ABSTRACT

Eunhee Shim, MICROBIAL METABOLISM OF PHTHALIC ACID ISOMERS (Under the direction of Takeru Ito) Department of Biology, August, 1975.

Three soil microorganisms (probably Pseudomonas), were isolated by enrichment culture technique, using either phthalic acid or isophthalic acid, or terephthalic acid, as a sole source of carbon.

Oxidative metabolism of three organisms was studied by determination of oxygen consumption of the organisms with various suspected metabolic intermediates using an oxygen electrode and by determination of spectra of intermediates using a spectrophotometer.

The following results were obtained from such studies. Terephthalic acid-grown cells converted terephthalic acid into protocatechuic acid, and isophthalic acid-grown cells oxidized isophthalic acid to the same product. They oxidized protocatechuic acid to beta-carboxy-cis,cis-muconic acid.

None of the tested compounds suspected to be the intermediates of phthalic acid degradation including protocatechuic acid were oxidized by phthalic acid-grown cells. A new, possible intermediate was postulated, which was 2,7-diketo-3,cis-5,cis-octadienoic-1,8-dicarboxylic acid.

MICROBIAL METABOLISM
OF
PHTHALIC ACID ISOMERS

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Eunhee Shim

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by

Eunhee Shim

APPROVED BY:

SUPERVISOR OF THESIS Takeru Ito

Dr. Takeru Ito

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

James S. McDaniel

Dr. James S. McDaniel

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette

Dr. Joseph G. Boyette

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INTRODUCTION

Microbial oxidative metabolism of numerous aromatic compounds by Pseudomonas species have long been investigated for several decades. Such studies have contributed to the knowledge of metabolic pathways and cellular regulatory mechanisms.

Oxidative metabolism of phthalic acid, in particular, by soil Pseudomonas has been studied since 1955 (Evans). Later evidence showed that phthalic acid was converted sequentially to 4,5-dihydroxy-phthalic acid, protocatechuic acid, beta-carboxy-muconic acid, and beta-keto-adipic acid (Ribbons and Evans, 1960), as seen in Fig. 10 and Fig. 12, APPENDIX E.

Despite the existence of one such known pathway of phthalic acid degradation by a soil microorganism described above, it would be of value to examine a possible existence of alternate oxidative metabolism of phthalic acid. In view of metabolic diversity and capacity of microorganisms, it would appear reasonable to expect various pathways for oxidative degradation of aromatic compounds. Oxidative degradation of catechol and protocatechuic acid is such an example. This will be discussed in the REVIEW OF LITERATURE section.

Secondly, phthalic acid esters being major industrial products and environmental contaminants, their biological removal from industrial wastes by soil microorganisms poses an interesting metabolic problem (Ribbons and Evans, 1960; Marx, 1972).

Thirdly, comparison of various metabolic pathways of related structural isomers, such as, phthalic acid, isophthalic acid, and terephthalic acid, would give some insight into general metabolic schemes by which microorganisms degrade aromatic compounds as an energy source.

For these reasons, this study was undertaken. This paper is, therefore, concerned with identification of pathways of oxidative metabolism of phthalic, terephthalic, and isophthalic acids by soil microorganisms (probably Pseudomonas), which can specifically grow on these isomers as a sole source of carbon.

REVIEW OF LITERATURE

Phthalic acid (o-benzene dicarboxylic acid), isophthalic acid (m-benzene dicarboxylic acid), and terephthalic acid (p-benzene dicarboxylic acid) are relatively stable and in general toxic to living organisms. For example, their anti-bacterial activity was shown against Mycobacterium tuberculosis (Roper et al., 1968). Their general toxicity may not pose serious environmental problems, since many microorganisms belonging to diverse groups such as Pseudomonas, Mycobacteria, Vibrio, aerobic sporeformers, and Actinomycetes, are capable of decomposing various aromatic compounds and capable of growing at the expense of such compounds as an energy source. However, a large amount of the phthalic acid isomers and their esters have indeed escaped into the environment as industrial effluents or as remnants of herbicides (Kashin, Kaufman, and Sikina, 1970).

Interestingly, phthalic acid showed a very strong metabolic inhibition in vivo of mammalian spermatozoa (Graves, Salibury, and Lodge, 1967).

Explanation seems due at the beginning of this review as to the technique used in this present study. The technique involves sequential enzyme induction, and has been extensively utilized in studies of microbial oxidative metabolism.

The idea of sequential induction (or simultaneous adaptation) technique was initially advanced in the early literature of microbiology several decades ago (Winogradsky, 1887; Fermi, 1891). The technique

was introduced to studies of bacterial metabolism by Spiegelman (1948), Karlsson et al. (1948), Stanier (1948), and Monad et al. (1951).

This technique is based on the ability of a living organism especially a microorganism to synthesize adaptive or inducible enzymes, when the organism is exposed to appropriate substrates or related substances, which are called inducers. The adaptive or inducible enzymes are not present in the organism in the absence of inducers. When the organism is exposed to a substrate A (an inducer), it induces an enzyme which catalyzes conversion of A to a metabolic intermediate B. B then serves as an inducer for a second enzyme, which now catalyzes conversion of B to C. C serves as an inducer for a third enzyme, and so on. In this manner, the 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 (Cowgill and Pardee, 1957).

When an aerobic microorganism, which can grow in a culture medium containing an aromatic compound, A, as a sole source of carbon, is grown in such a growth medium, it contains all the enzymes necessary to degrade A in order to capture energy for its growth. Most of the initial degradative or oxidative reactions are catalyzed by the enzymes, oxygenases, induced in this organism. If the organism can oxidize A successively to B, C, and D, it should consume oxygen when it is exposed not only to A, but also to B or C or D. Oxygen consumption by this organism in the presence of a particular compound is, thus, an evidence for this compound to be a possible metabolic intermediate of oxidation of A. Lack of oxygen consumption is obviously taken as an

evidence against this compound. Particular compounds to be tested are selected to determine whether they are possible intermediates. The selection is made by postulating possible sites of oxygen attack on the benzene ring carbons. For example, since oxygen attack on C-2 and C-3 of phthalic acid with concomitant carbon dioxide evolution would form 2,3-dihydroxy-benzoic acid, the latter compound, thus, would be a possible intermediate of phthalic acid oxidation.

In application of this technique for metabolic studies, however, the possible presence of a permeability barrier in bacterial cell membrane is an obvious limiting factor, since such a situation will prevent induction of adaptive enzymes even if the compounds are capable of inducing bacteria to form the enzymes within the cells (Ornston, 1971a). Nevertheless, the technique has been fruitful in elucidating pathways and regulation of metabolism of aromatic compounds in microorganisms.

Although microbial catabolism of aromatic compounds has been reviewed by Dagley (1972), the pathways related to this study will be given below.

The known microbial dissimilatory mechanisms of aromatic compounds are aerobic and involve oxidative attack not only on the initial benzene ring, but also on most of all the intermediates involved. Metabolic pathways of certain aromatic compounds have already been identified and serve as working models for other investigations. Others are yet to be found. Some known pathways related to this present study are summarized in APPENDICES.

In studies of K-vitamins *in vivo* in both dog (Pohl, 1909) and man (Pakendorfk, Kudrashev, and Lasareva, 1941), phthalic acid was found to be an oxidative decomposition product of the quinones of the vitamin K group. Also this acid was shown to be excreted unchanged in higher animals (Shemiakin and Schkina, 1944), unlike benzoic acid which formed the glycine conjugate (Knoop, 1905).

Some studies of oxidative degradation of naphthalene and its halogen derivatives suggested that phthalic acid was a degradative intermediate (Tasson, 1928; Gray and Thorton, 1928; Jacobs, 1931). But later researches with soil *Pseudomonas* showed otherwise (Strawinski and Stone, 1943; Walker and Wiltshire, 1953; Murphy and Stone, 1954; Fernley and Evans, 1964; Davis and Evans, 1964).

It is interesting to note that phthalic acid was prepared by chemical oxidation procedure from alpha-naphthol (Joo and Loren, 1970), from naphthalene (Ichinokawa and Sano, 1971), from o-xylene (Yasuhiro and Hirohiko, 1970; Yokoyama, 1969; Digrov and Nastyukova, 1968; Cudmore, Warner, and Olsen, 1969; List, Dodt, and Alfs, 1970), and from ditolylethane (Plaksunova *et al.*, 1971).

Evans (1955) identified by chromatography, the presence of 4,5-dihydroxy-phthalic acid. Ribbons and Evans (1960) revealed that 4,5-dihydroxy-phthalic acid and protocatechuic acid were immediately metabolized by the phthalic acid-grown soil *Pseudomonas*. They suggested that 4,5-dihydro-4,5-dihydroxy-phthalic acid may be formed initially, followed by its dehydrogenation to 4,5-dihydroxy-phthalic

acid, and that the decarboxylation of 4,5-dihydroxy-phthalic acid led to the formation of protocatechuic acid by the soil Pseudomonas.

Protocatechuic acid has often been shown as an intermediate compound in oxidative metabolism of aromatic compounds by various microorganisms. It was formed by the initial oxidative degradation of p-hydroxy benzoate and of phenol in Virio O_1 (Evans, 1947), and in Pseudomonas fluorescens (Sleeper and Stanier, 1950). It was also formed in the p-aminobenzoic acid-grown Neurospora (Gross, Gafford, and Tatum, 1956), in the p-cresol-grown soil Pseudomonas (Dagley and Patel, 1957) and Vibrio O_1 (Cain, 1961), in the p-hydroxy-benzoate-grown Pseudomonas (Stanier, Palleroni and Doudoroff, 1966) and Pseudomonas putida (Higgins and Mandelstam, 1972).

The oxidation of protocatechuic acid by Pseudomonas fluorescens was first studied by Stanier and Ingraham (1954). The oxidative product was proved to be beta-carboxy-cis,cis-muconic acid by MacDonald et al. (1954), and Dagley and Patel (1957). The product was extremely unstable. The half life was only 3 minutes at 100°C at pH 2.0 and 40 minutes at room temperature at pH 7.0. Protocatechuic acid oxidase, so termed by Evans (1947), which catalyzes conversion of protocatechuic acid into beta-carboxy-cis,cis-muconic acid, was extracted, purified, and crystallized by Hayaishi (1964).

Ornston and Stanier (1966) showed that oxidation of beta-carboxy-cis,cis-muconic acid led to the formation of beta-ketoadipate-enol-lactone via gamma-carboxy-muconolactone in Pseudomonas putida. Higgins and Mandelstam (1972) confirmed a similar pathway with the same organism.

The pathway was also demonstrated in Pseudomonas fluorescens (Ornston, 1971b), and in Moraxella calcoacetica (Canovas, Ornston and Stanier, 1967). The latter converted beta-ketoadipate-enol-lactone into beta-ketoadipyl CoA. Further fate of beta-ketoadipyl CoA was succinate and acetyl CoA (Ornston and Stanier, 1966), or succinate and acetate (Higgins and Mandelstam, 1972) (Fig. 11, APPENDIX E).

Another pathway of protocatechuic acid oxidation contains alpha-hydroxy-gamma-carboxymuconic semialdehyde, which is apparently formed by the bond cleavage between C-4 and C-5 of protocatechuic acid. Further conversion of this intermediate results in alpha-hydroxy-gamma-carboxy-alpha,beta-dihydro-muconic acid, and finally into pyruvate via oxaloacetate and lactate (Dagley, Evans, and Ribbons, 1960) (Fig. 9, APPENDIX E).

The possibility that 2,6-dioxa-3,7-dihydroxy-3,3,0-octane ("the dilactone") and beta,gamma-dihydroxyadipic acid are intermediates of oxidation of protocatechuic acid by Pseudomonas (Elsden and Peel, 1958) was excluded by Cain et al. (1961). These compounds are now not considered as intermediates of protocatechuic acid oxidation (Fig. 7, APPENDIX E).

Another intermediate compound was also identified in other stock cultures of Pseudomonas (Strain p-1) by Dagley, Stopher, and Trippett (1960) as 2,4-lutidinic acid (Fig. 8, APPENDIX E). However, it was proved that the surprising formation of pyridine nucleus as a consequence of benzene nucleus rupture was totally non-enzymatic reaction between the initial ring cleavage compound alpha-hydroxy-gamma-

carboxy-muconic semialdehyde and the ammonium ion accidentally present in the cell-free extract (Ribbons, 1960).

Catechol has also been shown frequently as an intermediate of oxidative metabolism of aromatic compounds in microorganisms.

Catechol was formed as an intermediate by benzoate-grown fluorescent Pseudomonas (Mar and Stone, 1961; Gibson, Koch, and Kallio, 1968; Canovas, Ornston, and Stanier, 1967), by m-cresol-grown soil bacteria (Hughes, 1951), by phenol-grown Pseudomonas (Kilby, 1948), by mandelate-grown and tryptophan-grown Pseudomonas putida (Ornston, 1966b), by o-cresol-grown Pseudomonas (Dagley and Stopher, 1957; Hayaishi et al., 1957; Dagley et al., 1964; Bayly and Dagley, 1968), and by naphthalene-grown Pseudomonas (Fernley and Evans, 1964; Mckenna and Kallio, 1965). Catechol was also an intermediate in salicylic acid metabolism by various Pseudomonas species. Proctor and Scher (1960) showed an oxidative pathway of benzoic acid by Rhodopseudomonas species which contained both protocatechuic acid and catechol as intermediates.

It is generally accepted that dihydroxylation is a pre-requisite for enzymatic fission of benzene ring (Gibson, 1968). Even benzene is oxygenated to catechol in Pseudomonas putida (Gibson et al., 1968).

In some species of Pseudomonas, catechol is cleaved by catechol 1,2 oxygenase to give rise to cis,cis-muconic acid (Evans et al., 1951; Hayaishi et al., 1957; Taniuchi et al., 1964; Ornston, 1966a; Farr and Cain, 1968), while in other species catechol is oxidized into alpha-hydroxy-muconic semialdehyde by catechol 2,3 oxygenase (Dagley, Stopher, and Trippett, 1960; Kojima et al., 1961), and eventually converted into acetate and pyruvate.

Catechol is oxidized successively to cis,cis-muconic acid, muconolactone, and to beta-ketoadipate-enol-lactone, which is a converging point in oxidation of catechol and protocatechuic acid. This common intermediate is converted to beta-ketoadipate which is eventually degraded to succinate and acetyl CoA (Dagley, 1972), as discussed previously (Fig. 11, APPENDIX E).

2,3-Dihydroxy benzoic acid was shown to be converted into alpha-hydroxy muconic semialdehyde and further into pyruvate and other compounds of tricarboxylic acid cycle either by C-1,C-2 cleavage or C-3, C-4 cleavage with the evolution of carbon dioxide (Ribbons, 1966; Ribbons and Watkinson, 1968).

The known pathways of oxidative metabolism of various aromatic compounds discussed here obviously provided the guides for studying oxidative pathways of phthalic acid isomers in the soil bacteria employed in this study.

MATERIALS AND METHODS

The methods described by Seaman (1962) were employed for many parts of this study including the preparation of the culture medium for agar plates, and for agar slants, and the isolation of the bacteria.

LIQUID CULTURE MEDIUM

For the isolation of the organisms which can grow at the expense of phthalic acid, terephthalic acid, or isophthalic acid, as a sole source of carbon, the following culture medium was used: 0.15g of dibasic potassium phosphate, 0.05g of monobasic potassium phosphate, 0.02g of magnesium sulfate, 0.75g of ammonium chloride, 0.05g of yeast extract, 0.2g of phthalic acid or isophthalic acid, or terephthalic acid, 1 ml of the Pseudomonas salt solution (See APPENDIX B for the salt composition), 1:10 mixture of tap water and distilled water to make final volume of 100 ml. (This culture medium contained 59mg Mg^{++}; 2mg Fe^{++}; 2mg Zn^{++}; 50µg Cu^{++} per liter.)

AGAR PLATES AND AGAR SLANTS

To 100 ml of the liquid culture medium was added 1.5g of the Difco agar, and the mixture was dissolved by gentle heating. The mixture was autoclaved and while in liquid state about 15 ml portions of the liquid were poured aseptically into petri dishes. The petri covers were maintained at an angle to protect the plates from the air-borne contamination during the transfer of the liquid. Agar slants were also prepared with the same ingredients as were the agar plates. Eight ml

portions of the dissolved warm mixture were dispensed into 16x150 mm tubes which then were plugged with cotton and autoclaved. These tubes, while still hot, were placed obliquely at an approximately 20^{o} from the bench top, cooled and stored.

STERILE SALINE SOLUTION

Twenty-four 13x150 mm test tubes each containing 5.4 ml of 0.9% sodium chloride solution were autoclaved and used for a sterile transfer of the soil bacteria.

ISOLATION OF BACTERIA

Approximately 0.6g of collected soil was added into a test tube containing the sterile saline solution described above. Six-tenths ml of the above suspension was then aseptically transferred into three test tubes containing the liquid culture medium described above. The tubes were incubated at room temperature overnight. Upon the appearance of the turbidity in the tubes, 0.1 ml portions of the medium were transferred aseptically into another three tubes containing 6.0 ml of the fresh culture medium and the tubes were incubated at room temperature overnight.

After conspicuous growth were observed, serial ten-fold dilution of the organisms from the best of the three tubes was carried out by preparing eight test tubes each containing 5.4 ml of the sterile 0.9% saline solution. From the tube, containing the organisms, 0.6 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, 0.6 ml

was aseptically transferred into the second tube, and from the second into the third, and from the third into the fourth and so forth. By repeating the ten-fold transfer, one million-fold dilution was accomplished in the very last tube. A 0.1 ml from each tube was aseptically transferred into a corresponding agar plate, and spread uniformly on the plate. The eight plates were incubated at room temperature for 24 hours.

The plates with low dilutions showed extensive growth over the agar plates. These plates which showed small numbers of large round discrete colonies were selected for further transfers. One such colony was aseptically transferred into a sterile tube containing the liquid medium by applying it to the sides of the tube just above the level of the liquid with a previously flamed bacteriological loop to make a uniform suspension. The tube was incubated for good growth. Stock cultures were made by streaking needle innoculation from the above liquid medium containing the good growth over the surface of the agar slants. The agar slants were incubated at room temperature until good growth appeared. The culture was then placed in a refrigerator for storage.

The above isolation procedure was followed, using three culture media, one containing phthalic acid, another terephthalic acid, and the third isophthalic acid.

HARVEST OF THE BACTERIAL CELLS

A two liter Erlenmeyer flask containing one liter of the liquid medium was plugged with cotton and autoclaved. After cooling, one loopful of bacterial cells from agar slants was added aseptically to

provide an inoculum and incubation was maintained with a continuous shaking at 37°C overnight. Cells were collected by centrifugation at 10,000g for 15 minutes. These packed cells were suspended uniformly into 0.05M potassium phosphate buffer, pH 7.0 to be washed. Washing of the bacterial cells was repeated two more times by suspending in the buffer and by centrifuging at 10,000g for 10 minutes. The cells were suspended uniformly in a small amount of the buffer to form a thick but freely flowing slurry.

The above procedure was followed, using three culture media, one containing phthalic acid, another isophthalic acid, and the third containing terephthalic acid and employing three corresponding organisms isolated.

DRY WEIGHT DETERMINATION

The dry weight of the washed cells was conveniently estimated turbidometrically at 540 nm, with optical density readings at the wavelength calibrated against the dry weight of the organism.

To construct the calibration curve, 1.0, 0.8, 0.5, 0.2, 0.1 ml portions of the cell slurry were transferred with a large orifice pipette into small pre-weighed pans made of aluminum foil, which were shaped on the mouth of 125 ml Erlenmeyer flask. While those were drying in an oven at 105°C, similar volumes of the suspension were pipetted into photometric tubes and diluted with the potassium phosphate buffer that suspended the cells to bring the volume to the 10 ml

mark. The cells were suspended evenly and optical density readings were made. A tube containing the potassium phosphate buffer was used as a reference.

The readings were plotted on linear graph paper against the weight of the corresponding dried samples. Dry weight of bacterial cells in any suspensions was read out of the graph, after optical density of the bacterial suspension at 540 nm was made (APPENDIX C).

OXYGEN CONSUMPTION DETERMINATION

The oxygen consumption was measured by a Clark oxygen electrode (Yellow Springs Instrument Co.). Theory of the electrode involves electroreduction of the oxygen to water at the platinium cathode surface, which was maintained at 0.8 volts. The reaction effects a flow of current proportional to the oxygen concentration at the cathode surface. The current is then amplified and recorded on chart paper (Kieley, 1963).

Three ml of a fresh shaken potassium phosphate buffer (0.05M pH 7.0) maintained at room temperature was added into the oxygen electrode vessel and the instrument was run for 3 to 5 minutes. Then 0.03 ml of freshly washed bacterial cells was added and the instrument was run for 3 to 5 minutes.

The vessel was completely washed with deionized water and 3 ml of 0.005M substrate solution in 0.05M potassium phosphate buffer, pH 7.0, was added in the vessel followed by the addition of 0.03 ml of the cells. The oxygen consumption was automatically recorded on recorder chart paper. The substrates tested were benzoic acid,

catechol, protocatechuic acid, salicylic acid, and 2,3-dihydroxy benzoic acid, in addition to one of the phthalic acid isomers, in the expense of which the organisms were isolated from the soil.

Oxygen content of the reaction medium was assumed equal to that in air-equilibrated pure water, and was approximately 258μM at 25°C (Kieley, 1963).

SPECTROPHOTOMETRIC DETERMINATION

Ultra-violet spectra of protocatechuic acid and possible intermediates were determined by a Coleman model 124 spectrophotometer with a model 165 recorder with silica cuvettes having a light path 1 cm at room temperature.

Samples of the bacterial slurry from both isophthalic acid and terephthalic acid-grown cells were sonically disrupted for 3 minutes at a 60 watt sonifier cell disrupter model W-140 (Bronson Co.) at an optimum operating current. The pre-chilled probe was cooled with ice bath during the sonic treatment. The samples were centrifuged at 10,000g for 10 minutes to remove undisrupted whole cells, and the supernatants were used for the source of enzymes.

The clear supernatant (0.2ml) was incubated for 2 minutes with 20 ml of the 0.01 M protocatechuic acid in 0.05 M potassium phosphate buffer, pH 7.0, with constant agitation on a magnetic stirrer. Two ml of 0.1 M perchloric acid was added to the incubation mixture to stop the enzyme reaction and the mixture was centrifuged at 15,000g for 10 minutes. The supernatant was neutralized with 0.1 M potassium hydroxide to pH 7.0. Potassium perchlorate, formed as a precipitate,

was removed by centrifugation at 15,000g for 10 minutes, and the supernatant was used for determination of ultra-violet spectrum for possible presence of degradative intermediates. For the determination of the spectrum of protocatechuic acid, the same procedure was followed except that 0.2 ml of the enzyme extract (supernatant) was replaced by 0.05 M potassium phosphate buffer, pH 7.0.

PROTEIN DETERMINATION

The method of Lowry et al. described by Layne (1957) was employed in protein determination. Crystalline bovine serum albumin was used as a standard. To six test tubes, bovine serum albumin (0.2 mg/ml) was added in 0.1 ml increments (0.0, 0.1, 0.2, 0.3, 0.4, 0.5). Each tube was brought to 1.0 ml by the addition of deionized water. In each tube, 5 ml of Reagent C was added. After 10 minutes, 0.1 ml of Reagent E was added. Each tube was allowed to stand for 30 minutes. The color was read by a Klett-Summerson photometer with red filter.

One ml of properly diluted enzyme extracts from bacterial cells was subjected to the same procedure, and the amount was determined by reading out of the standard curve (APPENDIX D).

Reagent C was freshly prepared by mixing 50 ml of Reagent A (2% Na_2CO_3 in 0.1N 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 one volume of commercial Folin-Ciocalteu reagent (2N) with one volume of water to make it 1N in acid. The Folin-Ciocalteu reagent was obtained from Fisher Scientific Co.

RESULTS AND DISCUSSION

The bacterial cells grown at the expense of either isophthalic acid or terephthalic acid as a sole source of carbon showed rapid oxygen consumption in the presence of protocatechuic acid (Fig. 1, 2, APPENDIX A). They showed little oxygen consumption with the other suspected intermediate compounds tested. From this experiment, it was concluded that protocatechuic acid was a possible intermediate in the oxidative degradation of both isophthalic acid and terephthalic acid by these organisms isolated from the soil.

However, cells grown on phthalic acid failed to show any appreciable oxygen uptake with all the suspected intermediate compounds tested, except of course, phthalic acid (Fig. 3, APPENDIX A). The phthalic acid-grown cells were either unable to metabolize these substrates, or impermeable to those compounds. Although a question of cell permeability remains, it is possible that the organism isolated here follows a different pathway than the one studied by Evans (1955), and Ribbons and Evans (1960), who showed that 4,5-dihydroxy-phthalic acid and protocatechuic acid were the initial intermediates of phthalic acid oxidation by the soil microorganisms they examined.

The failure to find any possible degradation products by the phthalic acid-grown cells in this study elicited postulation of a new intermediate, 2,7-diketo-3-cis,5-cis-octadienoic-1,8-dicarboxylic acid. This intermediate would be formed when an oxygen molecule attacks on C-1 and C-2, and the ring fission occurs between these two carbons.

Search in chemical and biochemical literature failed to uncover description of this compound (Fig. 13, APPENDIX E).

The cell-free extracts of both isophthalic acid- and terephthalic acid-grown cells were incubated with protocatechuic acid for 2 minutes. Both incubation mixtures showed disappearance of ultra-violet light absorption of protocatechuic acid and appearance of a rather broad absorption peak at near 255 nm (Fig. 4, APPENDIX A). The results indicated the presence of beta-carboxy-cis,cis-muconic acid in the incubation mixtures. The appearance of this absorption peak was rather surprising since one would expect beta-carboxy-cis,cis-muconic acid to be further degraded rapidly in the presence of such a crude enzyme preparation. It was probably due to either deterioration of enzymes which catalyze further degradation of beta-carboxy-cis,cis-muconic acid or slow reaction rates by the subsequent enzymes.

According to the above results, the initial conversion of both isophthalic acid and terephthalic acid appeared to be the consequence of the attack of molecular oxygen on C-3 and C-4 in isophthalic acid and on C-1 and C-2 in terephthalic acid.

The ring fission of protocatechuic acid to give rise to beta-carboxy-cis,cis-muconic acid was the result of opening benzene nucleus by "ortho" cleavage, i.e., the ring cleavage at the bond between the carbons bearing ortho-hydroxy groups of protocatechuic acid, as already shown (Dagley, 1972).

The enzyme which catalyzed the conversion of isophthalic acid or terephthalic acid into protocatechuic acid, and the enzyme which

catalyzed the formation of beta-carboxy-cis,cis-muconic acid from protocatechuic acid obviously are oxygen transferases (Mason, 1958), or dioxygenases (Hayaishi, 1964).

Initial oxidative metabolism of isophthalic acid and terephthalic acid is another example in which hydroxylation of benzene nucleus occurs prior to the ring-fission (Gibson, 1968). Oxidation of protocatechuic acid by the cells grown at the expense of isophthalic acid and terephthalic acid is similar to the one in the previous investigations done in several microorganisms (MacDonald, Stanier, and Ingraham, 1954; Gross et al., 1956; Dagley and Patel, 1957; Dagley, Evans and Ribbons, 1960; Stanier, Palleroni and Doudoroff, 1966; Higgins and Mandelstam, 1972).

Identification of these organisms employed in this study was not carried out, but it was assumed that they belong to Pseudomonas species, for the method employed here to isolate bacteria from the soil (enrichment culture technique) often yields those species.

In the taxonomic studies of Pseudomonas, Stanier, Palleroni, and Doudoroff (1966) found that the "ortho" cleavage of protocatechuic acid to give rise to beta-carboxy-cis,cis-muconic acid was characteristic of the entire fluorescent group of Pseudomonas and the organisms which carry out the "meta" cleavage of protocatechuic acid to produce gamma-carboxy-alpha-hydroxy-cis,cis-muconic acid semialdehyde belonged to non-fluorescent species, such as Pseudomonas acidovorans. If the organisms in this study can be assumed as Pseudomonas species, they may be classified in the fluorescent Pseudomonas group.

Further oxidative pathway of beta-carboxy-cis,cis-muconic acid was not studied. However, it may be assumed that further stepwise conversion is similar to the one previously studied by many investigators mostly with Pseudomonas species (Dagley, 1972).

Perhaps the most interesting part of this study is that evidence was obtained for possible existence of an alternate pathway of phthalic acid degradation by soil bacteria. Further studies are necessary to determine validity of the postulated intermediate, 2,7-diketo-3-cis,5-cis-octadienoic-1,8-dicarboxylic acid, of phthalic acid oxidation.

SUMMARY

Three types of bacteria were isolated from the soil by enrichment culture technique.

The soil was added to a liquid medium containing phthalic acid as a sole source of carbon. The bacterial cells grown in the medium were transferred into a fresh culture medium, and such a transfer was repeated several times. The cells grown in the final culture medium were properly diluted and transferred on agar plates containing phthalic acid as a sole carbon source. A single colony, which had the biggest diameter on the agar plate, was selected and eventually transferred on agar slants and allowed to grow for a day and stored in a refrigerator.

The bacterial cells were harvested after their growth overnight in the liquid medium, and collected by centrifugation and washed with a phosphate buffer for studies of oxidative metabolism of phthalic acid of this organism.

A number of compounds suspected to be intermediates of oxidative degradation of phthalic acid were individually mixed with the intact harvested cells. High oxygen consumption in such a mixture was considered as an evidence for the tested compound being an intermediate of phthalic acid degradation.

Oxygen consumption was determined by a Clark oxygen electrode.

Two other organisms were isolated by the same technique described above, except that phthalic acid was replaced by terephthalic acid, or isophthalic acid. Oxidative metabolism of terephthalic acid and isophthalic acid was studied in a similar manner as described above.

Those compounds, which were suspected to be intermediates of oxidative degradation of three phthalic acid isomers, and which were examined in this study, were catechol, protocatechuic acid, benzoic acid, salicylic acid, and 2,3-dihydroxy-benzoic acid.

The following results were obtained from the oxygen consumption studies. Terephthalic acid-grown cells converted terephthalic acid into protocatechuic acid, and isophthalic acid-grown cells produced the same intermediate from isophthalic acid.

Further oxidation of this intermediate was studied by examining ultraviolet absorption spectrum of the incubation mixture of protocatechuic acid and a soluble bacterial extract, from isophthalic acid-grown and terephthalic acid-grown cells. The spectra obtained from the incubation mixtures indicated that the oxidation product of protocatechuic acid was beta-carboxy-cis,cis-muconic acid. Further conversion of the intermediate into some members of the tri-carboxylic acid cycle was assumed according to the already known metabolic pathways of protocatechuic acid oxidation by microorganisms.

None of the suspected compounds tested showed appreciable oxygen consumption in phthalic acid-grown cells. A new intermediate was postulated, for the oxidative degradation of phthalic acid by this organism, which is 2,7-diketo-3,cis,5,cis-octadienoic-1,8-dicarboxylic acid. The postulate was not tested.

APPENDICES

APPENDIX A

Fig. 1. Oxygen consumption of isophthalic acid-grown bacterial cells determined by oxygen electrode.

A: Protocatechuic acid

B: Isophthalic acid

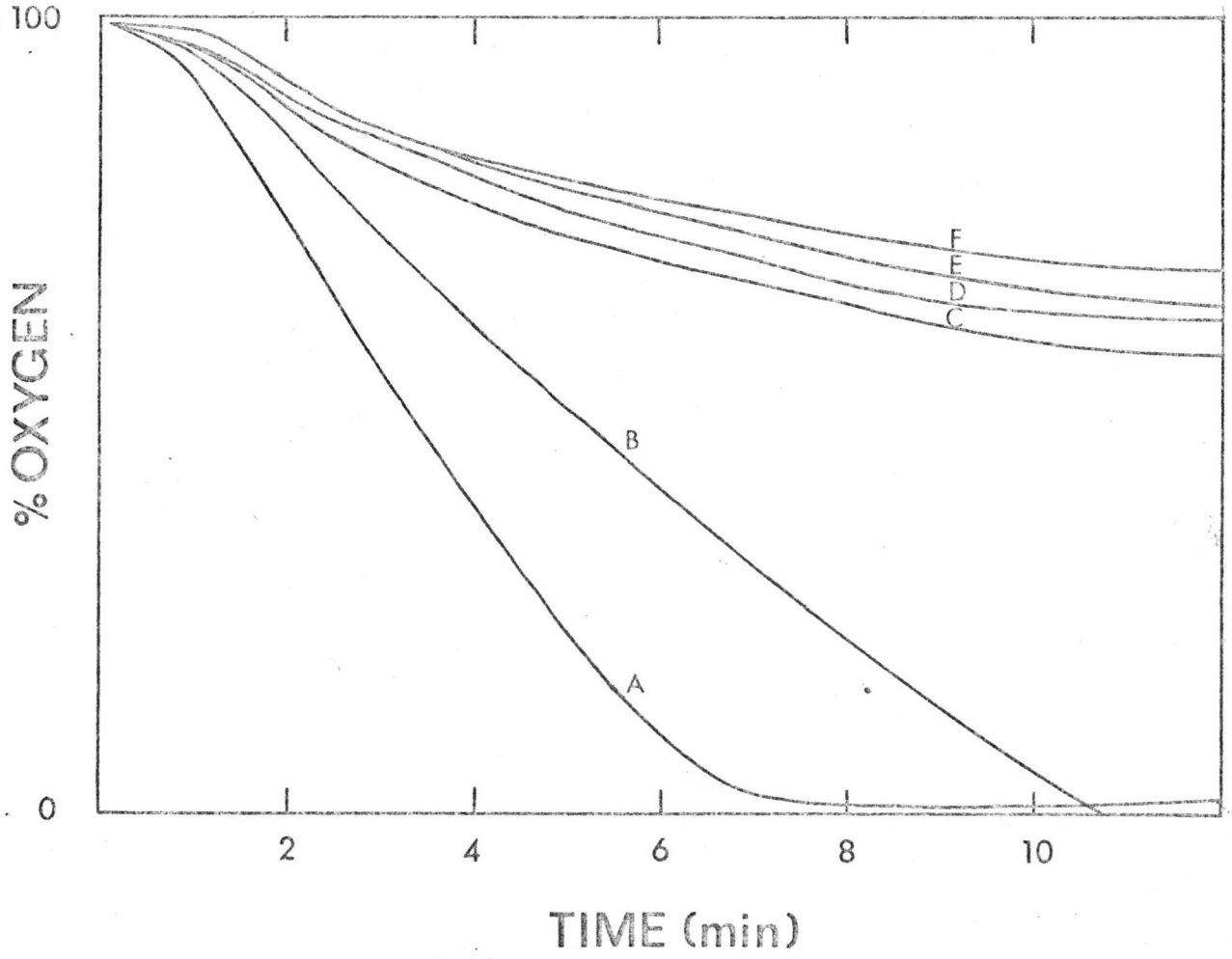
C: Catechol

D: Benzoic acid

E: 2,3-Dihydroxy-benzoic acid

F: No substrate

1.80 mg dry weight of crude cells was used per each experiment.


100
0
% OXYGEN
F
E
D
C
B
A
2
4
6
8
10
TIME (min)

APPENDIX A (Continued)

Fig. 2. Oxygen consumption of terephthalic acid-grown bacterial cells determined by oxygen electrode.

A: Protocatechuic acid

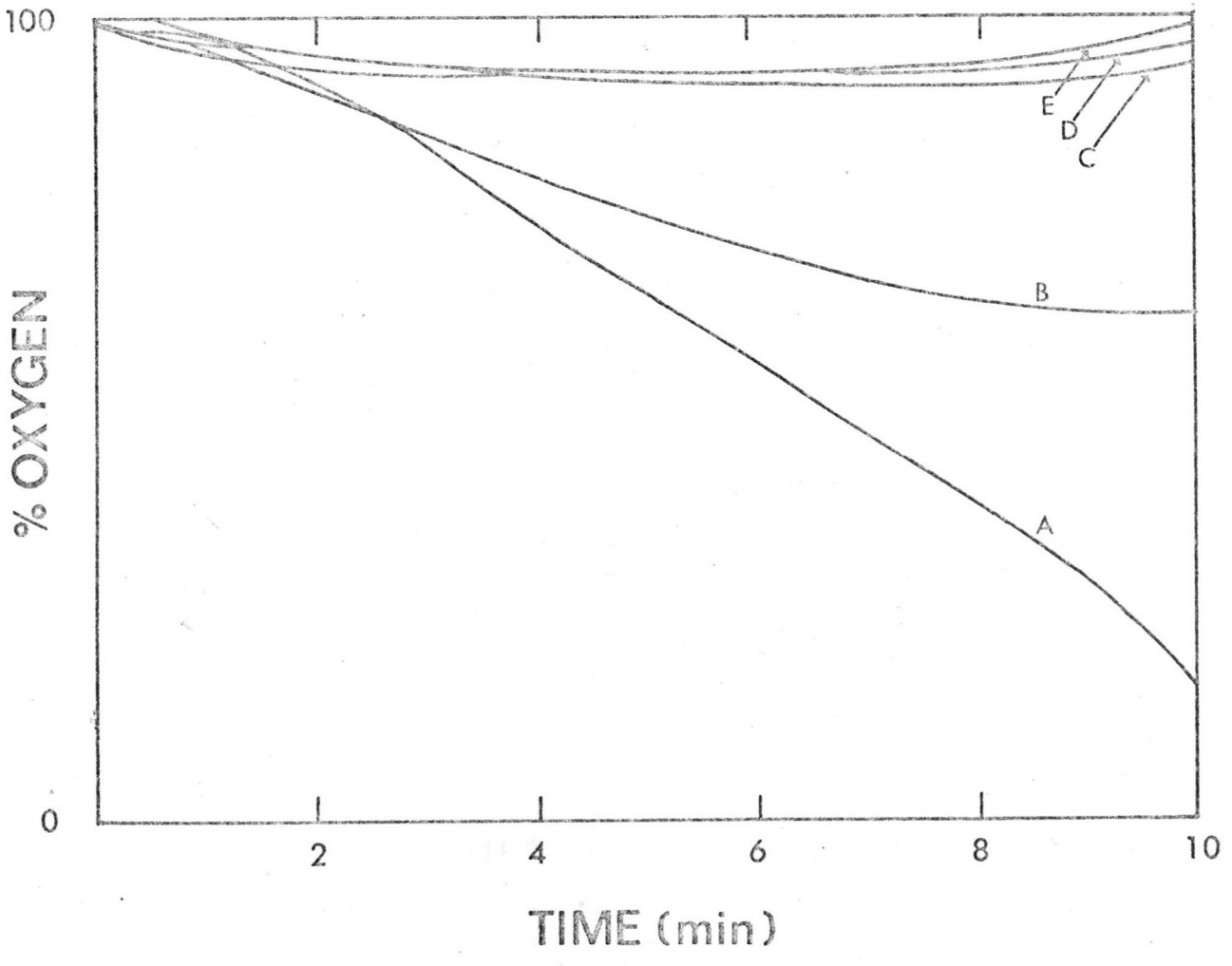
B: Terephthalic acid

C: Benzoic acid

D: 2,3-Dihydroxy-benzoic acid

E: No substrate

1.80 mg dry weight of crude cells was used per each experiment.


100
0
% OXYGEN
2
4
6
8
10
TIME (min)
A
B
C
D
E

APPENDIX A (Continued)

Fig. 3. Oxygen consumption of phthalic acid-grown bacterial cells determined by oxygen electrode.

A: Phthalic acid

B: Salicylic acid

C: Protocatechuic acid

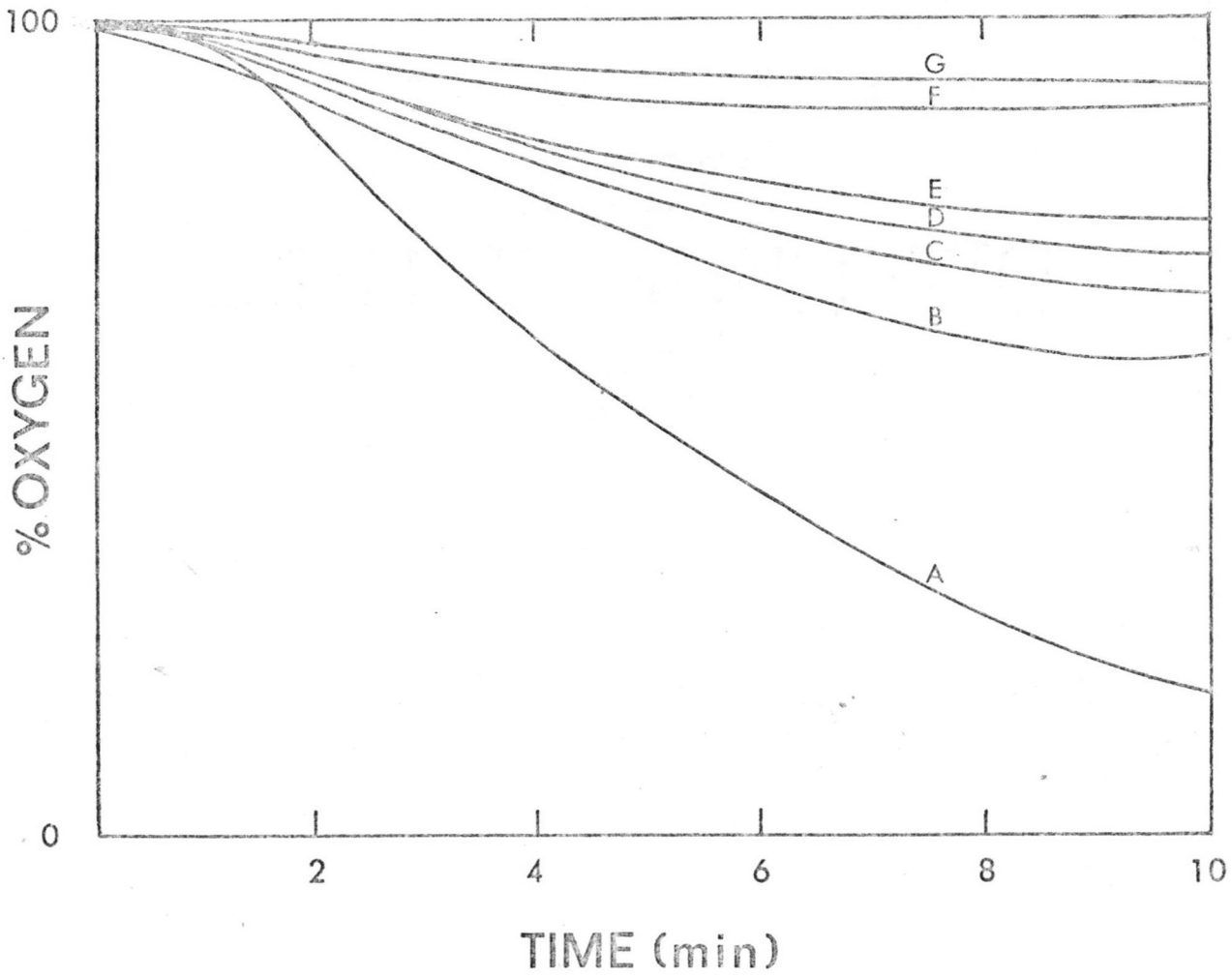
D: Benzoic acid

E: Catechol

F: 2,3-Dihydroxy-benzoic acid

G: No substrate

1.82 mg dry weight of crude cells was used per each experiment.


100
0
% OXYGEN
G
F
E
D
C
B
A
2
4
6
8
10
TIME (min)

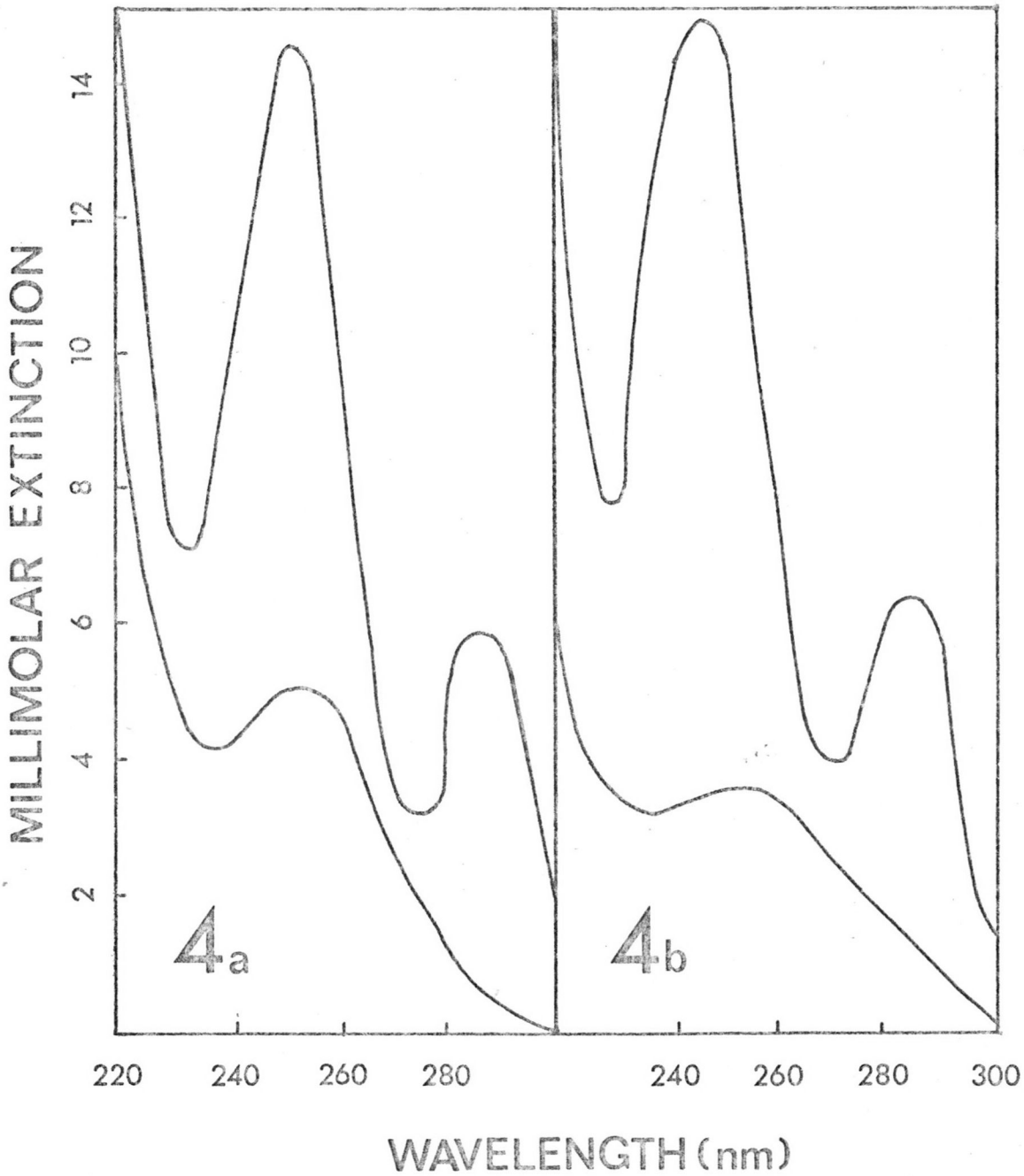
APPENDIX A (Continued)

Fig. 4a. Spectra of protocatechuic acid and of a possible intermediate, formed when protocatechuic acid and the cell-free extract of isophthalic acid-grown cells were mixed.

Fig. 4b. Same as 4a except that terephthalic acid-grown cells were used.

Upper curves: Protocatechuic acid

Lower curves: A possible intermediate, beta-carboxy-cis,cis-muconic acid


MILLIMOLAR EXTINCTION
2
4
6
8
10
12
14
4a
4b
220
240
260
280
240
260
280
300
WAVELENGTH (nm)

APPENDIX B

Pseudomonas Salt Solution

The following ingredients were mixed and dissolved. To this solution was added 0.1N HCl to the final volume of 250 ml.

12.5g of $MgSO_4 \cdot 7\ H_2O$
0.25g of $FeSO_4 \cdot 7\ H_2O$
50 ml of Zn AAS solution *
25 ml of 1:20 Mn AAS solution **
25 ml of 1:200 Cu AAS solution ***

One ml of the salt solution (containing 5 mg Mg^{++}, 0.2 mg Fe^{++}, 0.2 mg Zn^{++}, 5 µg Mn^{++}, 0.5 µg Cu^{++}) was used per 100 ml culture medium.

*AAS: Atomic Absorption Standard, obtained from Fisher Scientific Co. Zn AAS solution contained 1 mg Zn/ml in the form of ZnO in dilute HNO_3.

**1:20 Mn AAS solution was prepared by diluting one volume of Mn AAS solution to 20 volumes with water. Mn AAS solution contained 1 mg Mn/ml in the form of Mn metal in dilute HNO_3.

***1:200 Cu AAS solution was prepared by diluting one volume of Cu AAS solution to 200 volumes with water. Cu AAS solution contained 1 mg Cu/ml in the form of CuO in dilute HNO_3.

APPENDIX C

Dry Weight Determination

Table 1: Relationship between dry weight of bacterial cells and optical density

a. phthalate-grown cells

b. isophthalate-grown cells

c. terephthalate-grown cells

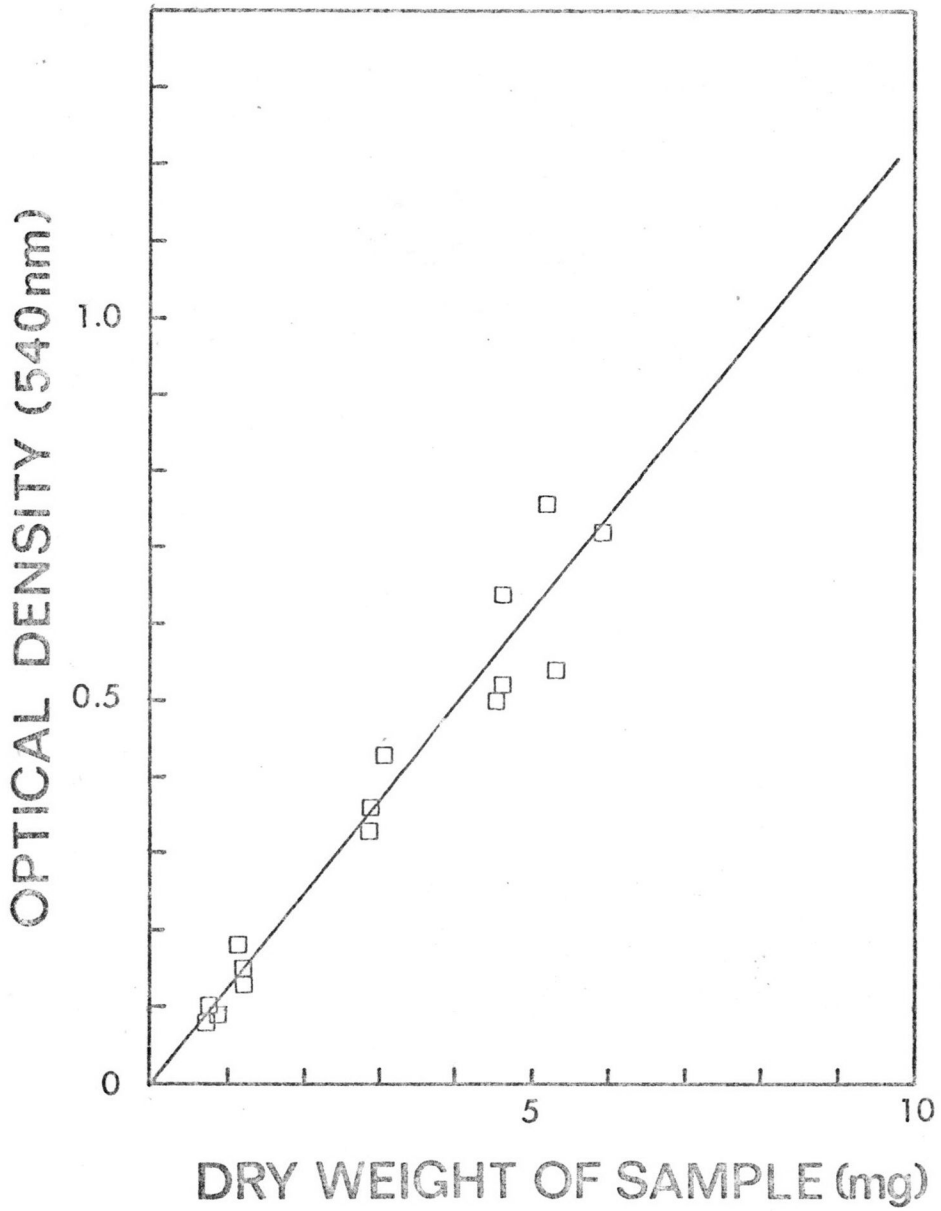
See the text for details.

Volume of bacterial slurry	Dry Weight (mg.)			Optical Density		
	a	b	c	a	b	c
0.1 ml	0.92	0.80	0.78	0.09	0.10	0.08
0.2 ml	1.25	1.17	1.24	0.15	0.18	0.13
0.5 ml	2.92	3.10	2.91	0.36	0.43	0.33
0.8 ml	4.63	4.60	4.69	0.52	0.50	0.64
1.0 ml	5.97	5.25	5.39	0.72	0.76	0.54

APPENDIX C (Continued)

Dry Weight Determination

Fig. 5. Relationship between dry weight of bacterial cells and optical density. Table 1 is plotted here.


OPTICAL DENSITY (540 nm)
1.0
0.5
0
5
10
DRY WEIGHT OF SAMPLE (mg)

APPENDIX D

Protein Determination

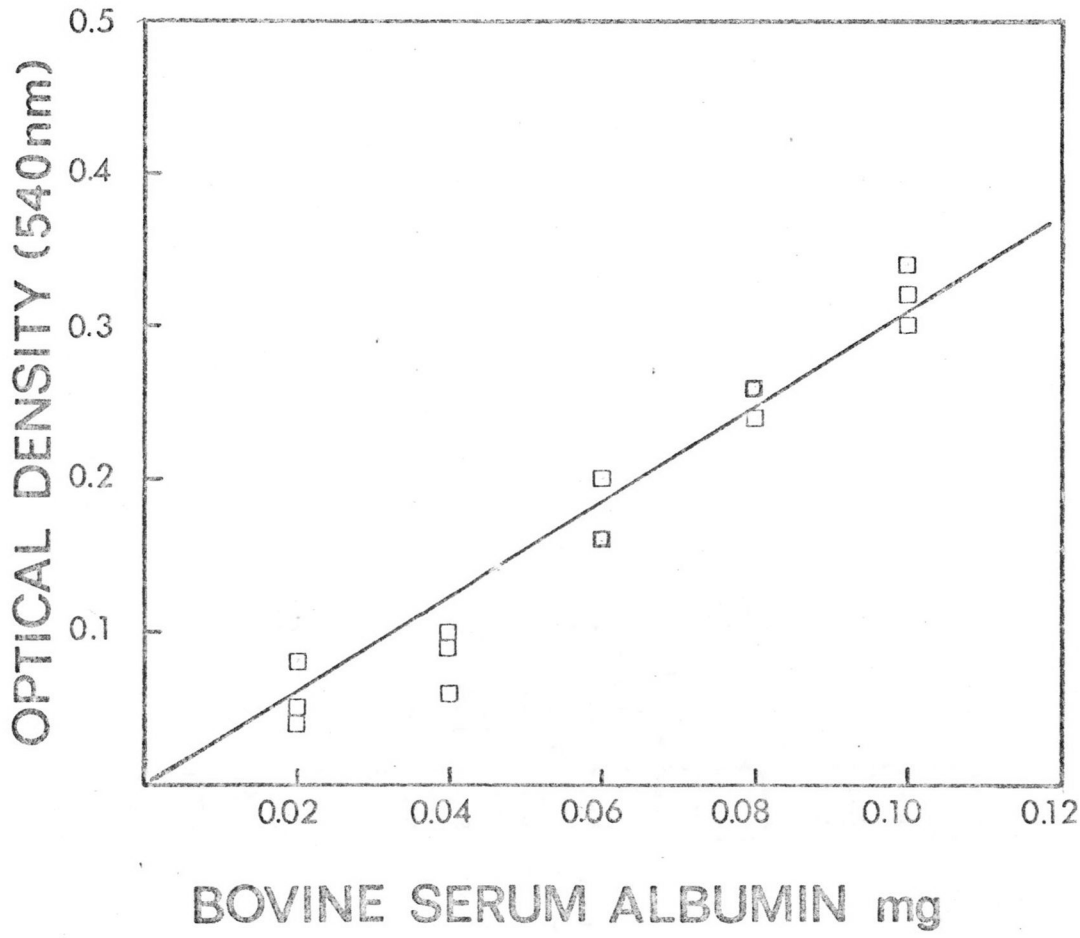
Table 2: Determination of protein in standard and in cell free extracts of bacteria

Protein	Optical Density		
0.00 mg crystalline bovine serum albumin	0.06	0.06	0.04
0.02 mg " " " "	0.10	0.11	0.12
0.04 mg " " " "	0.12	0.15	0.14
0.06 mg " " " "	0.22	0.22	0.24
0.08 mg " " " "	0.32	0.30	0.30
0.10 mg " " " "	0.36	0.40	0.36
1 ml of enzyme extract from phthalic acid-grown cells	0.09	0.11	0.11
1 ml of enzyme extract from isophthalic acid-grown cells	0.07	0.09	0.10
1 ml of enzyme extract from terephthalic acid-grown cells	0.07	0.10	0.08

APPENDIX D (Continued)

Protein Determination

Fig. 6. Standard curve for protein. Table 2 is plotted here. Optical density readings were corrected for the blank (0.00 mg protein).

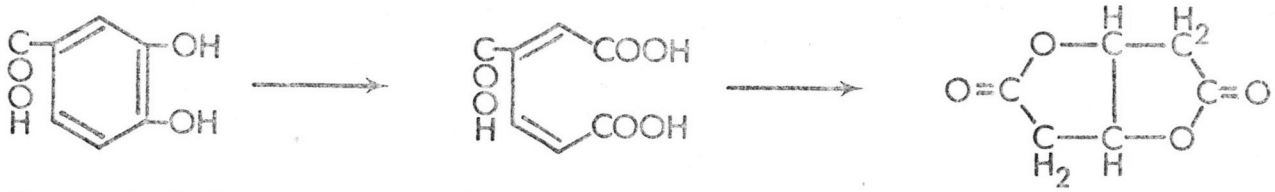

OPTICAL DENSITY (540nm)
0.5
0.4
0.3
0.2
0.1
0.02
0.04
0.06
0.08
0.10
0.12
BOVINE SERUM ALBUMIN mg

APPENDIX E

Metabolic Pathways

APPENDIX E (continued)

Fig. 7. Intermediates suggested by Elsden and Peel (1958) in Vibrio.



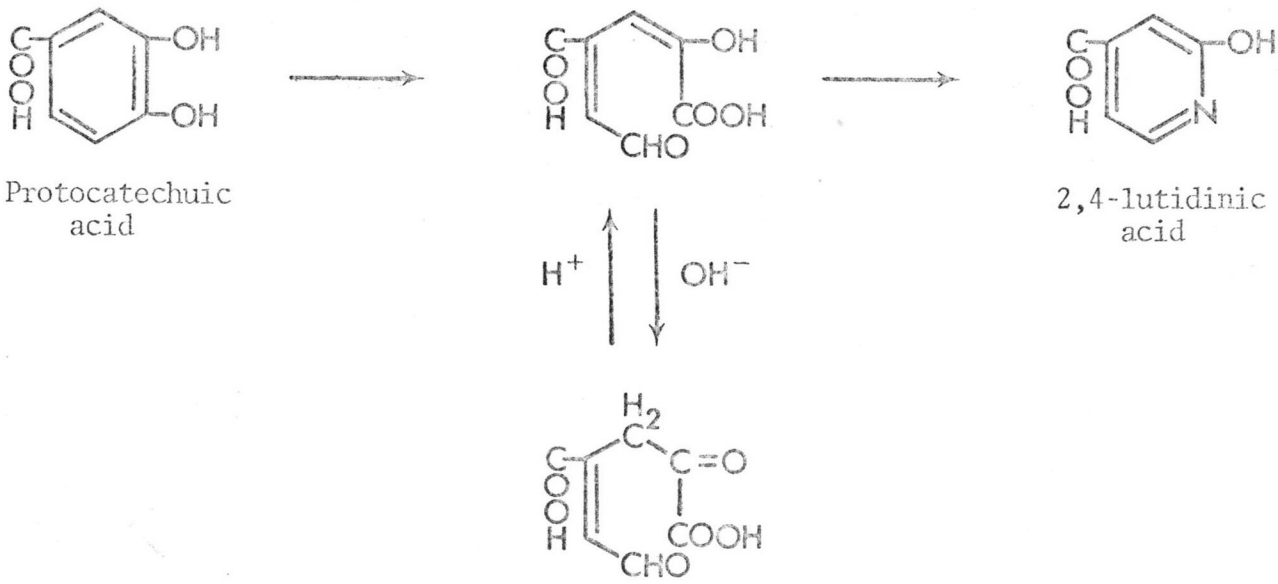
Protocatechuic acid

beta-carboxy cis,cis-muconic acid

"Dilactone"

APPENDIX E (continued)

Fig. 8. The non-enzymatic formation of 2,4-lutidinic acid in Pseudomonas species found by Dagley, Stopher, and Trippett (1960).



Protocatechuic acid

2,4-lutidinic acid

Enol and keto forms of alpha-hydroxy-gamma-carboxy muconic semialdehyde

APPENDIX E (continued)

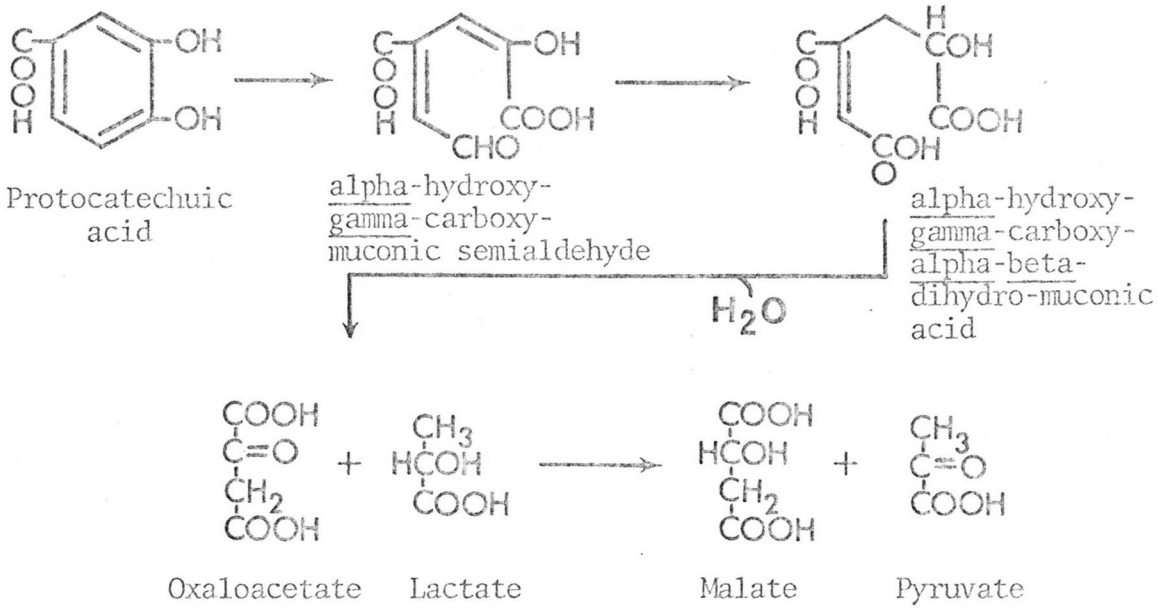
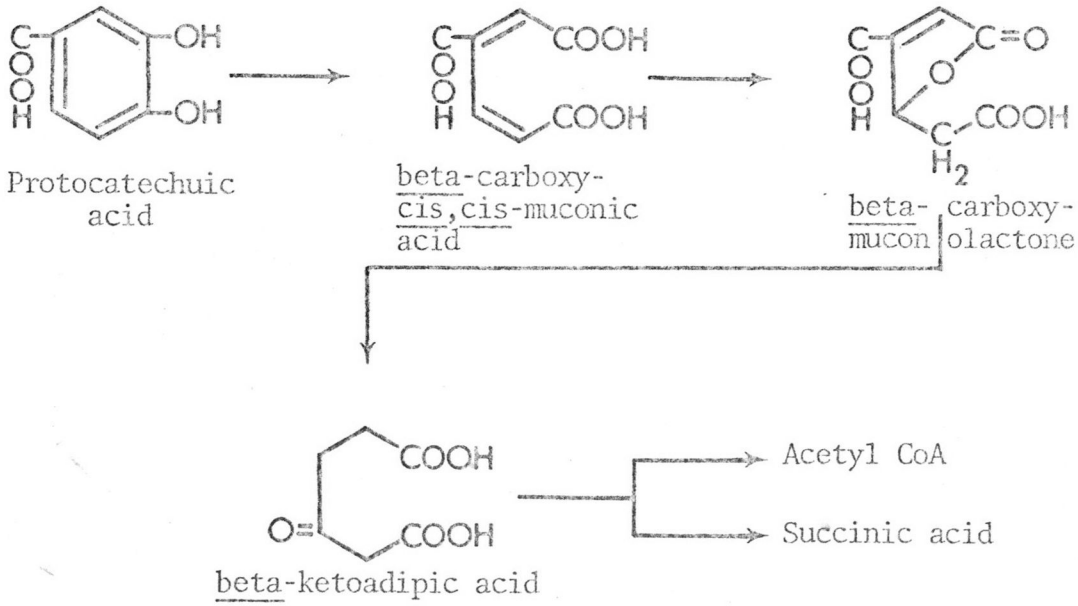


Fig. 9. Pathways of initial 4/5 split of protocatechuic acid (Dagley, Stopher, and Trippett, 1960).

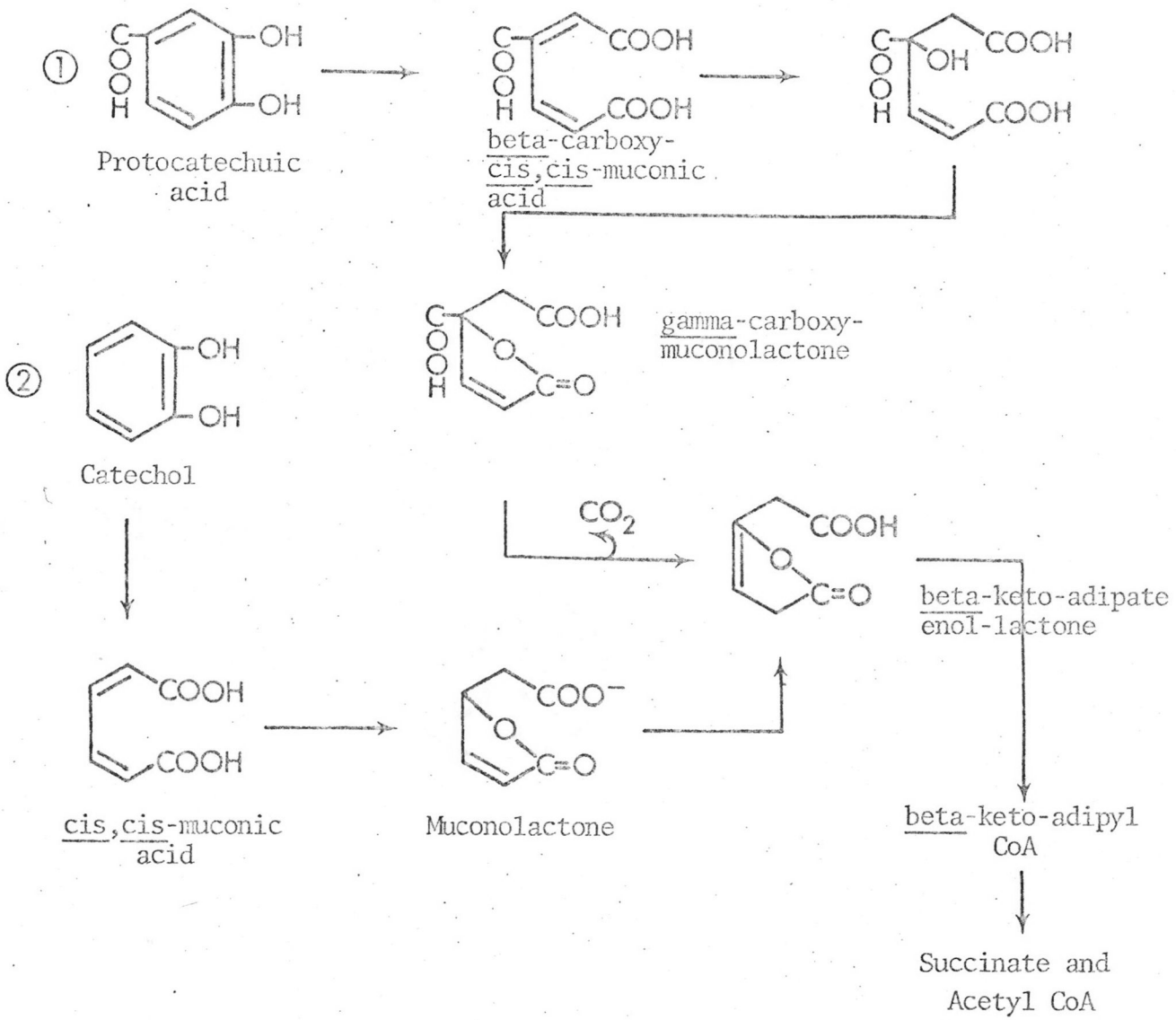
APPENDIX E (continued)

Fig. 10. Pathways of initial 3/4 split of protocatechuic acid (Ribbons and Evans, 1960).



APPENDIX E (continued)

Fig. 11. Pathways of protocatechuic acid and catechol oxidation in Pseudomonas putida by Ornston and Stanier (1966).



APPENDIX E (continued)

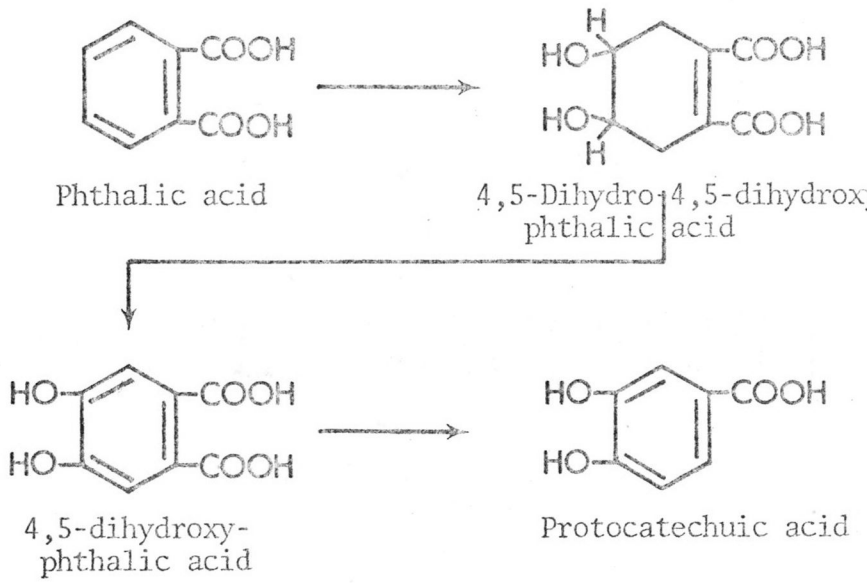
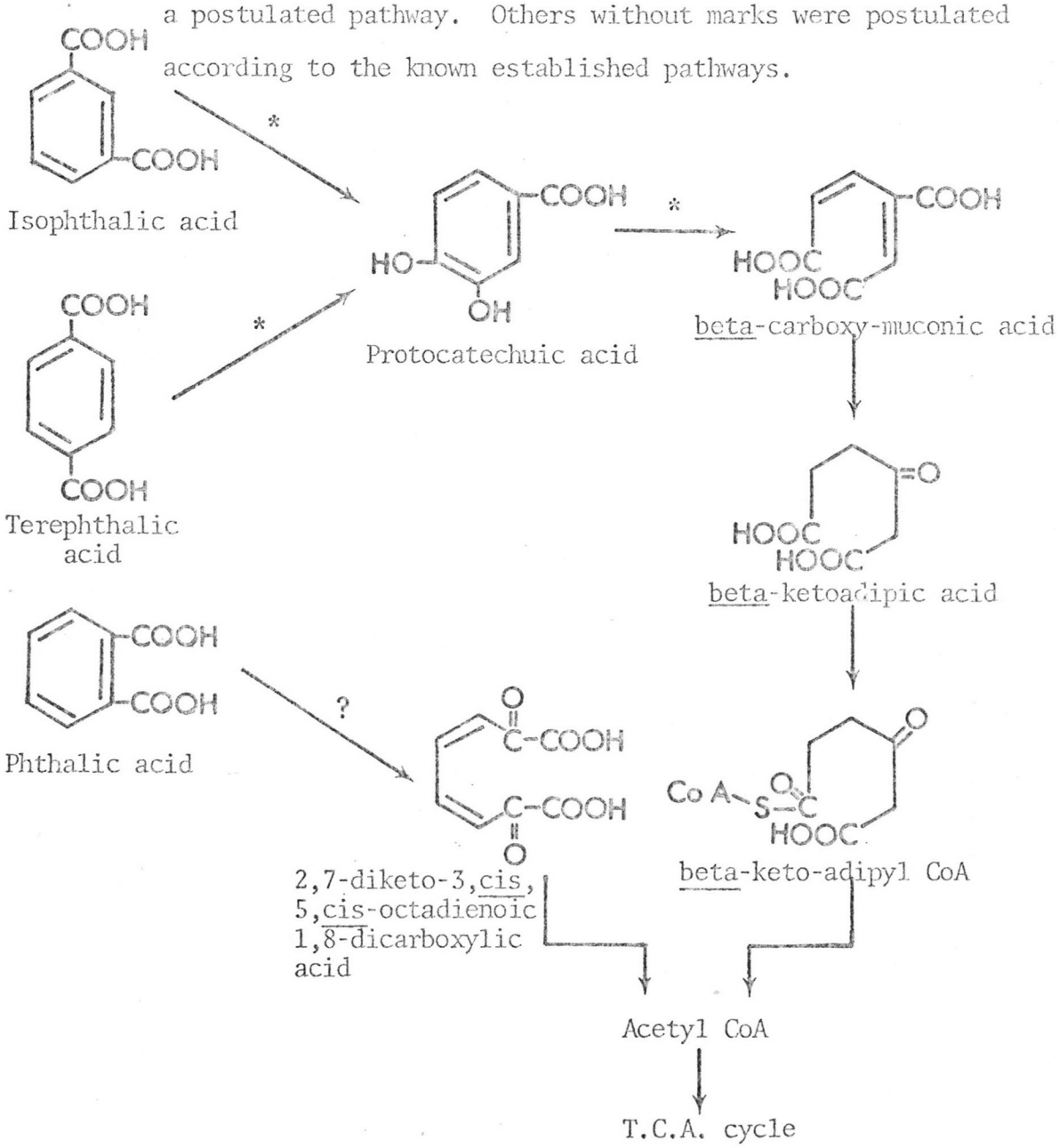


Fig. 12. Pathway of oxidative metabolism of phthalic acid indicated by Ribbons and Evans (1960).

APPENDIX E (continued)

Fig. 13. Pathways of oxidative metabolism of phthalic acid isomers in this work. The reactions marked with * were found in this work. The reaction marked with the question mark is a postulated pathway. Others without marks were postulated according to the known established pathways.



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