ABSTRACT

Alan Rogers Ayers. MECHANISMS OF ACTION OF 2,3-DIHYDROXYBENZOIC-3,4-DIOXYGENASE. (Under the direction of Takeru Ito) Department of Biology, February, 1978.

A soil bacterium (probably of the genus Pseudomonas) which could utilize 2,3-dihydroxybenzoic acid as a sole carbon source was isolated. The oxygenase which catalyzed the initial degradative reaction of 2,3-dihydroxybenzoic acid was purified and examined to determine whether the reaction was simultaneous or a two step process. Based on data obtained from ion-exchange chromatography, Warburg analysis, and studies of oxidative metabolism, it was determined that the oxidation of 2,3-dihydroxybenzoic acid was a one step process involving the simultaneous uptake of oxygen and release of carbon dioxide.

MECHANISMS OF ACTION OF 2,3-DIHYDROXYBENZOIC-3,4-DIOXYGENASE

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INTRODUCTION

Microbial oxidative catabolism of aromatic compounds has been and will continue to be an extremely important area of study for a more complete understanding of nature's mechanisms for degrading and releasing carbon atoms bound in these ringed chemical structures. Certain soil bacteria, such as Pseudomonas, possess the capability to catalyze the oxidation of numerous naturally occurring aromatic compounds using molecular oxygen. Further degradative reactions result in intermediates in general biochemical pathways such as the TCA cycle (Dagley 1972).

One aromatic compound, 2,3-dihydroxybenzoic acid (2,3-DOB), has been identified as a constituent of Populus balsamifera (Goris and Canal 1936) and was found to be degraded by a soil dwelling species of Pseudomonas (Ribbons 1966, Roof et al. 1953, Ribbons and Senior 1970). Initial cleavage of 2,3-DOB was achieved by an oxygenase to form the product α-hydroxymuconic semialdehyde (Ribbons 1966, Ribbons and Senior 1970). The enzyme was described as an intramolecular, extradiol dioxygenase which cleaved a C=C bond of the aromatic ring adjacent to a hydroxylated carbon atom (Ribbons and Senior 1970, Hayaishi and Nozaki 1969). In the cleavage reaction, one molecule (2 atoms) of oxygen was incorporated into the ring structure, and one molecule of carbon dioxide was released to produce α-hydroxymuconic semialdehyde. Simultaneous incorporation of oxygen and release of carbon dioxide was assumed to be catalyzed by a single enzyme (Ribbons and Senior 1970). The assumption was based on evidence collected from a crude enzyme extract of Pseudomonas. The present study was undertaken in order to test its validity using a

purified enzyme preparation. The evidence presented in this paper seems to support the assumption.

REVIEW OF LITERATURE

A vast number of organic compounds are continually being synthesized, transformed, and decomposed by living organisms of the biosphere. Among these are several aromatic compounds that are generally characterized by one or several chemical (R) groups attached to one or several benzene rings. Since the benzene ring has a relatively stable structure due to its large resonance energy, the aromatic compounds will not readily break down to release the bound carbon atoms for recycling in ecosystems (Gibson 1968). If benzene and its derivatives (natural and artificial sources included) were not degraded, a vast accumulation of carbon would occur in the environment and would not be avilable for recycling. However, it is well known that certain aerobic microorganisms (bacteria and fungi) are able to release these carbon atoms by oxidizing the aromatic compounds to intermediates in the TCA cycle and other central biochemical pathways (Dagley 1975).

It is generally accepted that dihydroxylation is a prerequisite for enzymatic fission of the benzene ring (Gibson 1968). This enables the microorganisms to activate and incorporate molecular oxygen into the aromatic structure by oxygenase enzymes (Hayaishi and Nozaki 1969, Dagley 1972). Many of these oxygenases are inducible enzymes which are present in the cell at extremely low concentrations until the cells are grown at the expense of a particular aromatic compound as a sole carbon source (Dagley 1975). In this situation, the carbon source induces the cell to produce large concentrations of the oxygenase for its initial degradative step. However, exposure of the initial substrate to the organism results

in induction of all the enzymes that are directly involved in the utilization of that substrate. This sequential induction theory has been cited frequently in studies of bacterial metabolism (Spieglman 1948, Karlsson et al. 1948, Stanier 1948, and Monad et al. 1951). Thus, induction of the enzymes by the cell initiates one and only one pathway for complete metabolism of the substrate to carbon dioxide and water (Dagley 1972).

The oxygenases may be classified as two major types. Dioxygenases catalyze reactions in which both atoms of molecular oxygen are incorporated into their substrates. However, monooxygenases catalyze incorporation of one atom of oxygen into a substrate with another oxygen atom being reduced to water (Hayaishi et al. 1975). Because of its relevance to the work described in this paper, dioxygenases will be discussed in regard to substrate degradation.

In cleavage of an aromatic ring, dioxygenases catalyze incorporation of molecular oxygen into two double-bonded carbon atoms. One or both carbon atoms of the double bond may be hydroxylated. When the cleavage occurs between carbon atoms which are both hydroxylated, the dioxygenase is termed an intradiol enzyme. However, if the cleavage occurs between carbon atoms with only one carbon atom being hydroxylated, the enzyme is an extradiol type (Hayaishi et al. 1975). For example, pyrocatechase (intradiol) oxidizes catechol to cis,cis-muconic acid, but meta-pyrocatechase (extradiol) oxidizes catechol to form α-hydroxymuconic semialdehyde (Gibson 1968). Both types of dioxygenases characteristically contain a non-heme iron (Fe^{+++} or F^{++}) as a cofactor for the activation of oxygen (Hayaishi et al. 1975). Extradiol and intradiol

dioxygenases possess the ferrous and ferric forms respectfully. Thus, the ability of aerobic microorganisms to activate oxygen with dioxygenases is very important in liberating carbon atoms utilized in metabolic pathways for the requirements of growth.

Catechol dioxygenases were cited previously due to their close resemblance in enzymatic activity to 2,3-dihydroxybenzoic 3,4-dioxygenase (Ribbons and Senior 1970). Catechol and 2,3-dihydroxybenzoic acid (2,3-DOB) are catalyzed by metapyrocatechase and 2,3-dihydroxybenzoic 3,4-dioxygenase respectively to form a common product, α-hydroxymuconic semialdehyde, which absorbs maximally at 373 nm under alkali conditions (Dagley 1960, Ribbons and Senior 1970). However, unlike catechol oxidation, 2,3-DOB oxidation yields one mole of carbon dioxide for the one mole of oxygen utilized in forming α-hydroxymuconic semialdehyde. From this step, evidence shows that α-hydroxymuconic semialdehyde is converted sequentially to α-keto-γ-pentenoic acid, α-keto-γ-valeric acid, and finally pyruvate and acetealdehyde (Dagley 1972) as seen in Figure 4 (Appendix A).

Ribbons and Senior (1970) determined the position of cleavage of 2,3-DOB to occur between the third and fourth carbon atom of the benzene ring (extradiol dioxygenase responsible), but also mentioned that incorporation of oxygen was accompanied by a simultaneous release of carbon dioxide from the compound. However, "separation of the oxidative and decarboxylative activities has not been achieved" (Ribbons and Senior 1970). Thus a possibility still exists that the 2,3-dihydroxybenzoate-3,4-dioxygenase consists of an oxygenase and a decarboxylative enzyme in the initial degradative step of 2,3-DOB. If there are two enzymes

responsible for this gaseous exchange, a complete purification of the enzyme(s) is necessary to determine the mechanism of action of this enzyme.

MATERIALS AND METHODS

STERILE SALINE SOLUTION

Test tubes (13 x 15 mm), each containing 5.4 ml of 0.9% sodium chloride solution were autoclaved and used for sterile transfer of the soil bacteria (Seaman 1962).

ENRICHMENT CULTURE MEDIUM

A culture medium containing 2,3-DOB as the sole carbon source was prepared for selective isolation of a soil bacterium. This medium was composed of: 1.50 g dibasic potassium phosphate, 0.45 g monobasic potassium phosphate, 3.0 g ammonium chloride, 0.10 g yeast extract, 0.10 g casein hydrolyslate, 0.20 g magnesium sulfate, 1.0 g 2,3-DOB, and a 1:1 mixture of tap water and distilled water to make a final volume of one liter (Seaman 1962). The pH of the medium was adjusted to 7.0 with 1 N potassium hydroxide. This solution was filtered aseptically through a millipore filter into a sterile 1.0 liter flask. The medium was not autoclaved because of the instability of the 2,3-DOB to high autoclave temperatures.

AGAR PLATES AND AGAR SLANTS

For preparation of agar plates, 100 ml of 3% molten agar (Difco) was mixed with 100 ml of sterile enrichment culture medium. This final mixture while in a liquid state was dispensed aseptically into sterile petri dishes in approximately 20 ml portions.

Agar slants were prepared in the same manner, however 10 ml aliquots of the dissolved, warm solution were delivered into sterile, 16 x 150 mm

screw cap tubes. These tubes, while still warm, were placed at an angle of approximately 20° from a flat surface, cooled, and stored (Seaman 1962).

ISOLATION OF BACTERIA

Approximately 0.5 g of soil obtained from a Pitt County forest was placed in a test tube containing 5.4 ml of sterile saline solution. After a period of 6 to 12 hours, when the soil settled down, 0.6 ml of the slightly turbid saline solution was aseptically transferred into three test tubes each containing 6 ml of enrichment culture medium. The tubes were incubated at room temperature overnight. Appearance of turbidity in the tubes indicated bacterial growth, and 0.10 ml aliquots of the liquid medium were aseptically transferred into another three tubes containing fresh culture medium. These tubes were incubated at room temperature overnight. As extensive turbidity was observed in the tubes, a serial ten-fold dilution of the microorganisms was made from the test tube indicating the most cells. Eight test tubes, each containing 5.4 ml of sterile saline solution, were used to make the ten-fold dilution. From the tube containing the most cells, 0.60 ml was transferred into the first saline tube with a sterile 1.0 ml pipette and suspended uniformly by shaking. From the first saline tube, 0.60 ml was aseptically transferred into the second saline tube, and from the second into the third and so forth. These ten-fold transfers resulted in a one hundred million-fold dilution in the last saline tube. From each saline test tube, 0.10 ml was transferred aseptically into a corresponding agar plate (Seaman 1962). Eight such agar plates were prepared. The transferred solutions

were spread uniformly on the plates, and the organisms were allowed to grow at room temperature for 72 hours. The plates with low dilutions exhibited extensive bacterial growth, whereas the most dilute plates showed small numbers of rather large, round, discrete colonies. One such colony was selected for purity and transferred into a tube containing culture medium. The tube was incubated for 24 hours at room temperature. Stock cultures were made from this tube by streaking loop transfers from the tube over the surface of the agar slants. The agar slants were incubated for 24 hours to insure adequate growth, and upon good growth the cultures were placed in a refrigerator for storage.

HARVEST OF THE BACTERIAL CELL

One liter of the sterile enrichment culture medium was aseptically divided equally into three sterile 1 liter covered flasks. From the surface of the stock culture slant, bacterial cells were washed off with 1 ml sterile culture medium. Then, 0.3 ml aliquots of the washing were aseptically pipetted into the three 1 liter flasks. After inoculation, the three flasks were incubated for 24 hours with continuous shaking on a gyratory shaker for good aeration.

Cells were then collected by centrifugation at 10,000 g's for 15 minutes. The supernatant was discarded, and the pellet of cells was suspended in potassium phosphate buffer (pH 7.8, 0.02 M), and centrifuged at 10,000 g's for 10 minutes. Washing was repeated twice more. The washed cells were suspended in approximately 6-8 ml potassium phosphate buffer (pH 7.9, 0.02 M) to form a thick suspension. This solution was stored in ice under refrigeration.

SUBSTRATE SOLUTION

A substrate solution was prepared by adding 154.0 mg 2,3-DOB into 50 ml 0.02 M potassium phosphate buffer (pH 7.8) and 30 ml distilled water. The solution was adjusted to pH 7.0 with 1 N potassium hydroxide. The final volume was adjusted to 100.0 ml in a 100.0 ml volumetric flask with distilled water which resulted in a 10 mM substrate solution.

OXYGEN CONSUMPTION DETERMINATION

Oxygen consumption was measured polargraphically by a Clark oxygen electrode (Yellow Spring Instrument Co.), that was accommodated to a glass reaction vessel (3-3.5 ml) designed by Ito. The reaction mixture consisted of 2.90 ml substrate solution maintained at 22-23°C and 0.03 ml whole cell suspension (or 0.06 ml crude or 0.10 ml purified enzyme extract). The reaction mixture for determination of the corresponding endogenous oxygen consumption was identical except the substrate solution was replaced by potassium phosphate buffer (pH 7.8, 0.02 M). Results were recorded automatically on chart paper. A typical record is shown in Fig. 6, Appendix A. Oxygen content of the reaction mixtures was assumed equal to that in air-equilibrated pure water and was approximately 274 uM at 22°C and 263 uM at 23°C.

SONIFICATION OF CELLS

Approximately 6-8 ml of whole cells suspended in potassium phosphate buffer (pH 7.8, 0.02 M) were sonified for 6 minutes with a 130 watt, model W-140D sonifier (Branson Co.) at an operating power setting of "3". 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's for 15 minutes to remove any undisrupted cells and large cell membrane particles. Enzyme activity was checked and found in the supernatant portion, and was stored in ice under refrigeration until later use. The supernatant portion was termed "crude extract".

SPECTROPHOTOMETRIC DETERMINATION

A visible and ultraviolet spectrum of the oxidation product of 2,3-DOB, α-hydroxymuconic semialdehyde, was determined by a Coleman 124 double beam spectrophotometer and a Coleman 165 recorder.

The substrate solution was diluted fifty-fold. One-tenth ml of the crude extract (sonified supernatant) was added to 2.90 ml of the diluted substrate solution in a quartz cuvette, mixed and incubated for 1-2 minutes to allow the enzyme to convert the entire substrate present in the reaction mixture to the product. After incubation, the cuvette was placed in the spectrophotometer and the spectrum was recorded from 500-300 nm (Dagley 1960).

PROTEIN PURIFICATION

Approximately 5-6 ml of the crude extract was ultracentrifuged for 90 minutes at 40,000 g's with the supernatant being termed "ultracentrifuged extract".

Ammonium sulfate fractionation was carried out to further concentrate the enzyme. To five tubes, each containing 1.0 ml of ultracentrifuged extract, ammonium sulfate was added to make them 0%, 20%, 40%, 60% and 80% saturation. Each tube was centrifuged at 10,000 g's for 10 minutes. The supernatant and precipitate were separated. Each precipitate

was resuspended in 1.0 ml potassium phosphate buffer. All the tubes were then tested with substrate solution for enzyme activity (oxygen consumption) with the oxygen electrode.

3.0 ml of the ultracentrifuged extract was subjected in ion-exchange chromatography for final purification. The ion-exchange adsorbent used to fractionate the proteins was a derivative of cellulose, diethylaminoethyl-cellulose (DEAE-cellulose), which contains positively charged groups at neutral pH's. The DEAE-cellulose (Sigma Corp.) was washed and prepared for use by a specific wash procedure (Himmelhoch 1971) described below:

1. Approximately 400 g of medium mesh DEAE-cellulose is placed in a 6 liter beaker and washed with distilled water.
2. Decant and wash with .1 N HCl.
3. Decant and wash with distilled water until a pH of 4.0.
4. Decant and wash with 1 N NaOH.
5. Decant and wash with distilled water until a pH of 10.
6. Decant and wash twice with .1 N HCl.
7. Decant and wash with distilled water until a pH of 5.
8. Titrate with 1 N NaOH until a pH of 7.8.
9. Decant and wash with potassium phosphate buffer (pH 7.8, 0.02 M) until a final pH of 7.8.

The DEAE-cellulose thus prepared, pH 7.8, was stored for chromatographic purposes.

The ion-exchange chromatography was carried out in a 1 x 30 cm glass column which has at one end a stopcock for dropwise elution. An automatic fraction collector (Fractomat) was used to collect fractions in 16 x 150 mm test tubes. A polystaltic pump (Buchler) was employed to

form a continuous salt concentration gradient. The pump was adjusted to deliver 1.0 M NaCl in potassium phosphate buffer (pH 7.8, 0.02 M) into a 0.20 M NaCl in the same buffer, and the resulting mixture into the column continuously at a constant rate. Thus, the salt concentration linearly increased from 0.20 M NaCl concentration to 1.0 M.

Approximately 150 ml of DEAE-cellulose suspension was poured into the column and allowed to settle by gravity for 24 hours. The height of the ion-exchange adsorbent ranged from 19.5 to 21.5 cm. The entire ion-exchange chromatography equipment was kept in a cold cabinet (Uni-Therm) at 2°C. A description of the procedure is as follows:

1. Column was washed and eluted with potassium phosphate buffer (pH 7.8, 0.02 M) until 1 ml of the buffer remained on the top of the column to prevent the column from drying.

2. 3.0 ml of the ultracentrifuged enzyme extract was added and allowed to flow until 1 ml of the extract remained on top of the column.

3. Potassium phosphate buffer (pH 7.8, 0.02 M) was added to wash and elute the column for the first 20 fractions in two experiments and for the first 15 fractions in the third experiment, before elution with the continuous salt gradient. Each fraction contained 5.4 ml of eluant and the elution rate was 1.35 ml/min.

4. After these fractions, the column was eluted with the continuous salt gradient described above.

5. 81 fractions were collected in one experiment and fifty-one fractions were collected in the other two.

Fractions were analyzed for proteins by reading absorbances at 280 nm and 260 nm on a Coleman 124 double beam spectrophotometer. Fractions containing proteins were tested for enzyme activity by the oxygen electrode and stored under refrigeration for further studies with Warburg manometer.

PROTEIN DETERMINATION

Protein concentration in the crude extracts and chromatographed preparations were determined by UV absorption (Layne 1957) at 260 nm and 280 nm or by the method of Lowry et al. (1951).

The method based on UV absorption at 260 nm and 280 nm was employed as a quick and fairly accurate estimate of the amount of protein in solution. The crude and ultracentrifuged extracts were diluted 1:50 with potassium phosphate buffer (pH 7.8, 0.02 M) before the absorbance was read at wavelengths of 260 nm and 280 nm. After the optical densities at 260 nm and 280 nm were recorded, the protein was estimated in the following manner (Layne 1957):

1. Calculate the ratio of optical density at 280 nm/260 nm.
2. Use this number to determine the corresponding value of F from the indicated graph (Fig. 11, Appendix A).
3. Calculate the protein concentration from the equation:

$$(O.D._{280})\ (F)\ (\tfrac{1}{d}) = \tfrac{mg}{ml}\ \text{protein}$$

The fractions obtained from ion-exchange chromatography were estimated for protein in the same manner, however no dilution of 1:50 was necessary.

In some cases, in order to check the accuracy of the UV absorption method, Lowry's procedure was performed. Crystalline bovine serum albumin was used to prepare a standard of 1.0 mg/ml concentration. To six test tubes, the standard was added in 0.1 ml increments (0.0, 0.10, 0.20, 0.30, 0.40, 0.50). Each tube was brought to 1.0 ml total volume by the addition of distilled water. To each tube, 5.0 ml of Reagent C was added. After 10 minutes, 0.1 ml of Reagent E was added, rapidly mixed, and allowed to react for 30 minutes. One-tenth milliliter of properly

diluted enzyme solution was brought to a 1.0 ml volume with distilled water and the Lowry reagents (C and E), as described for the standards, were added and allowed to react. The standards and unknown (enzyme solutions) were read at 500 nm and 750 nm with a Coleman 124 spectrophotometer. A second Lowry test was run in the same manner, however the standards were added in 0.05 ml increments (0.00, 0.05, 0.10, 0.15, 0.20, 0.25). The absorbance was read at 500 nm and 750 nm on a Coleman 111 spectrophotometer. Protein content in the unknowns was read from the standard curve.

Reagent C was prepared daily by mixing 50 ml of Reagent A (2% Na_2CO_3 in 0.1 N NaOH) and 1 ml of Reagent B (0.2% $CuSO_4 \cdot 5H_2O$ in 1% sodium potassium tartrate). Reagent E was prepared by diluting the commercially available Folin-Ciocalteu reagent 1:1 with distilled water to obtain a 1 N solution (Lowry et al. 1951).

DETERMINATION OF OXYGEN AND CARBON DIOXIDE BY WARBURG RESPIROMETER

Warburg constant volume respirometer was used to determine oxygen consumption and carbon dioxide evolution which resulted from the mixture of the substrate and a crude or a purified enzyme preparation (Umbreit et al. 1945).

One Warburg flask was used to determine oxygen consumption and two flasks were necessary for the determination of the volume of carbon dioxide evolved from the substrate. Substrate solution, 2.90 ml, was pipetted into the main chamber of a single-arm flask. The sidearm contained 0.10 ml of crude enzyme extract. Potassium hydroxide (6 N), 0.20 ml, was pipetted into the center well containing a filter paper

wick. The potassium hydroxide soaked filter paper served as an efficient absorbent for any carbon dioxide in the flask. Thus, the only gas being measured was oxygen. A second and a third flask with two sidearms were used in measuring carbon dioxide evolution. The main chamber contained 2.90 ml of substrate solution. One sidearm contained 0.10 ml of crude enzyme extract and the other sidearm contained 0.20 ml of 6 N sulfuric acid. No potassium hydroxide was added to the center well. An additional flask was the thermobarometer containing only 3.20 ml of distilled water. This flask was used to correct any change in manometric readings due to atmospheric pressure changes.

The four flasks with their gas vent sealed and closed were attached to the manometers and sealed and secured with springs. They were placed in a water bath maintained at 37°C with the manometer stopcock open for 5-10 minutes for temperature equilibration. After 10 minutes, all stopcocks were closed. The first flask was taken out of the water bath and the enzyme extract in the sidearm was added into the substrate solution and placed back in the water bath. For the second flask, the enzyme in a sidearm was mixed with the substrate, and immediately the sulfuric acid in the other sidearm was added into the mixture. For the third flask, the enzyme in a sidearm was added, but the sulfuric acid in the other sidearm was not tipped into the mixture until the end of incubation. The manometers were read just before the enzyme additions and every 5-8 minutes afterward for 56-49 minutes of incubation. At the end of incubation, flask three was removed from the bath and the sulfuric acid was added to the reaction mixture. Readings were continued every 5-8 minutes for an additional 15 minutes on all four flasks.

For the purified enzyme preparation, 2.80 ml of substrate and 0.20 ml of enzyme preparation were used. More purified enzyme preparation was used because of its greatly reduced protein concentration (Fig. 10).

ELECTRON MICROSCOPY TECHNIQUES

Whole Mount Bacteria

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 and observed with a Hitachi HS-8 transmission electron microscope.

Sectioned Bacteria

Bacterial cells were fixed in Kellenberger's standard fixative (Kay 1967), dehydrated in a graded ethanol series and embedded in Araldite 6005. Sections ($\sim$600 Å thick) were double stained with lead and uranium salts and observed with the transmission electron microscope.

RESULTS AND DISCUSSION

In order to better understand the structure and morphology of the bacterium used in the present study, whole mounts of intact cells and thin sections were examined with the transmission electron microscope. Figures 1 and 2 (Appendix A) are whole mount preparations showing the bacterium to be a monotrichous rod. Thin sections of the bacterium (Fig. 3, Appendix A) show it to have typical bacterial internal structure. These results were not meant to be a detailed analysis of the ultrastructure of the bacterium but were used only to determine its prokaryotic nature.

Regarding metabolic studies, bacterial cells were tested for oxygen consumption in the presence of a 2,3-DOB solution. The cells exhibited rapid oxygen uptake in the presence of the substrate, but little oxygen consumption in the presence of potassium phosphate buffer (pH 7.8, 0.02 M) alone (Fig. 6, Appendix A). These results indicated that the cells contained the necessary enzymes to oxidize 2,3-DOB.

The concentration of proteins in the crude extract obtained from sonification varied from day to day (Table 2, Appendix A). This may be due to variations such as the amount of cell suspension, the depth the sonifier tip was dipped into the cell suspension, and other unknown factors that varied from day to day, although an identical power setting and time period were employed for sonification.

The reaction between the substrate and a crude extract yielded a very distinct yellow color that is a characteristic of α-hydroxymuconic semialdehyde (Ribbons 1966, Ribbons and Senior 1970). The product from

the mixture of the crude extract and 2,3-DOB absorbed between 370-378 nm (Fig. 5, Appendix A), indicating that it was α-hydroxymuconic semialdehyde.

Ribbons (1966) and Ribbons and Senior (1970) reported that a dioxygenase was responsible for the simultaneous incorporation of oxygen and evolution of carbon dioxide in the initial cleavage of 2,3-DOB. Assaying crude enzyme extracts of Pseudomonas in constant volume respirometers, Ribbons and Senior (1970) concluded that 2,3-DOB was cleaved by 2,3-DOB-3,4-dioxygenase incorporating one molecule of oxygen to the substrate and releasing one molecule of carbon dioxide simultaneously. Since, this crude extract certainly would contain many enzymes, one can not exclude a possibility that the oxygen incorporation and the carbon dioxide release are catalyzed by two separate enzymes. Indeed, there may exist three possible methods of cleavage for the degradative process (Fig. 4, Appendix A). Among these, conversion of 2,3-DOB to catechol probably does not occur, since catechol is not oxidized by the crude enzyme extract (Ito, personal communication). Thus, the question is whether there was an oxygenase and a second decarboxylative enzyme as seen in the two remaining pathways (Fig. 4, Appendix A) or just one oxygenase enzyme as described by Ribbons and Senior (1970). In order to determine whether one or two enzymes are responsible for the initial cleavage of the substrate, it is essential that the enzyme preparation be pure. Thus, purification of the crude extract was attempted.

Crude extracts (sonified) were subjected to ultracentrifugation for further concentration of the enzyme. Tests for enzyme activity showed the enzyme to be in the soluble fraction. Specific activity increased

in the ultracentrifuged fraction in two of the three extracts. In the other, specific activity decreased upon ultracentrifugation (Table 2, Appendix A). This might be due to a possible occlusion of the enzyme in the particles in the crude extract. Such occlusion would be possible if the enzyme is particle-bound loosely but strong enough to stay with the particles upon insufficient sonification. Removal of inhibitors during ultracentrifugation might be a possible explanation for the increased activity after the ultracentrifugations. These possibilities were not tested.

Ammonium sulfate fractionation was attempted with the ultracentrifuged extract. However, a decrease in specific activity of the enzyme was quite obvious (Table 1, Appendix A). Evidently, the enzyme to be purified was inactivated in greater amounts than the other proteins. Thus, in subsequent studies, ammonium sulfate fractionation was not attempted.

Three ultracentrifuged enzyme extracts were subjected to DEAE-cellulose (ion-exchange) chromatography as a final fractionation step. One of the DEAE-cellulose chromatography experiments (Fig. 7, Appendix A) showed three fractions containing enzyme activity. Specific activity was shown to be greatest in fraction 25, thus indicating the greatest purification of the enzyme. In two other experiments (Figs. 8 and 9, Appendix A), only two fractions were found to contain enzyme activity. Specific activity was greater in fraction 31 for both experiments. Analyzing the results of ion-exchange chromatography (Table 2, Appendix A), one can clearly see that great amounts of unwanted proteins were removed by the chromatography, thus greatly increasing the specific activity of the

fractions containing the enzyme. The first ion-exchange experiment (Fig. 7, Table 2, 8/30/77) increased specific activity 80-fold, whereas the second and third experiments (Table 2, 9/15/77 and 10/2/77) increased specific activity some 11-fold. These fractions with the greatest specific activity were selected for an additional assay by Warburg Respirometer to determine if the enzyme was responsible for oxygen uptake only or both oxygen uptake and carbon dioxide evolution.

Both crude and pure enzyme extracts were assayed by Warburg respirometer (as described in Materials and Methods) for oxygen uptake and carbon dioxide evolution in order to determine whether or not the purification procedure (ion-exchange chromatography) separated the two functions. Typical results on oxygen uptake are given in Fig. 10, on carbon dioxide evolution in Table 3, Appendix A.

As seen in Table 3, Appendix A, the ratios of oxygen consumed to carbon dioxide released by the crude extract ranged from 0.86-1.15 indicating an approximate 1:1 ratio. The crude extract used contained 0.9 mg of protein which is a large amount with comparison to the purified preparations. The oxygen/carbon dioxide ratio obtained from the purified preparations (3 from chromatography fractions) (Table 3, Appendix A) ranged from 0.90-1.23, again indicating an 1:1 ratio. Those used were 0.060, 0.073, and 0.078 mg protein which is a tremendous decrease in protein used as compared to the crude extract. Yet, oxygen/carbon dioxide ratios were essentially the same in both cases. In spite of the fact that purification was 80-fold in one experiment and 11-fold in the other two, the ratio of oxygen consumption to carbon dioxide evolution remained approximately 1:1 in the purified extract as well as in the

crude preparation. Thus, it is quite likely that one oxygenase was responsible for both the oxygenation and decarboxylation (Fig. 4, Appendix A). There still remains an unlikely possibility that the reaction involves two separate enzymes, since the purified enzyme preparations may not be a single protein as evidenced by the shape of the protein profile in the area where the enzyme activity was found (Figs. 7, 8, and 9, Appendix A). However, it may be now safely concluded that the oxidation of 2,3-DOB was catalyzed by an oxygenase which simultaneously oxygenates and decarboxylates the substrate, as proposed by Ribbons and Senior (1970).

SUMMARY

A soil bacterium (probably Pseudomonas) was isolated by the enrichment culture technique. The bacterial cells were grown in a liquid culture medium containing 2,3-DOB as a sole carbon source. After several transfers of cells into fresh culture media, the cells were properly diluted and transferred to agar plates containing 2,3-DOB as the carbon source. A single, discrete colony was selected for purity and transferred to agar slants and stored under refrigeration.

Bacterial cells were grown in 1 liter quantities of culture media for 24 hours and harvested by centrifugation for metabolism studies.

A substrate solution containing 2,3-DOB as the sole carbon source was prepared to test the cell's ability to oxidize the substrate. A Clark oxygen electrode was used to measure this oxygen consumption by the cells.

Whole cells were sonified and centrifuged to collect the cell-free crude extract. This crude extract was ultracentrifuged and the soluble extract was purified by ion-exchange chromatography to isolate the enzyme responsible for the oxidation of the substrate to α-hydroxymuconic semialdehyde. At each purification step, the activity (oxygen consumption) was measured with a Clark oxygen electrode.

The enzymatic oxidation of 2,3-DOB to α-hydroxymuconic semialdehyde involved incorporation of two oxygen atoms by a dioxygenase into the ring structure and release of carbon dioxide. Both a crude and a pure enzyme extract were assayed by the Warburg manometric method to determine if there was a decarboxylative enzyme in addition to the dioxygenase

responsible for the oxidative cleavage of 2,3-DOB. Results from the Warburg assay indicated that in both crude and pure enzyme extracts there was a simultaneous uptake of oxygen and release of carbon dioxide in a 1:1 molecular ratio.

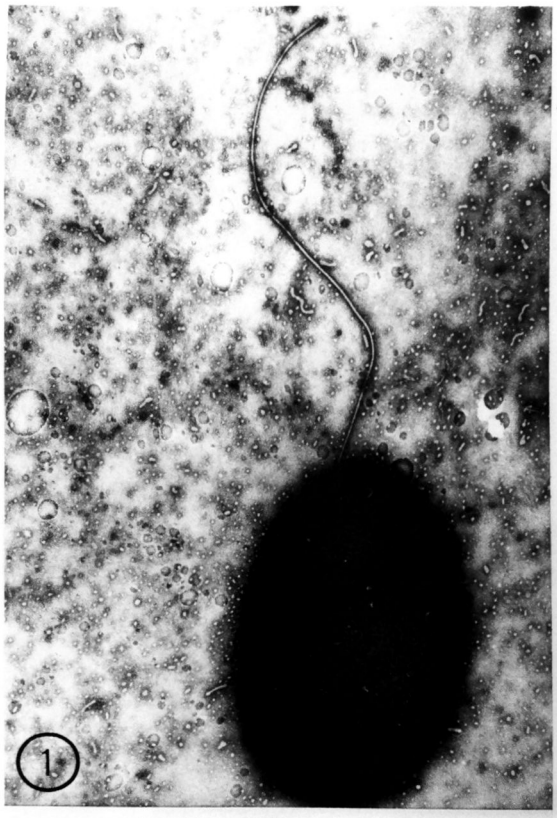
The purification of the crude enzyme extract did not separate oxidative and decarboxylative functions of the enzyme. Thus, one enzyme, 2,3-DOB-3,4-dioxygenase, was responsible for the simultaneous uptake of oxygen and release of carbon dioxide.

APPENDICES

APPENDIX A

Figs. 1 and 2. Negatively stained whole mount preparations of the soil bacterium X 45,000.

Fig. 3. Electron micrograph of sectioned soil bacterium X 82,500.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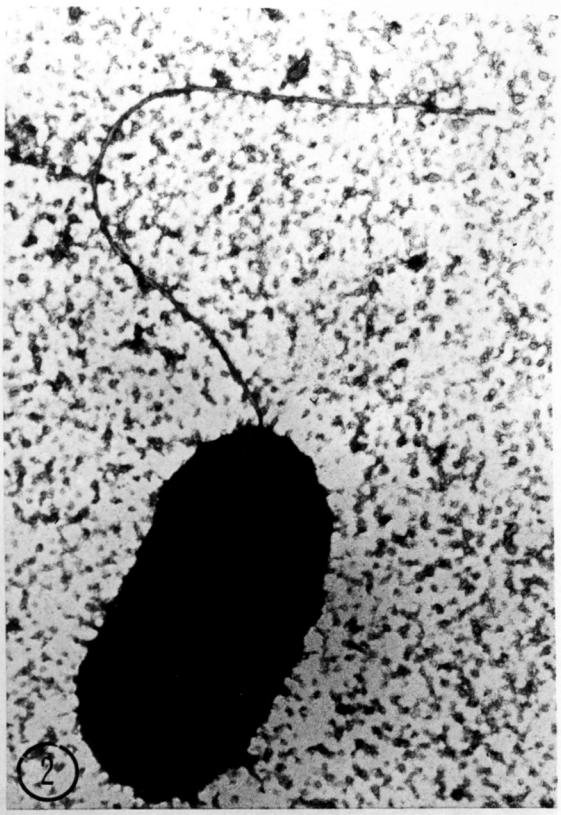
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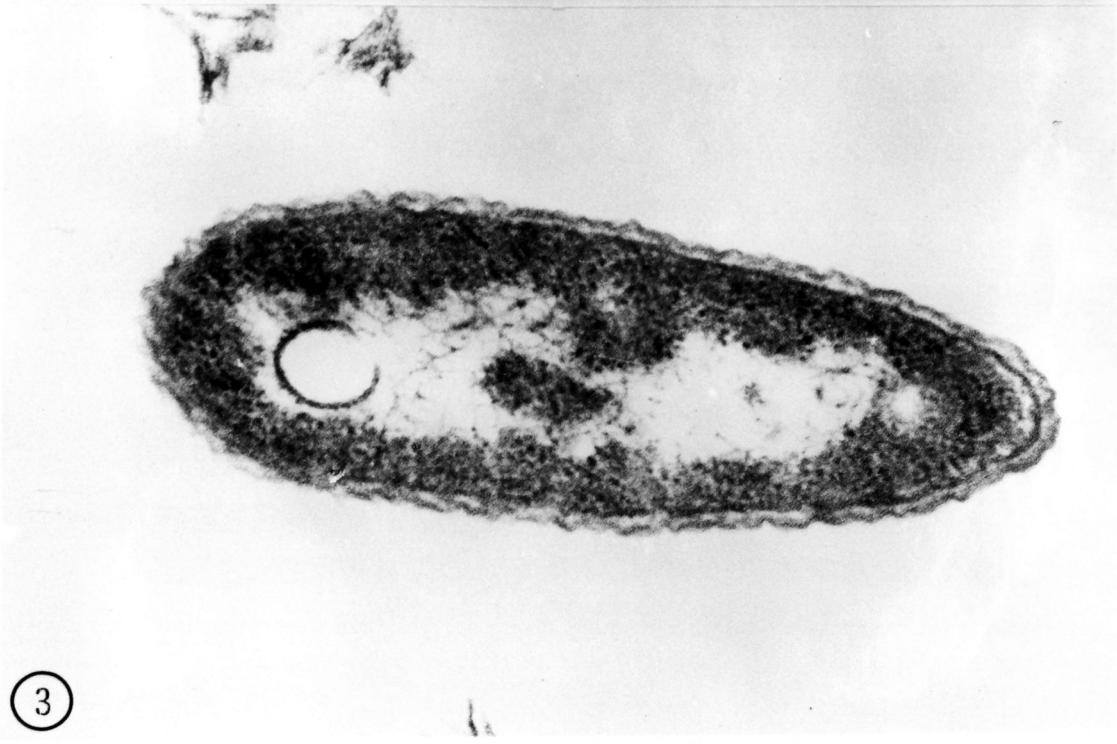
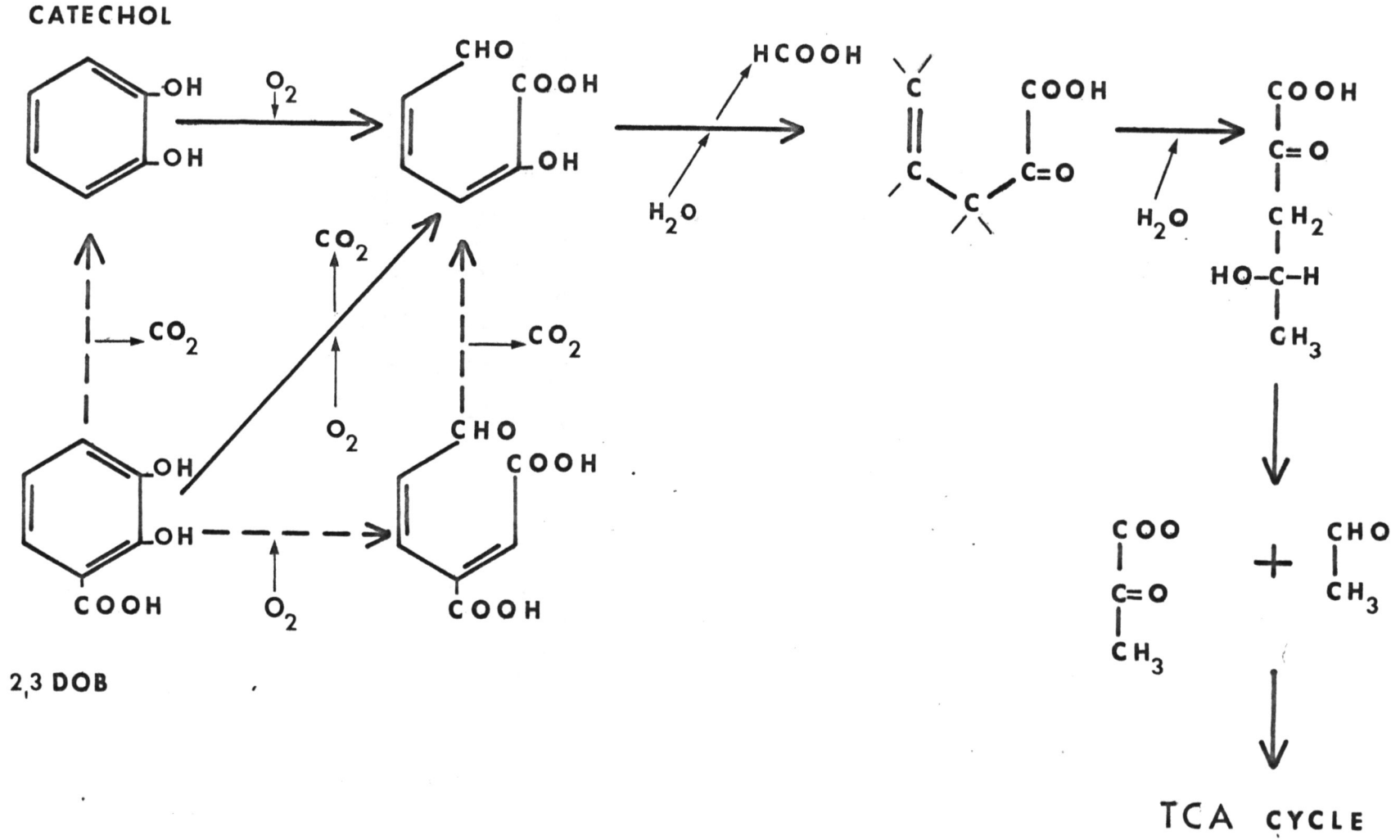

3

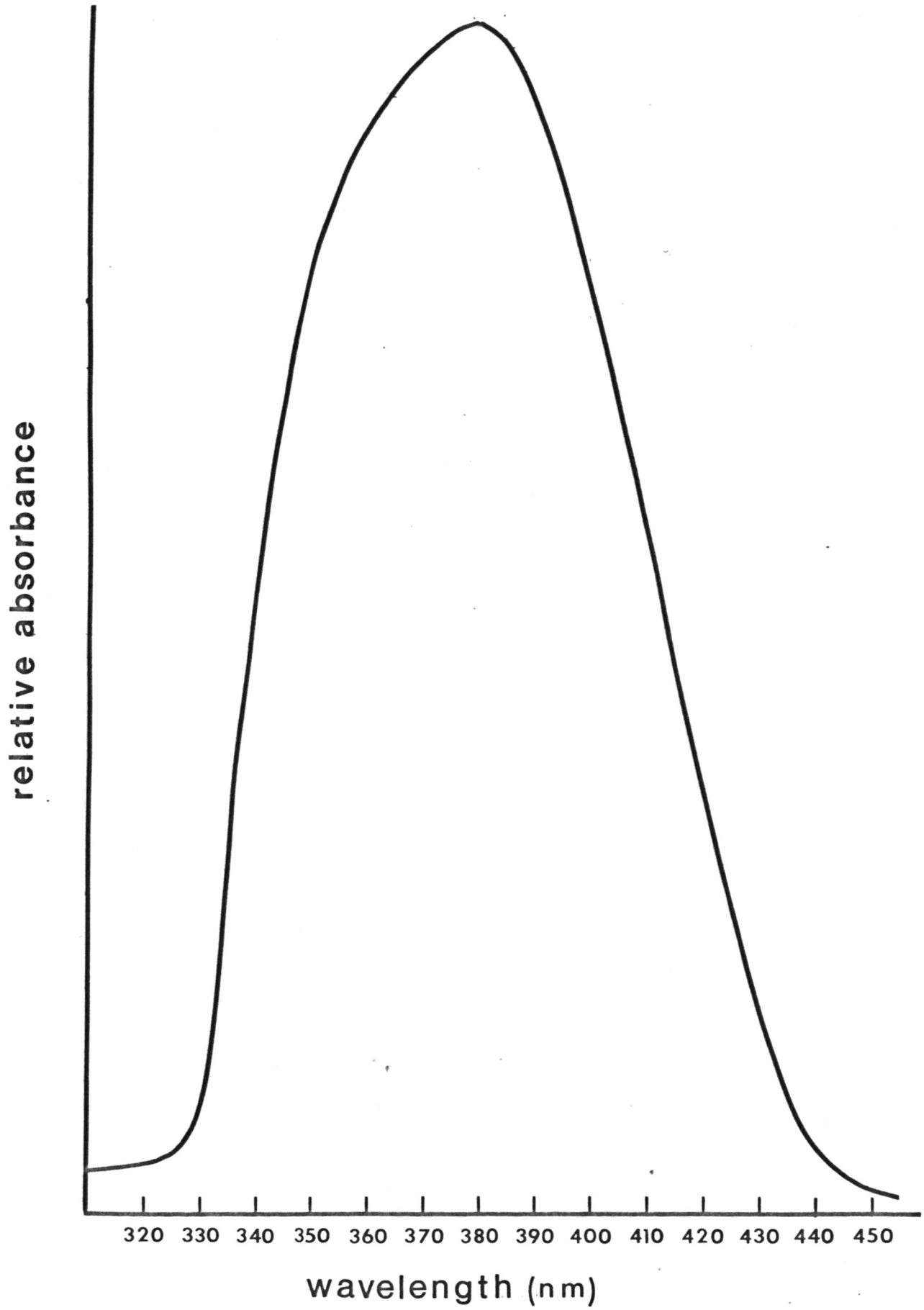
APPENDIX A (Continued)

Fig. 4. Pathways of catechol and 2,3-DOB oxidation by Pseudomonas. The initial degradation of 2,3-DOB as indicated by broken lines were postulated and tested as possible pathways of oxidation by the bacterium isolated in this study. Results indicated the pathway as being a one step mechanism as illustrated by the solid line.


CATECHOL
OH
OH
O_2
CHO
COOH
OH
HCOOH
H_2O
C
C
C
COOH
C=O
H_2O
COOH
C=O
CH_2
HO–C–H
CH_3
CO_2
CO_2
CO_2
O_2
O_2
OH
OH
COOH
CHO
COOH
COOH
COO
C=O
CH_3
+
CHO
CH_3
TCA CYCLE
2,3 DOB

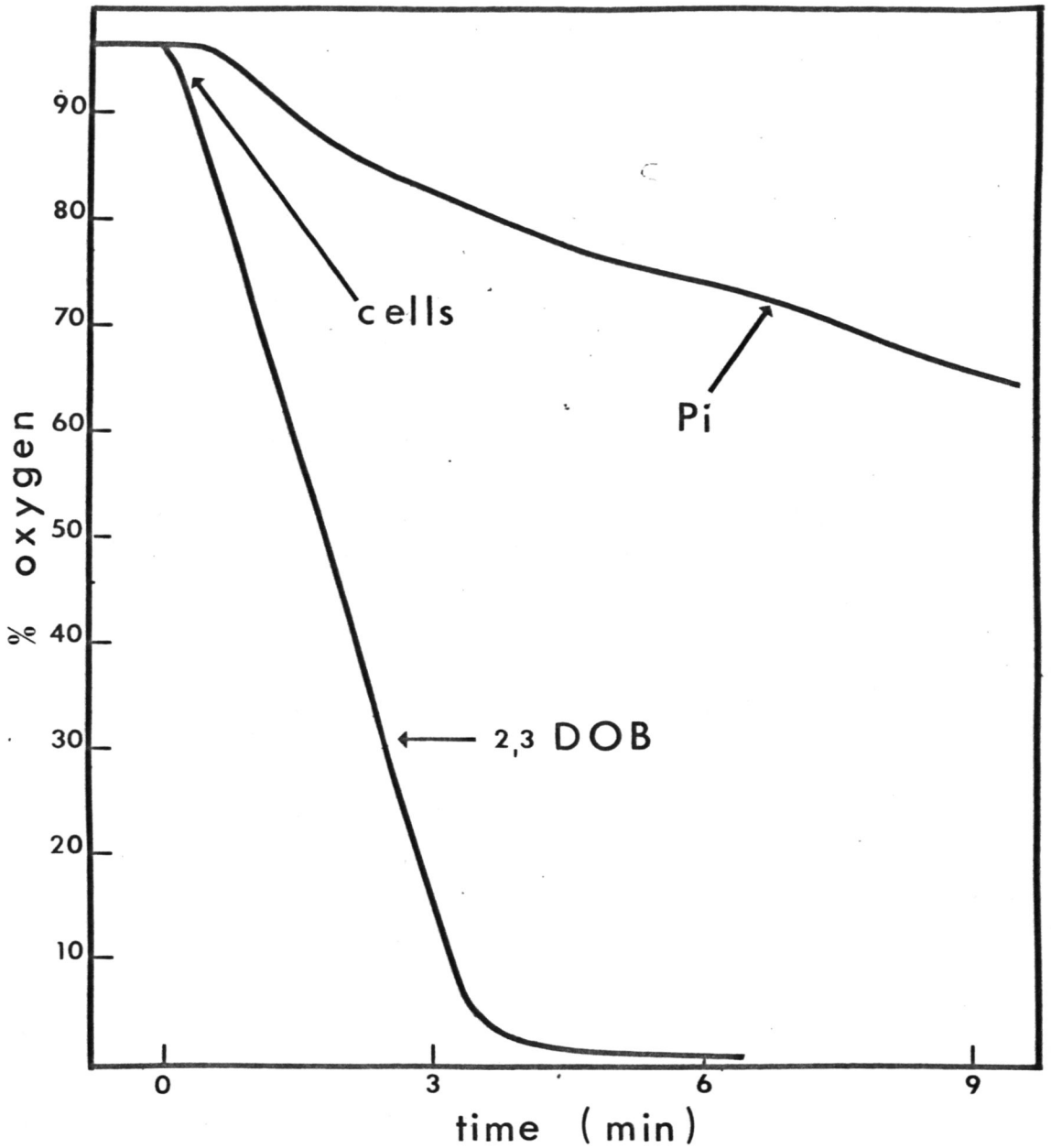
APPENDIX A (Continued)

Fig. 5. Spectrum of the product formed when 2,3-DOB substrate solution and a cell-free extract of 2,3-DOB grown cells were mixed. The spectrum resembles that of α-hydroxymuconic semialdehyde.


relative absorbance
320 330 340 350 360 370 380 390 400 410 420 430 440 450
wavelength (nm)

APPENDIX A (Continued)

Fig. 6. Oxygen consumption of 2,3-DOB grown cells when mixed with 2,3-DOB solution and potassium phosphate buffer determined by oxygen electrode.


90
80
70
60
50
40
30
20
10
% oxygen
cells
Pi
2,3 DOB
0
3
6
9
time (min)

APPENDIX A (Continued)

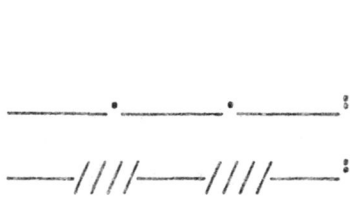
Table 1: Determination of specific activity of enzyme after treatment with ammonium sulfate. Specific activity was calculated from oxygen consumption results recorded on an oxygen electrode at room temperature. The specific activity was calculated from the recorder's results and defined as nmoles of oxygen consumed per min per mg protein.

Ammonium Sulfate Fractionation

% $(NH_4)_2SO_4$ Saturation	Specific Activity (nmoles/min/mg)	
	Supernatant	Precipitate
0	1084.08	------
20	842.14	658.30
40	0	671.67
60	0	502.57
80	0	248.75

APPENDIX A (Continued)

Figs. 7, 8, and 9 (in sucessive order).



Fractions were assayed with oxygen electrode for 2,3-DOB oxygenase. Figs. 7, 8, and 9 are for enzyme extracts labeled 8/30/77, 9/15/77, and 10/2/77 in Table 2.

absorbance
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
80
fraction
specific activity (nm/min/mg x 10^2)
55
50
45
40
35
30
25
20
15
10
5

absorbance
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
50
fraction
specific activity (nm/min/mg x 10²)
55
50
45
40
35
30
25
20
15
10
5

absorbance
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
specific activity (nm/min/mg x10²)
55
50
45
40
35
30
25
20
15
10
5
5
10
15
20
25
30
35
50
fraction

APPENDIX A (Continued)

Table 2: Summary of enzyme purification.

ENZYME	SAMPLE	PROTEIN (mg/ml)	TOTAL VOL. PROTEIN (ml)	TOTAL ACTIVITY (nmoles/min)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (nmoles/min/mg)	YIELD (%)
8/30/77	crude	42.00	5.0	16,522.80	210.00	78.68	100
	ultracent.	38.90	3.0	4,449.77	116.70	38.13	27
	fraction 4	6.25	5.9	0	1.49	0	---
	6	.64	5.9	0	3.77	0	---
	10	.01	5.8	0	.001	0	---
	23	.42	5.4	0	2.25	0	---
	24	.41	5.4	1,523.74	2.19	695.77	9
	25	.30	5.4	5,277.71	1.63	3,237.86	32
	26	.28	5.4	1,290.67	1.51	854.75	8
9/15/77	crude	15.39	8.6	28,481.72	132.35	215.20	100
	ultracent.	6.11	6.6	13,627.35	40.32	337.98	48
	fraction 7	.29	5.4	0	1.56	0	---
	10	.36	5.4	0	1.96	0	---
	19	.01	5.0	0	.001	0	---
	29	.19	4.6	0	.86	0	---
	30	.15	4.6	0	.69	0	---
	31	.36	4.6	6,419.28	1.67	3,843.88	23
	32	.08	4.6	143.26	.35	400.16	.5
10/2/77	crude	16.20	8.0	19,810.66	129.60	152.86	100
	ultracent.	10.80	5.0	9,962.46	54.00	184.49	50
	fraction 4	.20	5.6	0	1.12	0	---
	5	.14	5.6	0	.78	0	---
	6	.01	5.4	0	.04	0	---
	28	.28	4.8	0	1.34	0	---
	29	.33	4.8	0	1.59	0	---
	30	.39	4.8	2,257.18	1.88	1,200.63	11
	31	.11	4.8	1,083.89	.52	2,084.42	6
	32	.21	4.8	0	1.02	0	---

APPENDIX A (Continued)

Table 3: Oxygen Consumption and Carbon Dioxide Evolution.

Date: the date of measurement of oxygen consumption and of carbon dioxide evolution by the enzyme preparation with 2,3-DOB.

Enzyme: the date of the enzyme preparation from freshly harvested cells.

Oxygen Consumption and Carbon Dioxide Evolution

DATE	ENZYME	TYPE	PROTEIN (mg/ml)	TOTAL PROTEIN USED (mg)	TIME OF INCUBATION (minutes)	X_{O_2} (umoles)	X_{CO_2} (umoles)	X_{O_2}/X_{CO_2}
6/10/77	6/10/77	crude	9.00	.90	58	10.04	9.23	1.08
6/10/77	6/10/77	crude	9.00	.90	46	5.50	4.80	1.15
6/14/77	6/10/77	crude	9.00	.90	56	8.64	8.78	0.98
6/14/77	6/10/77	crude	9.00	.90	56	5.80	6.71	0.86
6/16/77	6/10/77	crude	9.00	.90	69	10.25	9.58	1.06
6/16/77	6/10/77	crude	9.00	.90	69	8.36	8.48	0.99
9/02/77	8/30/77	pure	.302	.060	51	4.02	4.31	0.93
9/02/77	8/30/77	pure	.302	.060	51	3.40	3.78	0.90
9/15/77	9/15/77	pure	.364	.073	65	5.03	4.09	1.23
9/15/77	9/15/77	pure	.364	.073	65	5.15	4.28	1.20
10/02/77	10/02/77	pure	.392	.078	63	4.83	4.24	1.14
10/02/77	10/02/77	pure	.392	.078	63	4.08	3.77	1.08

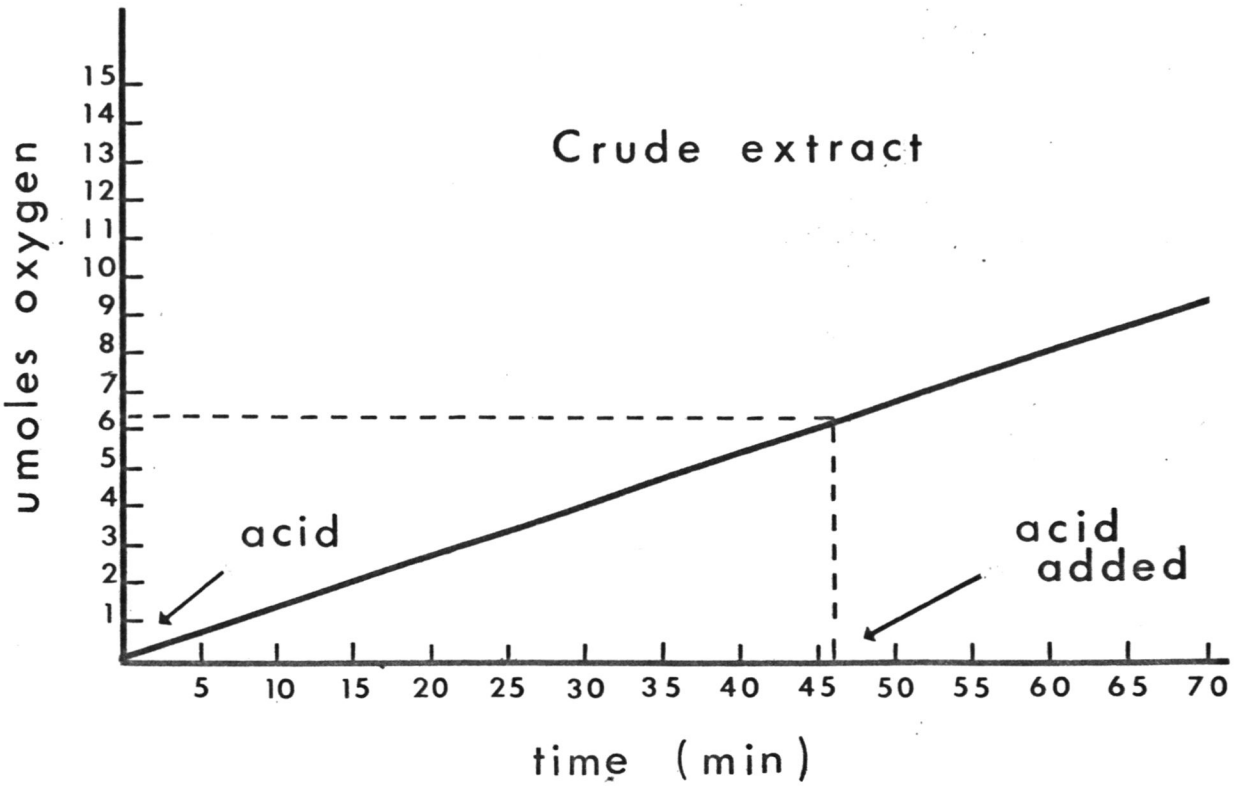
APPENDIX A (Continued)

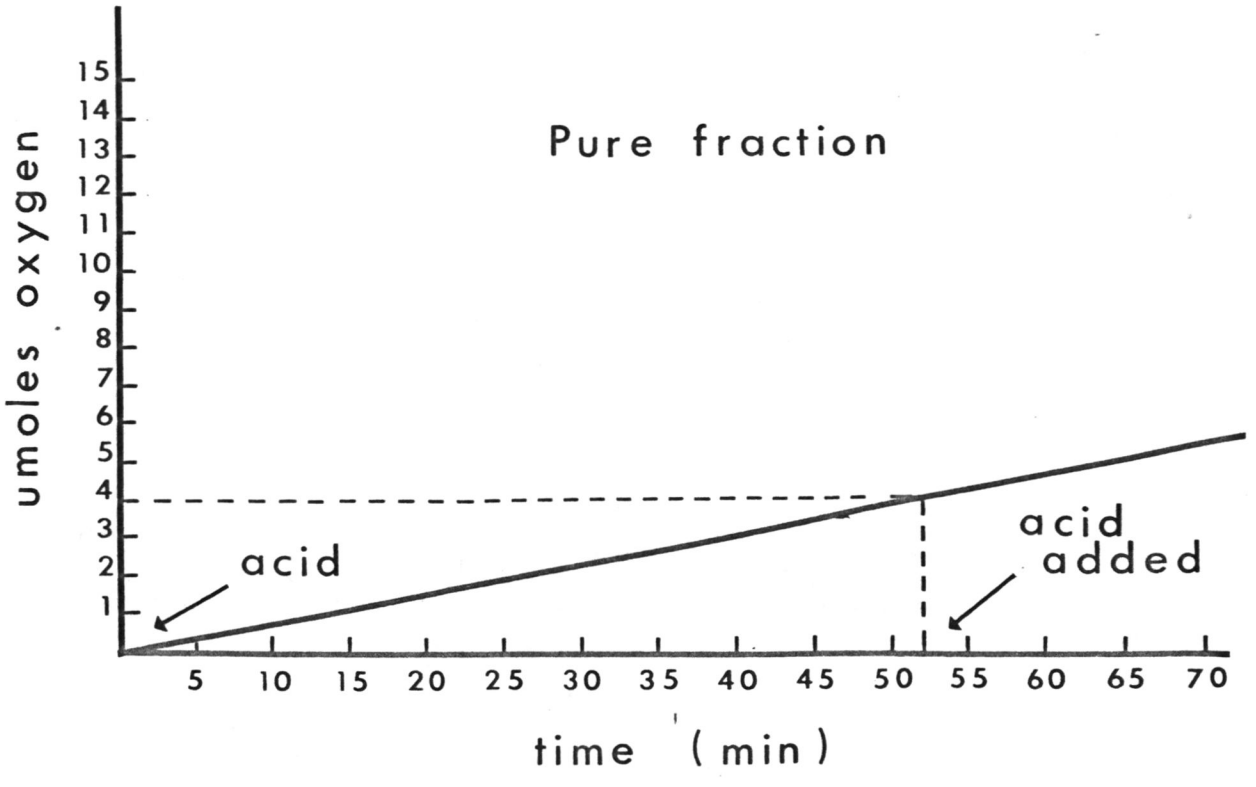
Fig. 10. Oxygen Uptake by Enzyme Extracts Determined by Warburg Respirometers

Oxygen uptake was determined in one flask. Acid was added to another flask at time zero, and to a third flask at the end of incubation as indicated by the broken line. The difference in carbon dioxide released between last two flasks was the carbon dioxide released due to the enzyme reaction. See Table 3 for the carbon dioxide data.

Crude extract (6/10/77) assayed on 6/10/77.

Purified extract (8/30/77) assayed on 9/2/77.


Crude extract
umoles oxygen
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
acid
acid
added
5
10
15
20
25
30
35
40
45
50
55
60
65
70
time (min)


Pure fraction
umoles oxygen
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
acid
acid
added
5
10
15
20
25
30
35
40
45
50
55
60
65
70
time (min)

APPENDIX A (Continued)

Fig. 11. Graph used in protein estimation (Tach et al. 1972)

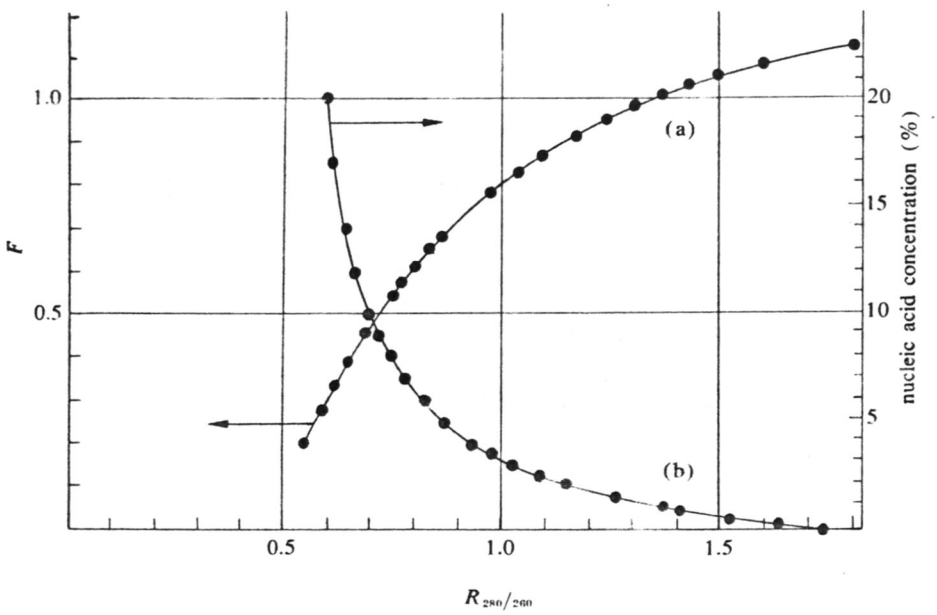


Figure 2-6. Estimation of protein and nucleic acid concentration. Calculate the ratio of ODs at 280 mμ and 260 mμ. From this number (i.e., the $R_{280/260}$) determine the corresponding value of F from the graph (curve (a)). Calculate protein concentration from the equation

$$OD_{280} \cdot F \cdot \left(\frac{1}{d}\right) = \text{milligrams protein per milliliter}$$

where d is the path length of the absorption cell in centimeters.

The nucleic acid concentration may be determined as a percentage of the protein concentration. The appropriate percentage value is also a function of $R_{280/260}$, and may be read from curve (b). (The data for this plot are taken from Layne, 1957.)

APPENDIX B

LIST OF ABBREVIATIONS

2,3-DOB	2,3-dihydroxybenzoic acid
TCA	Tricarboxylic acid cycle

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