THE DEGRADATION OF AROMATIC COMPOUNDS BY *ARTHROBACTER* SPECIES

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Abstract

Some yellow and red pigmented species of *Arthrobacter* have been shown to possess the ability to attack several of the 41 aromatic compounds. The capacity of the red pigmented species to grow in catechol has been demonstrated. From growth experiments and manometric studies of cells harvested from glucose, phenol, hippuric acid, benzoic acid, *m*- and *p*- hydroxybenzoic acid, and catechol media it has been shown that *Arthrobacter* species bring about the oxidation of benzoic acid via *m*- and/or *p*- hydroxybenzoic acids, protocatechuic and/or gentisic acids to catechol and to pyruvic acid.

Introduction

The degradation of aromatic compounds by ring cleavage is an essential biochemical step in the release of organic carbon locked up in benzenoid structures of plant tissues. Microorganisms are in main the agents known to possess the ability to degrade a variety of such compounds present in different environments. Although they are most versatile in this aspect only a few genera are known to carry out this process as may be gathered from the reviews of Rogoff (1961), Evans, (1963), Treccani (1963), Mckenna and Kallio (1965), van Der Linden and Thijsse (1965) and the recent report from this laboratory (Bhat, 1967).

In the light of present knowledge, microbial attack on the aromatic ring appears to be dependent on induced enzymes formation. Only a few studies, however, have so far been carried out with a view to determine the effect of chemical structure of these compounds on the ability of bacteria adapted to given aromatic compound to oxidize related compounds. Czekalowski and Skarzynski (1948) investigated the relationship between chemical structure and the use of aromatic compounds by one phenol – tolerant strain of *Achromobacter*. Kramer and Doetsch (1950) used *Achromobacter*, *Micrococcus* and *Vibrio* species capable of utilizing phenols to study their ability to grow on related aromatic compounds. Tabak *et al* (1964) reported to have isolated *Xanthomonas*, *Pseudomonas*, *Achromobacter* and *Flavobacterium* species capable of utilizing phenol and related aromatic compounds. The oxidation of

benzene by bacteria has been studied by Marr and Stone (1961) and that of toluene by Claus and Walker (1964). Some aspects of the subject has been investigated in this laboratory by Jayasankar and Bhat (1966).

In the oxidation of benzoic acid by soil pseudomonads catechol was found to be a key intermediate (Evans *et al*, 1949; Sleeper and Stanier, 1950). This was demonstrated by sequential induction experiments and by the isolation of catechol from culture filtrates. Sleeper (1951) carried out tracer experiments with benzoic acid labelled with C^{14} in carboxyl or 1-carbon positions and reached the conclusion that catechol formed by the bacteria from carboxyl carbon labelled benzoic acid was inactive whereas that formed from the 1- carbon labelled compound retained the activity showing thus the decarboxylation of benzoic acid to catechol A detailed study of benzoic acid metabolism by *Pseudomonas convexa* var. *hippuricum* was carried out by Bhat *et al* (1959). They pointed out the involvement of salicylic acid as an intermediate in the oxidation of benzoic acid to catechol. They suggested the possibility of successive introduction of hydroxyl groups on the benzene molecule to yield salicylic acid first and then catechol. Oxidative decarboxylation of salicylic acid to catechol has also been subsequently demonstrated by other workers recently, *e. g.*, Tsukamura (1965) in *Mycobacterium fortuitum*. However, Yano and Arima (1958) reported that several strains of *Pseudomonas* showed an alternative pathway via gentisic acid. Walker and Evans (1952) indicated other pathways by which bacteria metabolized monohydroxy derivatives of benzoic acid. In this communication are presented some of the results of the experiments conducted on the degradation of the aromatic compounds by several strains of *Arthrobacter* species isolated from soil samples. An indication of the ability of these bacteria to attack aromatic compounds can be obtained from the earlier reports from here (1966*a*, 1967) and elsewhere (Stevenson, 1967).

MATERIALS AND METHODS

Bacterial cultures employd: Sixteen of 170 strains of *Arthrobacter* tested were observed to possess the ability to utilize aromatic compounds. Seven of them were yellow pigmented and ureolytic (*Arthobacter ureovorous* n.sp.) while seven other were of the red-pigmented species (*Arthrobacter ruber* n. sp. and nonureolytic. One strain tentatively identified as *A. tumescens*, along with another strain which appeared to be a non-pigmented variant thereof, were also included in this investigation. All the strains were examined for their ability to attack 41 aromatic compounds chosen for this work but only two (No. 4 and 63) were more closely examined for their growth, enzymatic and respiratory behaviour. The cultures during the course of the work were maintained on basal salts-hippurate-yeast extract agar slants.

Growth experiments: The various growth substrates were tested in pyrex test tubes (1.8 × 15 cm) containing 10 ml of the medium. The compounds or their solutions, sterilized by heat or filtration, were incorporated into the basal medium used by Khambata *et al* (1960) at desired concentrations. All the substances were tested at 0.05% level; except catechol, phloroglucinol, *m*-cresol, *p*-cresol, benzene, orcinol, resorcinol and gallic acid which were tested at 0.02% level, in every case 0.05% ammonium sulphate served as a nitrogen source and the medium was supplemented with 0.001% yeast extract. Appropriate control tubes were also maintained side by side; incubation was at room temperature (20-28 °C). The growth was measured over a period of 10 days with a Bausch and Lomb 'Spectronic 20' colorimeter at 540 $m\mu$. In the shake flasks experiments also growth was measured by removing aliquots at stipulated time intervals and the residual substrate was estimated simultaneously.

Preparation of the resting cells for manometric studies: Specific adaptation of cells of *Arthrobacter* cultures (4 and 63) to the substrate was achieved by growing them on a mineral medium containing individually those specific compounds or other related compounds intended to serve as the sole source of carbon as: 0.5% hippuric acid, 0.1% benzoic acid, 0.1% *p*- or *m*- hydroxybenzoic acids, 0.1% benzaldehyde, 0.1% benzylalcohol, 0.2% tyrosine, 0.05% phenol or 0.02% catechol. When the medium contained hippuric acid, no other nitrogen source was incorporated; in others 0.05% ammonium sulphate along with 0.001% yeast extract was incorporated. For harvesting cells unadapted to aromatic compounds, glucose + 0.05% ammonium sulphate were added to an otherwise mineral base. Details of the procedure for the preparation of the resting cells have been provided by Mullakhanbhai and Bhat (1966). Acetone dried cells were prepared by the method recommended by Youmans *et al* (1956).

Chemical methods used for identifying the intermediate products: To detect the intermediates formed in the cultures growing in hippuric acid, the cultures fluids were tested, after 24 hours, for the presence of benzoic acid – a resultant product of the hydrolysis of hippuric acid with a 10% solution of ferric chloride in water which gave a buff coloured precipitate indicating its presence. The presence of glycine was detected by the circular paper chromatographic technique, (Giri *et al* 1953).

After 30 hr of growth the culture medium was proved to contain catechol by the sensitive colour test recommended by Evans (1947). For the detection and estimation of hippuric acid in the present study a unidimensional paper chromatographic method proposed by Gaffney *et al* (1954) and catechol by the method recommended by Nair and Vaidyanathan (1964) were used.

Measurement of oxidation: The investigations on the breakdown products of hippuric acid by *Arthrobacter* were carried out by the sequential

induction technique of Stanier (1947). The details of the method followed were those given by Umbreit *et al* (1957). The aerobic oxidation experiments were carried out in the conventional Warburg vessels using KOH paper in the centre well to absorb CO_2, air as the gas phase and at the temperature of 30°C. Total volume in each flask was 3.2 ml which included 0.2 ml of 20% KOH, 1.5 ml M/15 phosphate buffer pH 7.0 containing 100 mg, wet weight of cells. Substrates in 0.2 ml were always tipped off from the side arm in concentration of 5 micromoles and distilled water to make up the volume. Oxygen uptake was measured at the intervals of 10 minutes. Blank determinations were made without substrate and corrections were made for the endogenous respiration before their representation in graphs.

RESULTS AND DISCUSSION

Utilization of aromatic compounds: The red pigmented *Arthrobactor* species (Table I) grew very well indeed in a basal mineral medium containing 0.001% yeast extract as well as hippuric acid, *m*- and *p*- hydroxy benzoic acids, phnylacetic acid, gentisic acid, phenol, benzaldehyde, benzyl alcohol, catechol, *p*-cresol, and tyrosine. Other compounds like benzene, phloroglucinol, orcinol, resorcinol, *m*-cresol, *p*, *m* and *o*- aminobenzoic acids and gallic acid also supported their growth to a limited extent. *o*- hydroxy benzoic acid could not support growth. All others, *viz* pyrogallol, *p*-nitrophenol, *m*- nitrophenol, 2:4 dinitrophenol, *o*- and *p*-aminophenol, *o* and *m*-toluidine, quinol, hydroquinone, indole, tryptophane, phenylalanine, pthalic acid, 1:3 dihydroxy naphthalene, 2:7 dihhydroxynaphthalene and naphthol, also failed to support growth. The yellow pigmented ureolytic *Arthrobacter* species differed from the previous group only in one minor aspect, *viz* they could not attack catechol and gentisic acid, though they could attack phenylalanine (Table 2).

A. tumescens strains were even more interesting in the sense that they grew very well on *p*- and *m*- hydroxybenzoic acids and *o*- aminobenzoic acid and to a lesser extent an *m*- and *p*- aminobenzoic acids but not on *o*- hydroxy benzoic acid Benzylalcohol, benzaldehyde and *p*- cresol, which supported good growth of the other 14 strains, also failed to support their growth though phenylacetic acid served exceedingly well as a growth substrate (Mullakhanbhai and Bhat, 1966a).

Growth experiments: The response of the of the yellow pigmented *Arthrobacter* species (strain, 63) in relation to the utilization of hippuric acid is illustrated in Fig. 1. The disappearance of the substrate was followed by the simultaneous formation and utilization of glycine. Hippuric acid was utilized completely after 60 hrs of the growth period. Glycine was detectable and estimable after 10 hr of growth and upto 40 hr its formation at 20 hr being the highest. The figure offers a clear and typical picture of hippuric acid hydrolysis and formation and utilization of glycine by the *Arthrobacter* species.

TABLE I

Utilization of aromatic compounds by *Arthrobacter species*

	A. ruber strains							*A. tumescens*
	1	2	3	4	5	6	7	102
	Growth response: 100—% transmittance							
Hippuric acid	42	68	46	45	60	50	41	25
Benzoic acid	62	63	68	71	60	69	64	20
m- Hydroxybenzoic acid	65	57	69	65	63	58	67	64
p- Hydroxybenzoic acid	52	52	56	62	50	55	57	52
Phenylacetic acid	46	49	44	49	45	40	42	38
Protocatechuic acid	41	40	40	43	41	40	42	25
Gentisic acid	65	61	62	67	60	65	63	10
Phenol	66	68	40	74	60	62	64	57
Benzaldehyde	60	48	57	42	45	50	56	0
Benzylalcohol	65	59	66	48	60	58	57	0
Catechol	46	44	41	40	44	40	42	0
p- Cresol	53	57	57	56	52	55	49	0
Phenylalanine	0	0	0	0	0	0	0	29
Tyrosine	73	66	66	58	70	64	60	57
Benzene	25	28	24	32	26	24	28	21
Phloroglucinol	20	17	20	20	18	20	20	10
Orcinol	25	21	20	22	20	25	20	10
Resorcinol	20	29	22	30	27	25	20	10
m- Cresol	35	30	28	32	30	32	27	10
p- Aminobenzoic acid	15	20	18	20	15	15	18	17
m- Aminobenzoic acid	15	18	20	20	15	15	15	15
o- Aminobenzoic acid	20	20	20	25	18	18	20	40
Gallic acid	20	20	16	20	15	20	20	10

o-hydroxybenzoic acid, pyrogallol, *p*-nitrophenol, *m*-nitrophenol, 2:4-dinitrophenol, *o*-, and *p*-aminophenol, *o*- and *m*- toluidine, hydroquinone, hydroxyhydroquinone, indole, tryptophane, phthalic acid, 1:3-dihydroxynaphthalene, 2:7-dihydroxynaphthalene, and β-naphthol did not support any growth.

TABLE II

Utilization of aromatic compounds by *Arthrobacter* species

	A. ureovorous strains							*A. tumescens*
	57	58	59	60	61	62	63	103
	Growth response : 100—% transmittance							
Hippuric acid	71	66	62	54	60	58	78	50
Benzoic acid	50	55	52	50	57	58	60	40
m-Hydroxybenzoic acid.	40	42	40	41	41	40	44	56
p-Hydroxybenzoic acid.	39	40	38	40	40	38	40	55
Phenylacetic acid	50	58	60	50	52	67	56	86
Protocatechuic acid	46	45	47	44	45	42	50	30
Gentisic acid	0	0	0	0	0	0	0	30
Phenol	40	38	41	40	41	38	40	34
Benzaldehyde	50	45	50	47	46	45	44	0
Benzylalcohol	38	39	38	40	40	40	40	0
p-Cresol	38	38	35	36	39	35	40	0
Phenylalanine	30	29	25	30	32	29	35	42
Tyrosine	35	30	32	35	30	32	35	60
Benzene	20	20	20	15	18	15	20	23
Phloroglucinol	20	18	20	20	18	15	20	10
Orcinol	20	15	20	19	20	18	21	10
Resorcinol	20	25	21	27	20	20	30	10
m-Cresol	15	15	15	15	15	15	15	10
p-Aminobenzoic acid	15	15	15	15	15	15	15	17
m-Aminobenzoic acid	18	15	15	20	15	15	15	15
o-Aminobenzoic acid	20	21	24	20	20	20	25	35
Gallic acid	20	20	18	20	20	20	18	10

o-hydroxybenzoic acid, catechol, pyrogallol, *p*-nitrophenol, *m*-nitrophenol, 2:4-dinitrophenol, *o*-and *p*-aminophenol, *o*-and *m*-toluidine, hydroquinone, hydroxyhydroquinone, indole, tryptophane, phthalic acid, 1:3-dihydroxynaphthalene, 2.7-dihydroxynaphthalene and β-naphthol did not support any growth.

The red-pigmented *Arthrobacter* species exhibited a remarkable property to utilize catechol as a growth substrate. Very few bacterial strains have so far been reported to possess the ability to utilize catechol as growth substrate. Bacteria are known to oxidise catechol in Warburg vessels at a very high rate but because of its toxic effect it inhibits completely the multiplication of bacteria in the culture medium. Red-pigmented *Arthrobacter* possessed the ability to grow in it as well as to oxidize it at a very high rate in the resting cell preparations. This property is illustrated in figure No. 2. It represents a growth curve of the strain 4 of red-pigmented *Arthrobacter* species. The rate of disappearance of catechol was followed at various time intervals and catechol was found to disappear from the medium within 60 hr of incubation.

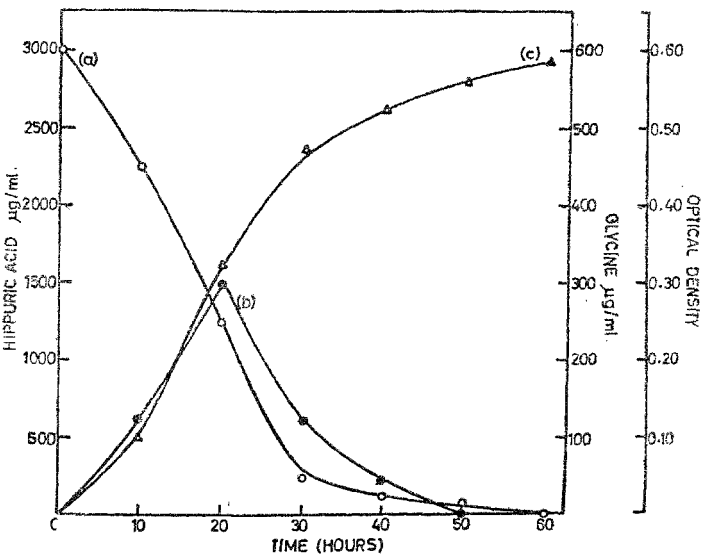

FIG. 1

The rate of disappearance of Hippuric Acid (*a*) and the formation and utilization of glycine (*b*) with reference to growth (*c*) of Arthrobacter species Strain 63 in a hippuric acid culture medium.

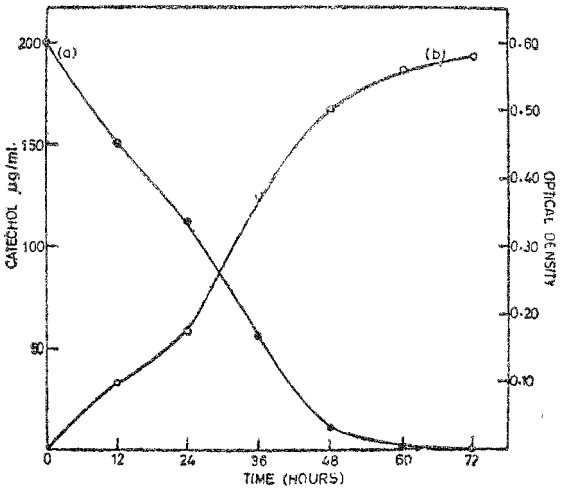

FIG. 2

The rate of disappearance of Catechol (*a*) and the rate of growth of Arthrobacter sp. strain 4 (*b*) in a Catechol culture medium.

Oxygen uptake experiments: The probability of certain compounds as involved in the benzoic acid catabolism was ascertained by the application of the sequential induction experiments using for this purpose washed cell suspensions from cultures grown on different substrates.

Glucose grown organisms: These were used to show that the benzoic acid catabolism by bacteria was an induced phenomenon. For the glucose grown cells of red-pigmented *Arthrobacter* species oxidised glucose without lag but could do so the aromatic compounds only after a relatively long lag phase. With the yellow-pigmented *Arthrobacter* species there was absolutely no oxygen uptake with aromatic compounds even after 2 hrs for which the test lasted.

Phenol grown organism: The yellow-pigmented *Arthrobacter* species almost immediately oxidized phenol and catechol but not hippuric acid, benzoic acid, *m*- hydroxy benzoic acid and protocatechuic acid; *p*- hydroxy benzoic acid was however slowly metabolized after a considerably long lag

period (Fig. 3). With the red-pigmented *Arthrobacter* species also there was an immediate oxygen uptake with phenol and catechol whereas *m*- hydroxy and *p*-hydroxybenzoic acids showed an oxygen uptake after 50 minutes of lag period. No oxygen uptake was recorded with protocatechuic acid, though gentisic acid showed an uptake after 70 minutes of lag period. This again was suggestive of the mechanism of benzoic acid oxidation as an induced phenomenon.

Hippuric acid grown organisms: Oxygen uptake was immediate with hippuric acid, benzoic acid, *m*-hydroxy benzoic acid, *p*-hydroxy benzoic acid, protocatechuic acid, catechol, pyruvic acid and acetate (Figs. 4 and 5). The red-pigmented species could also oxidize gentisic acid. The yellow variety elaborated at low oxygen tension a soluble dark brown pigment which gave a positive catechol test. With acetone dried cells there was an immediate and rapid uptake of oxygen with catechol and its utilization was complete within 140 minutes of incubation at room temperature (20-28°C). The results are presented in Fig. 6.

Benzoic acid grown organism: The red pigmented *Arthrobacter* species utilized immediately benzoic acid, *m*- and *p*- hydroxy benzoic acids protocatechuic acids, gentistic acid and catechol (Fig. 7). With the exception of gentisic acid others were oxidized by the yellow pigmented *Arthrobacter* species as well. The yellow pigmented species, when cultivated in benzoic acid containing medium under low oxygen tension, elaborated a dark brown soluble pigment and gave a positive catechol test when the culture fluid was tested.

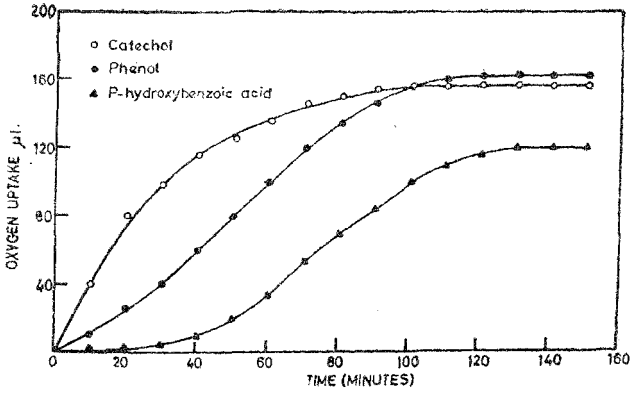

FIG. 3

Oxygen uptake from the oxidation of phenol, catechol and p-hydroxybenzoic acid by washed intact cells of Arthrobacter Strain 63 grown on phenol.

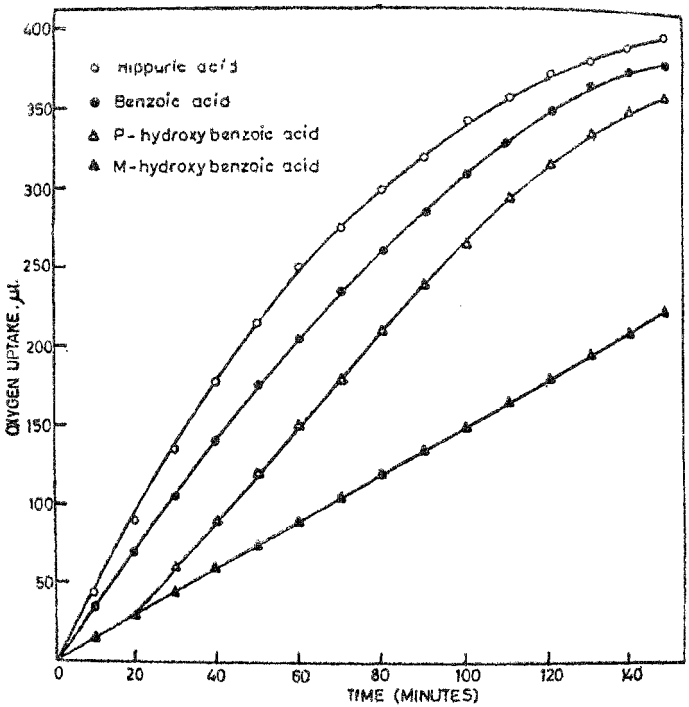

FIG. 4

Oxygen uptake from the oxidation of hippuric acid, benzoic acid p-hydroxybenzoic acid and m-hydroxybenzoic acid by washed intact cells of Arthrobacter strain 63 grown on hippuric acid.

m-Hydroxy benzoic acid grown organisms : Whereas the oxygen uptake was immediate with *m*- hydroxy benzoic acid, gentisic acid and catechol by the red pigmented species, benzoic acid and *p*- hydroxy benzoic acid were oxidized after a lag period (Fig. 8). The yellow species did not take up any oxygen with gentistic acid but protocatechuic acid got oxidized immediately It elaborated a soluble dark brown pigment which gave a positive catechol test.

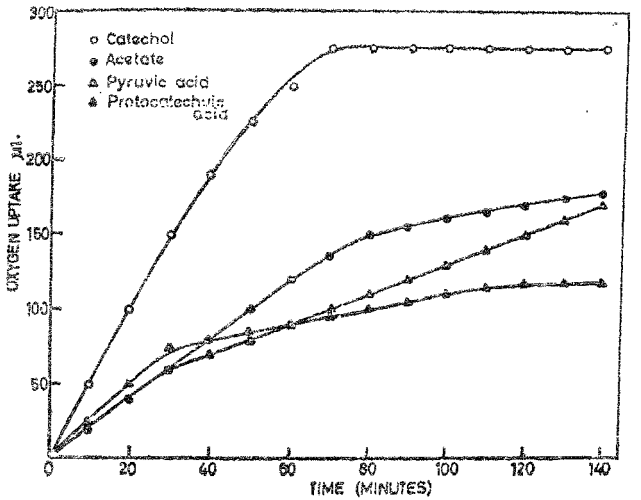

FIG. 5

Oxygen uptake from the oxidation of protocatechuic acid, catechol pyruvic acid and acetate by washed intact cells of Arthrobacter Strain 63 grown on hippuric acid.

p- Hydroxybenzoic acid grown organisms: The yellow variety showed an immediate oxygen uptake with *p*-hydroxy benzoic acid, protocatechuic acid and catechol but benzoic acid was oxidized only after a lag period (Fig. 9).

Catechol grown organisms: The red pigmented *Arthrobacter* species immediately oxidized catechol but all the other aromatic compounds only after a lag period (Fig. 10).

The fact that all the 16 isolates of 3 different *Arthrobacter* species studied could grow on *m*- and *p*- hydroxy derivatives of benzoic acid seems to indicate that there is a successive introduction of one hydroxyl group in benzoic acid molecule during oxidation either to meta or para position but not at ortho position as ortho hydroxy benzoic acid (salicylic acid) is neither utilized as a growth substrate nor oxidized by the resting cells in the Warburg vessels. This rules out the possibility of *o*- hydroxy benzoic acid as an intermediate in the catabolism of benzoic acid by *Arthrobacter* species. The introduction of second hydroxyl group and the formation of protocatechuic

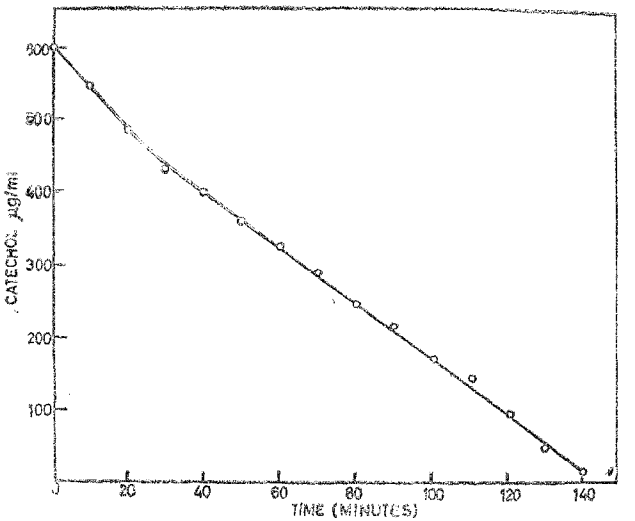

FIG. 6

The rate of disappearance of catechol from the reaction mixture incubtaed with acetone washed cells of Arthrobacter Strain 4.

acid seems to be more logical as benzoic acid adapted cells got simultaneously adapted to both protocatechuic acid and catechol. A simple step, *viz* decorboxylation would lead to the formation of catechol, a compound known to be the key intermediate in the catabolism of benzoic acid. Furthermore, the catechol cleavage product, *viz*., pyruvic acid is a metabolite par excellence for growth. The formation of this keto acid is equally interesting as in other bacterial species *cis-cis* muconic acid is known to be the product of catechol breakdown. Though the formation of this keto acid is not equivocally established, the evidences adduced are in favour of its occurrence. In the light of the present results of the sequential induction experiments, the degradation pattern of benzoic acid by *Arthrobacter* species seems to operate through the pathways indicated in Fig. 11.

ACKNOWLEDGEMENT

The author wishes to thank Dr. J. V. Bhat for the suggestion of this problem, guidance and help in this investigation.

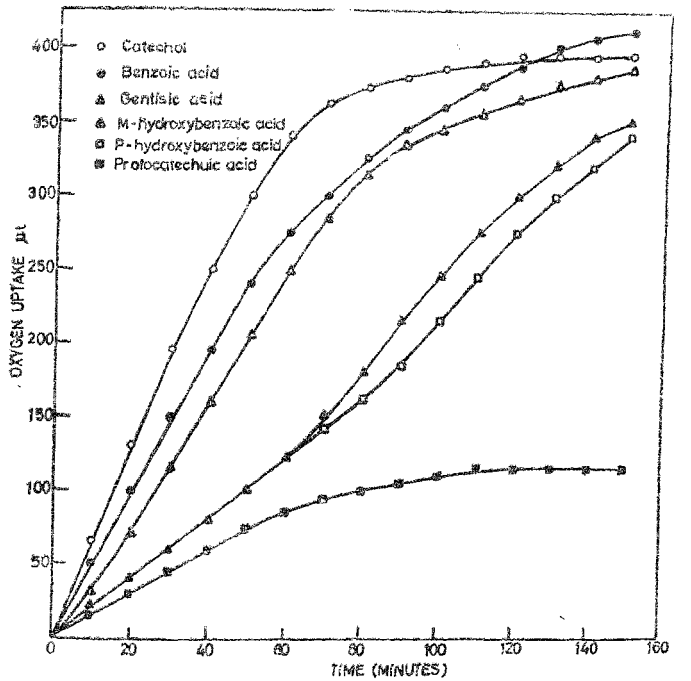

FIG. 7

Oxygen uptake from the oxidation of benzoic acid, p-hydroxybenzoic acid, m-hydroxybenzoic acid, gentisic acid, protocatechuic acid and catechol by washed intact cells of Arthrobacter Strain 4 grown on benzoic acid.

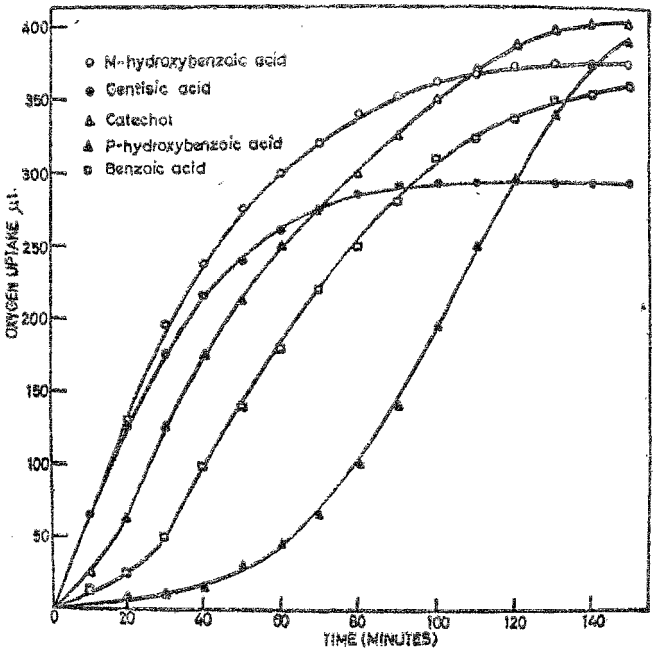

FIG. 8

Oxygen uptake from the oxidation of benzoic acid, p-hydroxybenzoic acid, m-hydroxybenzoic acid, gentisic acid and catechol by washed intact cells of Arthrobacter Strain 4 grown on m-hydroxybenzoic acid.

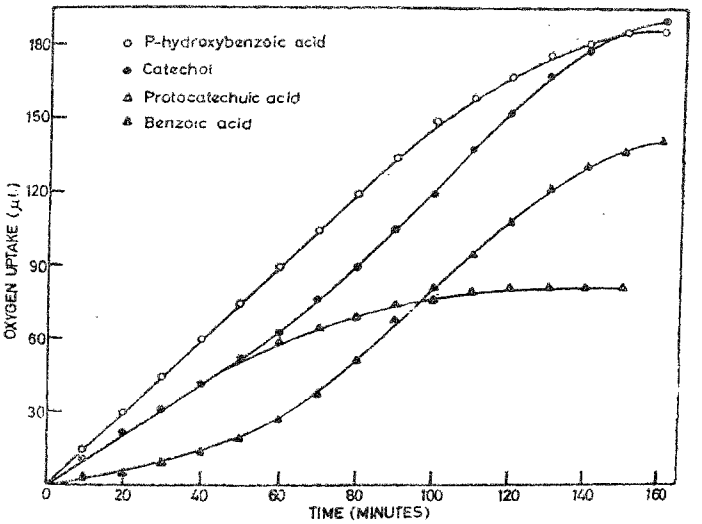

FIG. 9

Oxygen uptake from the oxidation of benzoic acid, p-hydroxybenzoic acid, protocatechuic acid and catechol by washed intact cells of Arthrobacter Strain 63 grown on p-hydroxybenzoic acid.

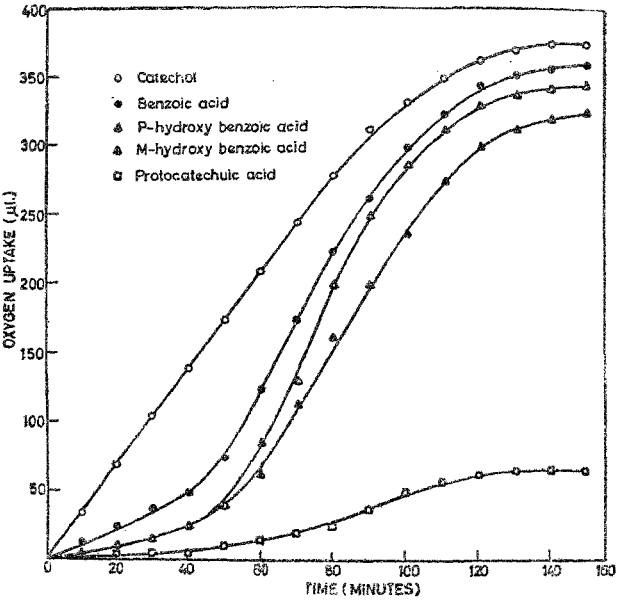

FIG. 10

Oxygen uptake from the oxidation of benzoic acid, p-hydroxybenzoic m-hydroxybenzoic acid, protocatechuic acid and catechol by washed intact cells of Arthrobacter Strain 4 grown on catechol.

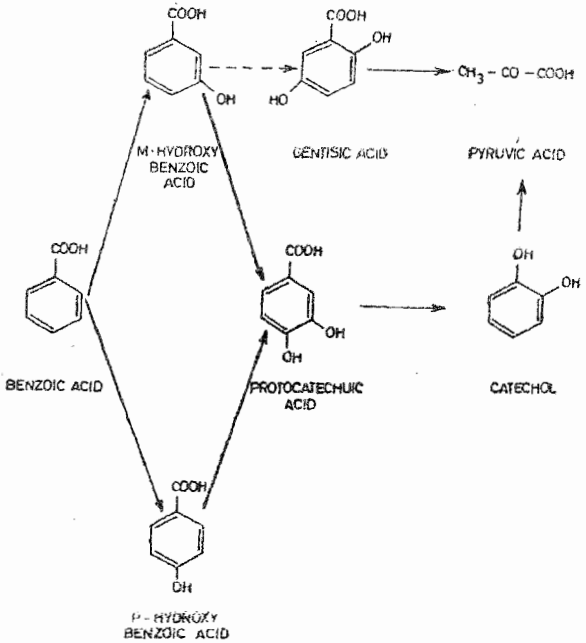

FIG. 11

Metabolic pathway of benzoic acid by Arthrobacter Species

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