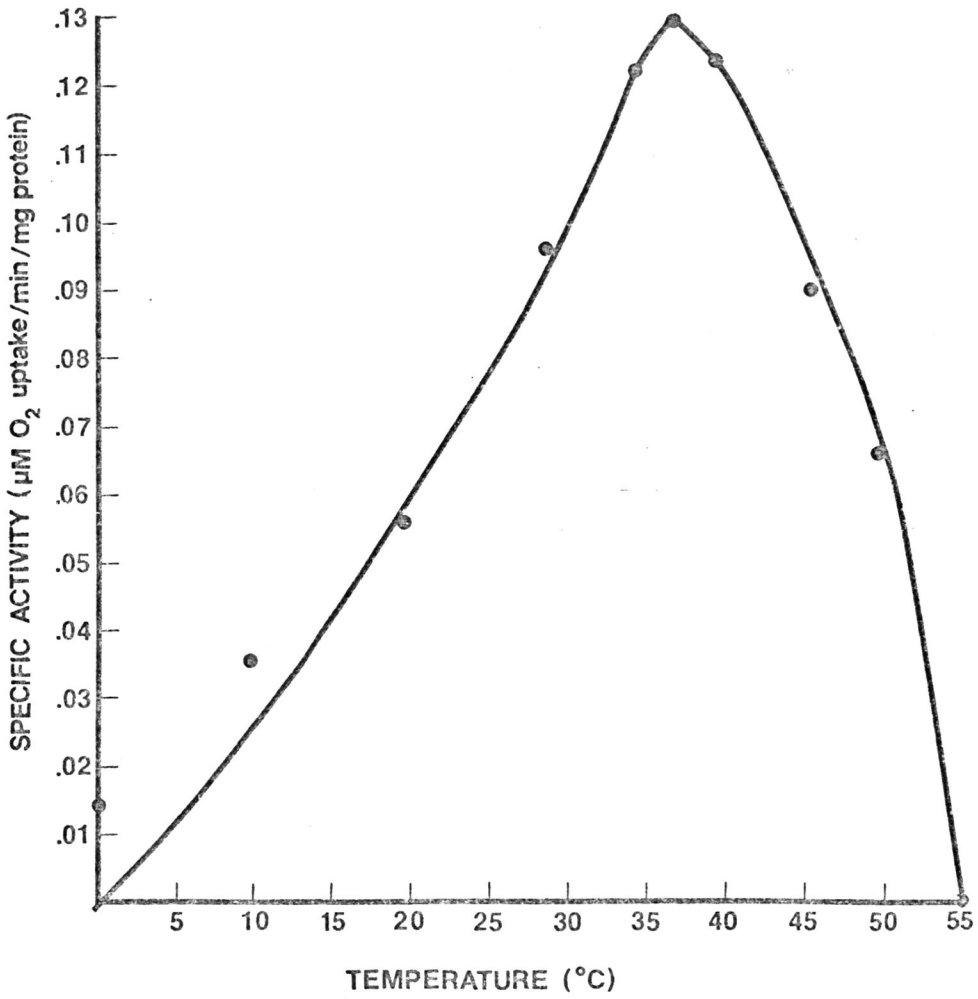
The specific activity was expressed in micro-moles oxygen uptake per minute per milligram cells dry weight.


SPECIFIC ACTIVITY (μM O_2 uptake/min/mg)
.4
.3
.2
.1
0
0
.04
.08
.12
.16
.20
GRAMS 2,3-DOB PER LITER GLUTAMATE MEDIUM

APPENDIX (Continued)

Fig. 7. Temperature optimum of 2,3-DOB oxygenase.

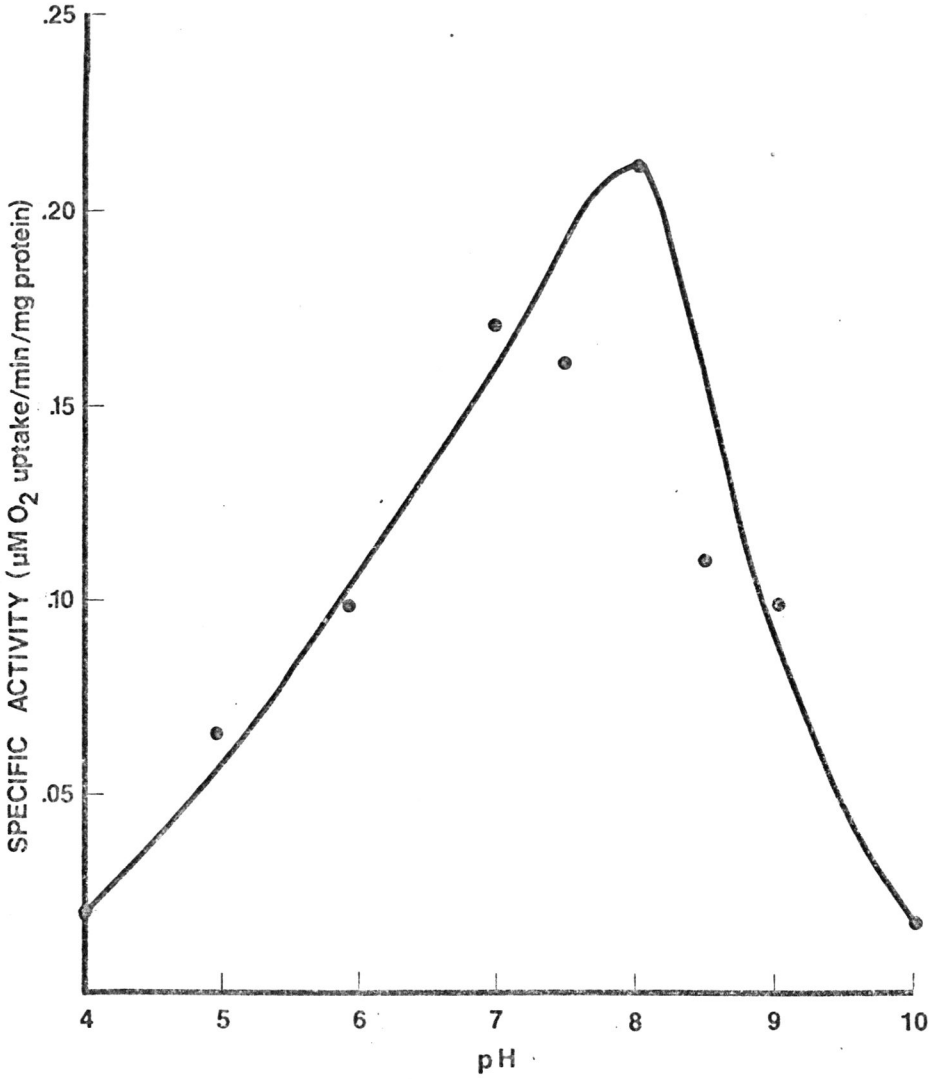
A crude extract obtained from cells grown in the standard glutamate medium with 2,3-DOB (0.4 g/liter) was used.


SPECIFIC ACTIVITY (μM O_2 uptake/min/mg protein)
.13
.12
.11
.10
.09
.08
.07
.06
.05
.04
.03
.02
.01
5
10
15
20
25
30
35
40
45
50
55
TEMPERATURE (°C)

APPENDIX (Continued)

Fig. 8. The pH optimum of 2,3-DOB oxygenase.

A crude extract of cells grown in the standard glutamate medium with 2,3-DOB (0.4 g/liter) was used.


.25
.20
.15
.10
.05
SPECIFIC ACTIVITY (μM O_2 uptake/min/mg protein)
4
5
6
7
8
9
10
pH

APPENDIX (Continued)

Fig. 9. Transport of 2,3-DOB into the bacterial cell.

- - - - - - - - - : Starved cells.

2,3-DOB was added at T_o, and the suspending medium was spectrophotometrically assayed at 305 nm at 30 minutes and each hour thereafter.

_____ : Growing cells.

2,3-DOB was added to growing cultures at T_o, and the suspending medium was spectrophotometrically assayed at 305 nm each hour. For specific details, see the text. Data for dry cell weight at each time for both starved and growing cells is provided in Fig. 10.

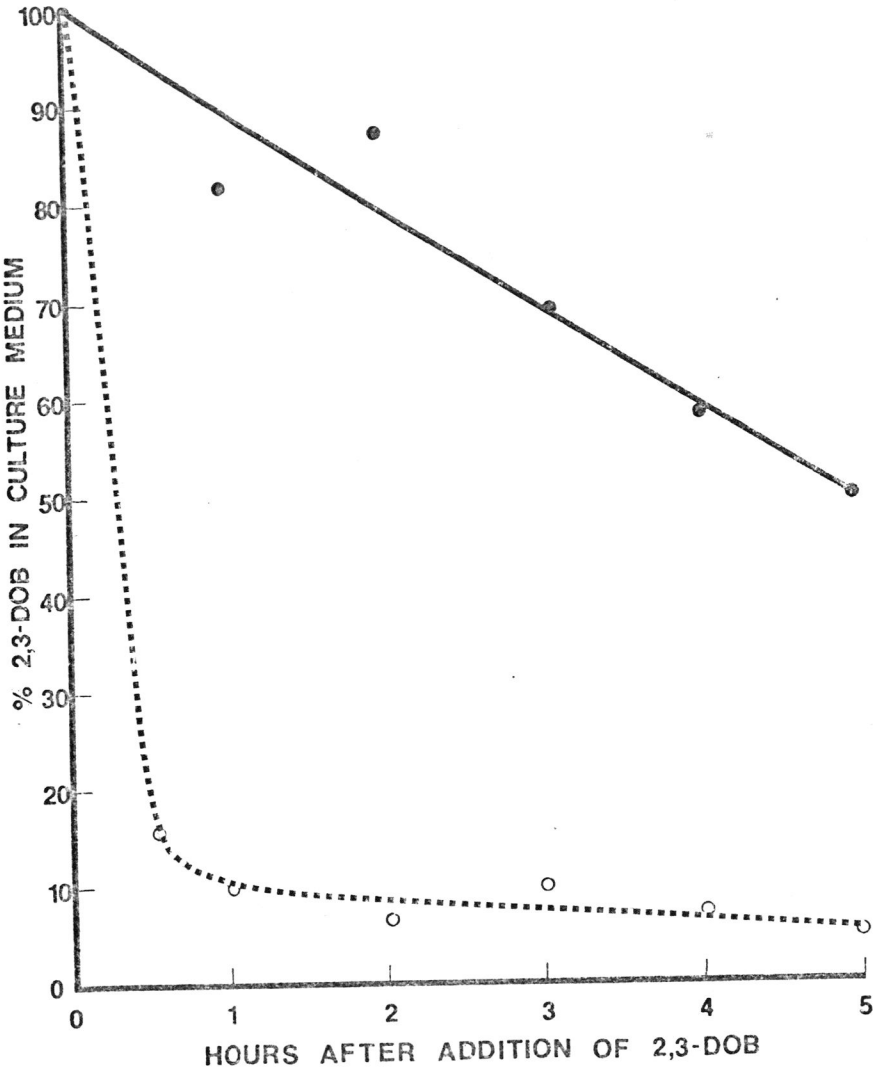

100
90
80
70
60
50
40
30
20
10
0
% 2,3-DOB IN CULTURE MEDIUM
0
1
2
3
4
5
HOURS AFTER ADDITION OF 2,3-DOB

Fig. 10. Induction of 2,3-DOB oxygenase in unadapted cells after addition of 2,3-DOB to the medium.

2,3-DOB was added at T_o and specific activity of the enzyme was determined with the oxygen electrode each hour. Specific activity was expressed in millimoles oxygen uptake per minute per milligram cells dry weight.

_____ : Specific activity of the enzyme in a growing culture.

- - - - - - - - - : Cell density of the growing culture.

——////——/// : Specific activity of the enzyme produced by starved cells in potassium phosphate buffer (0.02 M, pH 7.8).

— - — - — - — : Cell density of the non-growing culture.

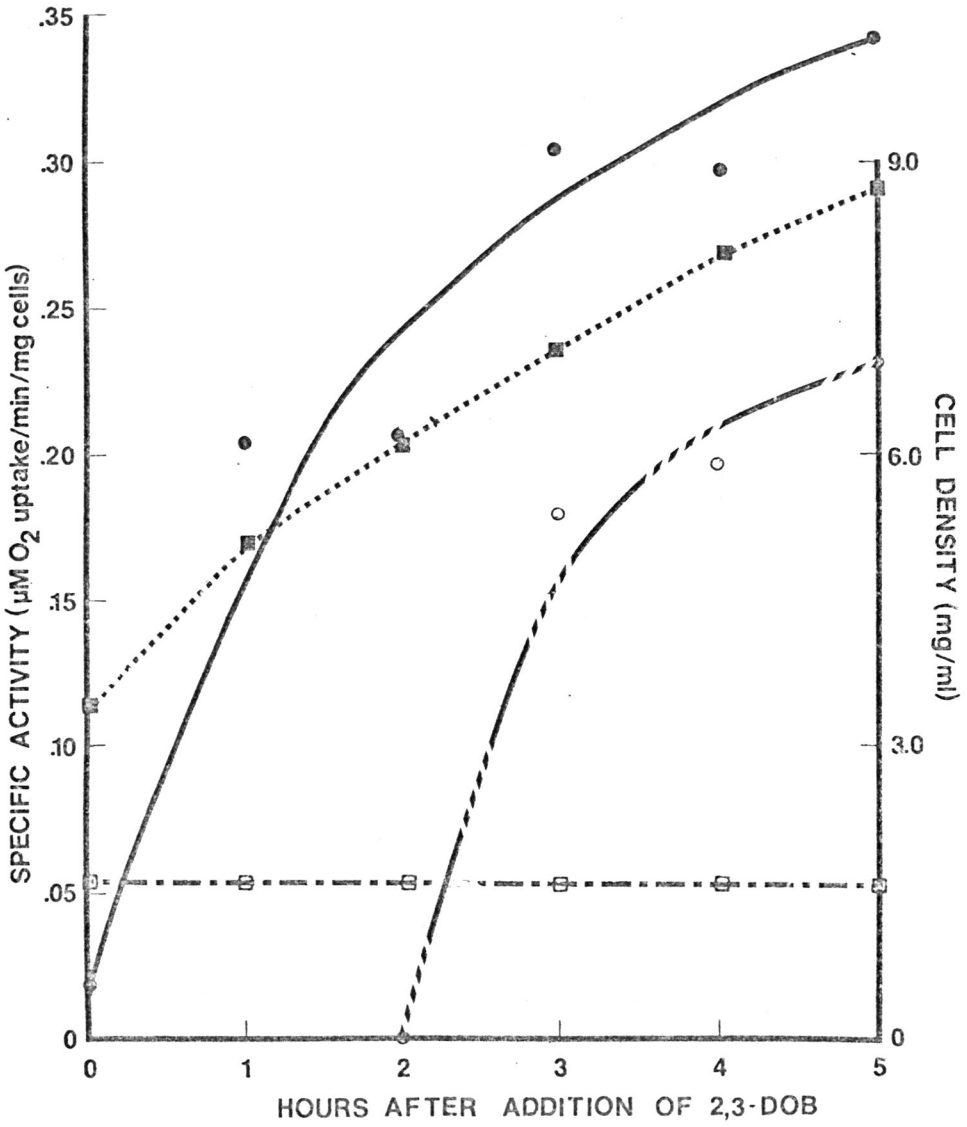

.35
.30
.25
.20
.15
.10
.05
0
SPECIFIC ACTIVITY (μM O_2 uptake/min/mg cells)
9.0
6.0
3.0
0
CELL DENSITY (mg/ml)
0
1
2
3
4
5
HOURS AFTER ADDITION OF 2,3-DOB

Fig. 11. Stability of 2,3-DOB oxygenase.

Crude, ultracentrifuged, and phosphatide-treated extracts were treated as described below and specific activity (expressed in micromoles oxygen uptake per minute per milligram protein) was checked immediately, at 1, 3, 7, and 10 days after the extracts were prepared, The specific activity was finally converted to % activity remaining.

—/—/—/—/—/— : Crude extract (fragmented cells spun for 10 minutes at 10,000 g). Concentration was 4.6 mg/ml.

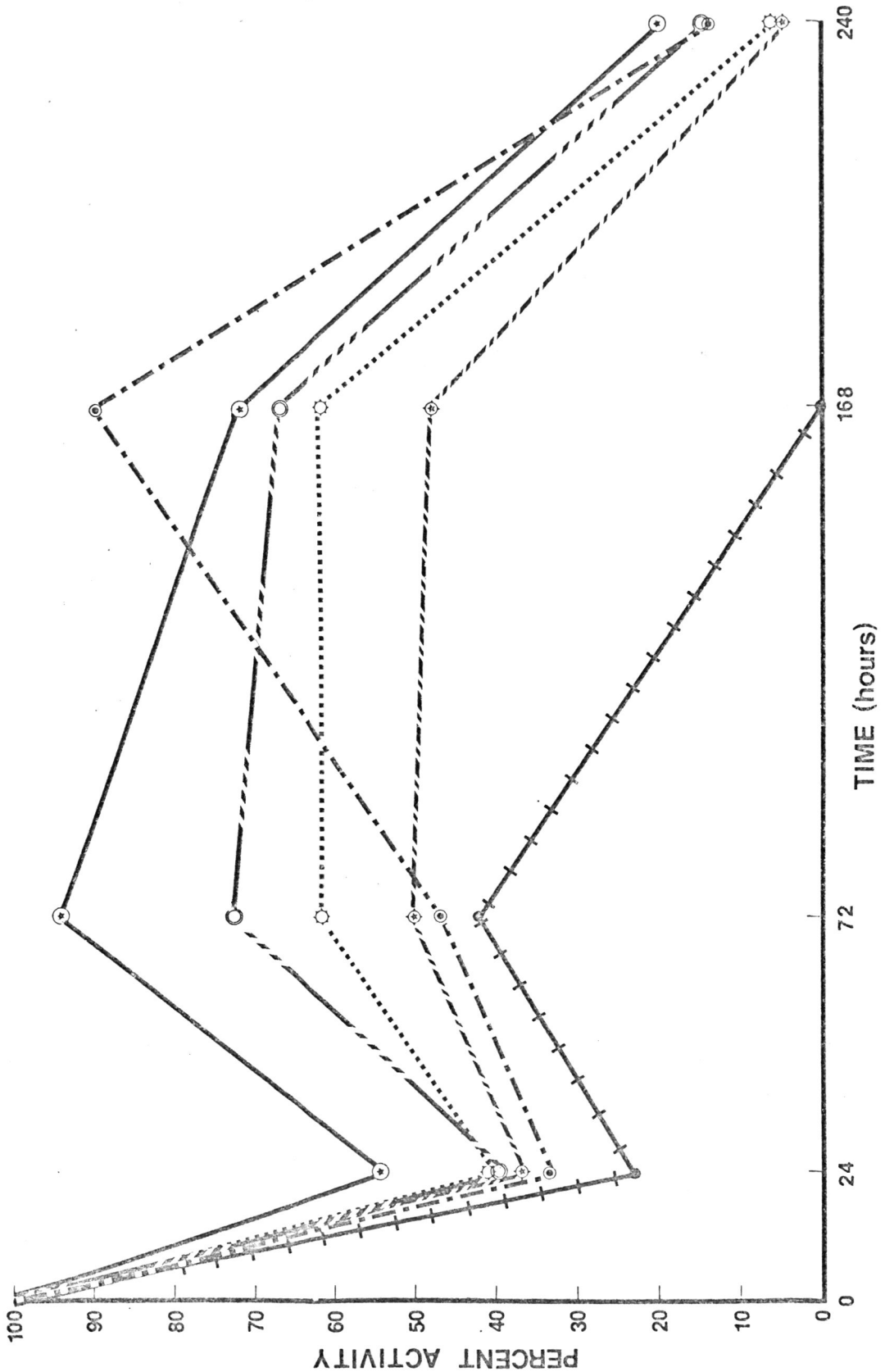
—+—+—+—+—+— : UC extract (crude extract ultracentrifuged 1 hour at 100,000 g). Concentration was 4.5 mg/ml.

_____ : Sonified UC extract. Concentration was 4.5 mg/ml.

—///—///—///— : PC extract (UC extract with phosphatidylcholine added and then sonified). Concentration was 4.5 mg/ml.

- - - - - - - - - - : PE extract (UC extract with phosphatidylethanolamine added and then sonified). Concentration was 4.5 mg/ml.

— - — - — - — : (PC + PE) extract (UC extract with both phosphatides added and then sonified). Concentration was 4.4 mg/ml.


PERCENT ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
0
24
72
168
240
TIME (hours)

APPENDIX (Continued)

Table 3. Induction pattern of various enzymes including 2,3-DOB oxygenase in P. fluorescens.

The numbers represent specific activity (micromoles oxygen uptake per minute per milligram cells dry weight) of the washed whole cell suspensions in potassium phosphate buffer (0.02 M, pH 7.8) prepared from P. fluorescens grown in the medium containing the compounds listed in the top row. Each cell suspension was tested for oxidation of the substrates listed in the first column.

SUBSTRATES	INDUCERS IN GLUTAMATE MEDIUM: 2,3-dihydroxybenzoic acid	benzoic acid	catechol	para-hydroxybenzoic acid	protocatechuic acid	ortho-vanillin	ortho-vanillic acid	4-methylcatechol	3-methylcatechol	
2,3-dihydroxybenzoic acid	0.300	0.032	0	0	0	0.053	0.017	0.060	0.046	
benzoic acid	0.017	0.713	0	0						
catechol	0	0.216	0.194	0.023	0					
para-hydroxybenzoic acid	0	0.084	0	0.581						
protocatechuic acid	0	0.049	0.249	0.516	2.388					
ortho-vanillin	0					0.028	0.021	0	0	
ortho-vanillic acid	0.029					0	0.028	0	0	
4-methylcatechol	0	0				0.022	0.026	0.027	0.029	
3-methylcatechol	0	0				0.028	0.027	0	0.044	

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