METABOLISM OF RESORCYLIC ACID AND ITS CORRESPONDING ALDEHYDE BY SOME SOIL ISOLATES

by

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ABSTRACT

Two soil isolates, tentatively identified as a Rhodotorula sp. and a Micrococcus sp. both able to utilize resorcylic acid as sole carbon and energy source, were used to investigate the mechanism of degradation of this substrate. Manometric experiments with both isolates showed that resorcylic acid was metabolized through resorcinol and that the metabolism of these aromatic compounds is under induced enzyme control. Resorcylic aldehyde failed to serve as a suitable growth substrate for either organism but in both cases it was oxidized by resting cell suspensions to the homologous acid by what appears to be a non-specific enzyme system. Evidence obtained from employing both paper chromatographic and manometric techniques indicate that the mechanism: resorcylic aldehyde → resorcylic acid → resorcinol is operating in both organisms.

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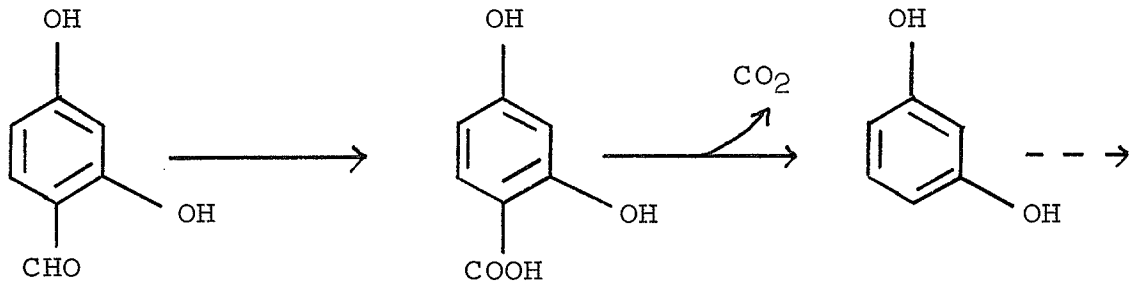
INTRODUCTION

Recently Halvorson (20) isolated an Aspergillus niger strain which was able to carry out the oxidation of resorcylic acid and its corresponding aldehyde according to the following mechanism:



resorcylic aldehyde | resorcylic acid | resorcinol

This mechanism is most interesting for two reasons. Firstly, the hydroxyl groups in these compounds are meta to one another; usually in the majority of dihydroxyphenolic compounds which are naturally occurring the hydroxyl groups are ortho or para to one another. Secondly, the carboxyl group of resorcylic acid is removed to form resorcinol; usually in compounds which contain a carboxyl group attached to an aromatic moiety, the carboxyl group remains intact prior to cleavage of the

aromatic ring. Consequently, this study was undertaken in order to determine whether this pathway is operative in other microbial systems or whether the mechanism in the Aspergillus sp. is a rare and isolated case.

HISTORICAL

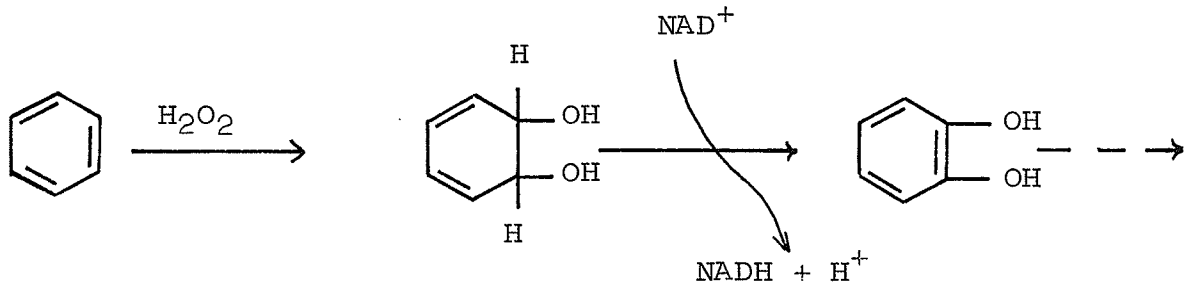
Many bacteria and to a lesser extent yeasts and fungi are known to degrade aromatic compounds. Bacteria active in this respect have been found in soils, in decomposing plant residues, in sewage and in faeces and have been characterized as members of the families Coccaceae, Mycobacteriaceae, Pseudomonadaceae, Spirillaceae, Bacteriaceae and Bacillaceae (13, 15). Many fungi, including species of Aspergillus, Penicillium and Neurospora, are able to utilize phenolic compounds as the sole source of carbon and energy (21, 43). Saccharomyces cerevisiae, Candida tropicalis, Rhodotorula glutinis and several other yeasts can grow on catechol, resorcinol and other more complex aromatic substrates (23, 26, 29, 30).

In heterotrophic organisms the energy required for growth is derived from the degradation of organic substrates. The energy bound up in the benzeneoid structures of aromatic compounds is made available to the organism at the expense of ring fission. These microorganisms produce mainly by induction, a whole sequence of enzymes which convert aromatic

substrates into an ortho or para dihydroxy derivative, prior to cleavage of the ring to form aliphatic acids; these ring fission products are funnelled into the Krebs cycle through various pathways, depending on the organism and cultural conditions (15).

A wide variety of aromatic compounds exists in nature. These compounds may have one or more phenolic rings and the manner of degradation of these compounds varies with the structure. In order to exemplify mechanisms of oxidation of aromatic compounds, some of the less complex monophenolic compounds have been chosen for illustrative purposes.

It is well established that ring fission occurs only when two hydroxyl groups have been substituted into the aromatic nucleus (15). In the bacterial oxidation of benzene (34) two hydroxyl groups are simultaneously introduced into the benzene ring to form catechol.

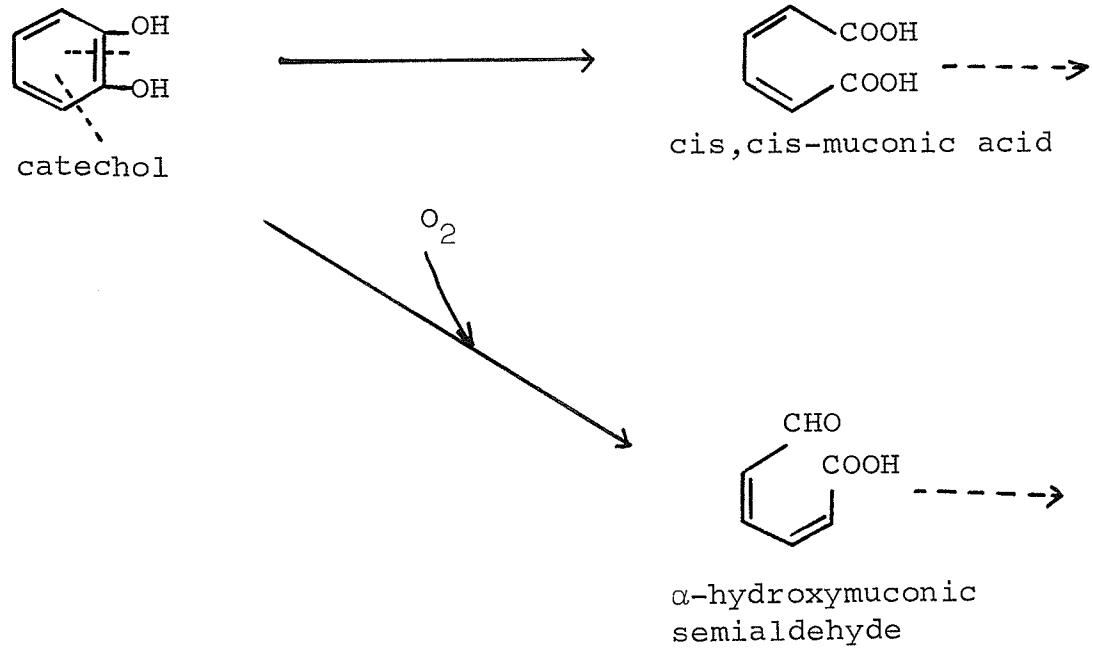


benzene

3,5-cyclohexadiene, 1,2-diol

catechol

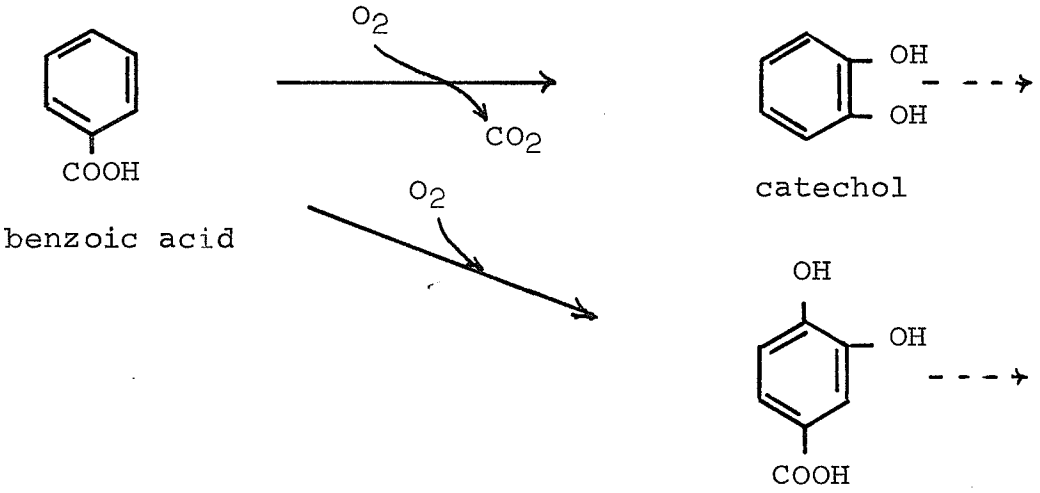
The resultant catechol is known to be metabolized by at least two different mechanisms. The first mechanism involves cleavage across the bond between the two hydroxyl groups as in the oxidation of catechol to cis,cis-muconic acid (10). In this case, as in all microbial degradations of aromatic compounds, a direct oxidation occurs in which molecular oxygen of atmospheric origin is the obligatory oxidant (11-13). The oxidation of protocatechuic acid to cis,cis-β-carboxymuconic acid follows the same pattern (14). The second mechanism involves cleavage across the bond adjacent to one of the hydroxyl groups. For example, catechol is oxidized through α-hydroxymuconic semialdehyde (11, 12, 36). These results are summarized below:



protocatechuic acid

cis,cis-β-carboxymuconic acid

When a carboxyl group is the only substituent into the aromatic nucleus metabolism still proceeds by the introduction of two hydroxyl groups prior to cleavage. Benzoic acid is converted to catechol by direct oxidative decarboxylation in the majority of cases (43, 49) but exceptions are known. In some photosynthetic anaerobic bacteria, it has been reported that benzoic acid is converted to catechol via protocatechuic acid (37) but the exact mechanism is not clear. These reactions are shown below:

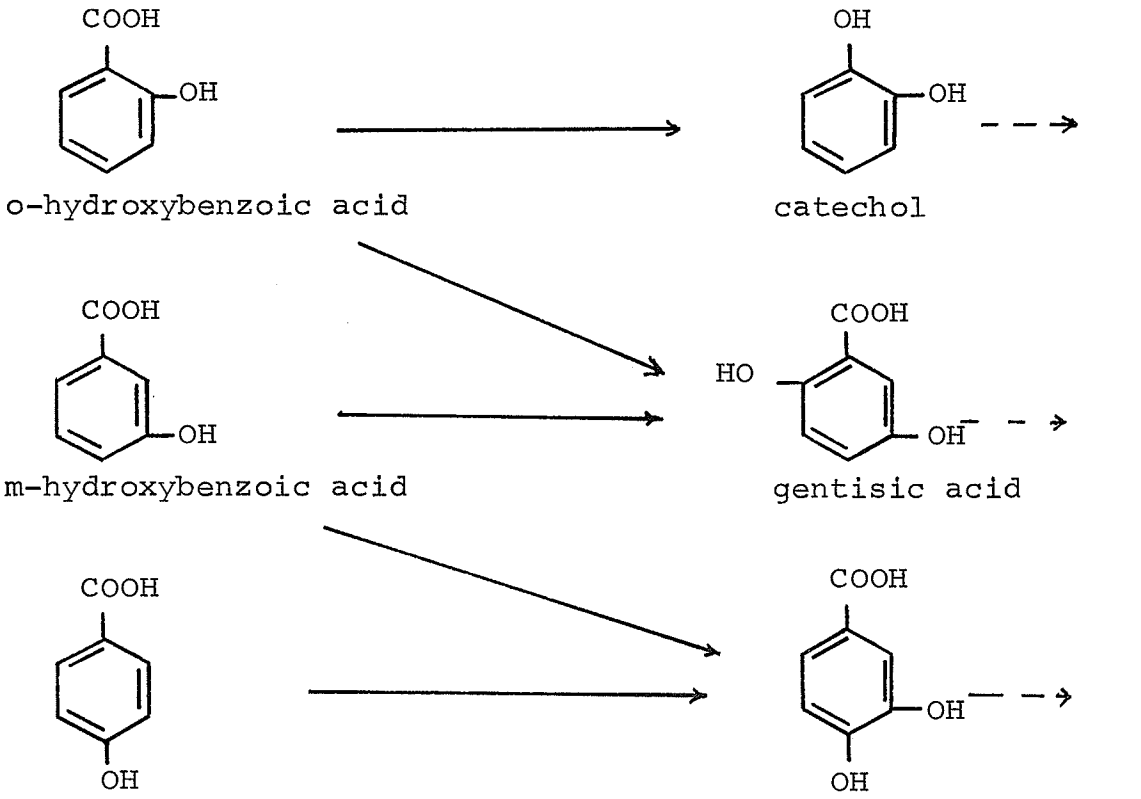


benzoic acid

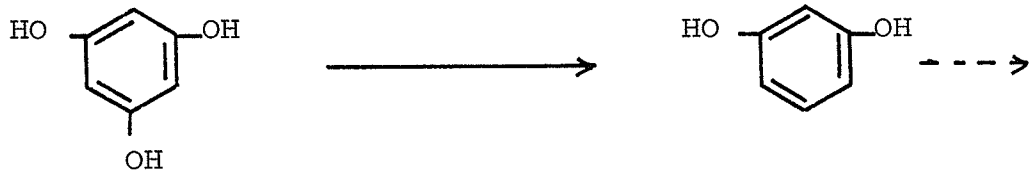
catechol

protocatechuic acid

When one carboxyl and one hydroxyl group are substituted into the aromatic ring, the method of cleavage depends upon the relative position of the substituent groups. o-Hydroxybenzoic acid is metabolized by two pathways, one through catechol and the other through gentisic acid. m-Hydroxybenzoic acid is also metabolized by two pathways, one through protocatechuic acid (49) and the other through gentisic acid. p-Hydroxybenzoic acid is also degraded through protocatechuic acid (31, 42). These results are summarized below:

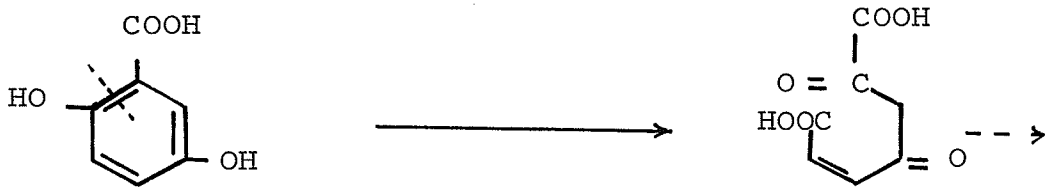


When three substituents are introduced into the benzene ring the mechanism of cleavage again depends both upon the nature of the groups and their relative positions to one another. For example, phloroglucinol (1,3,5-trihydroxybenzene) is converted to resorcinol before ring fission occurs (39).



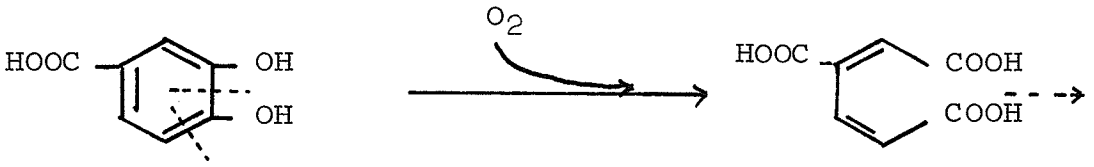
phloroglucinol resorcinol

While the exact mechanism involved is not known, phloroglucinol loses one of its hydroxyl groups. Gentisic acid (2,5-dihydroxybenzoic acid) undergoes direct cleavage to yield maleylpyruvate without the loss of any of its substituent groups (32, 46). Protocatechuic acid may also undergo direct cleavage to form β-carboxymuconic acid or α-hydroxy-γ-carboxymuconic semialdehyde depending on the site where fission occurs (12, 47). The following reactions show these schemes:



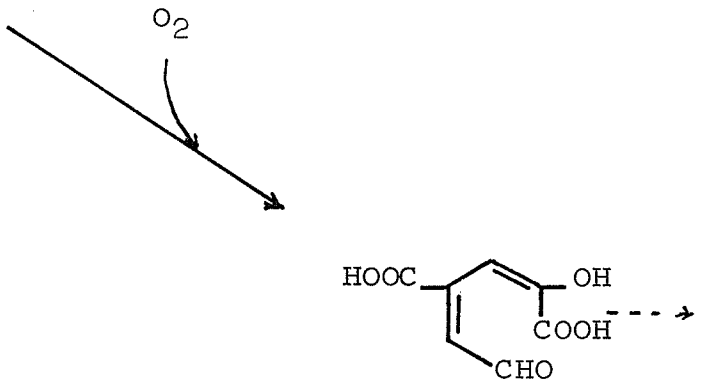
gentisic acid

maleylpyruvic acid



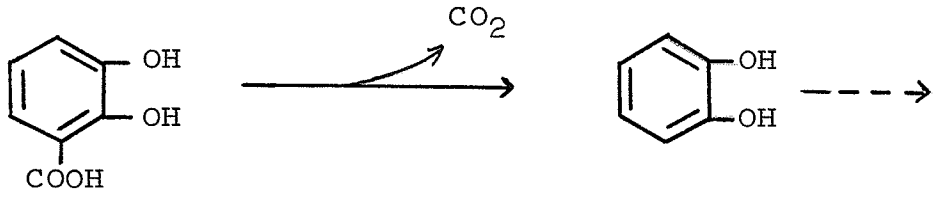
protocatechuic acid

β-carboxymuconic acid



α-hydroxy-γ-carboxy-
muconic semialdehyde

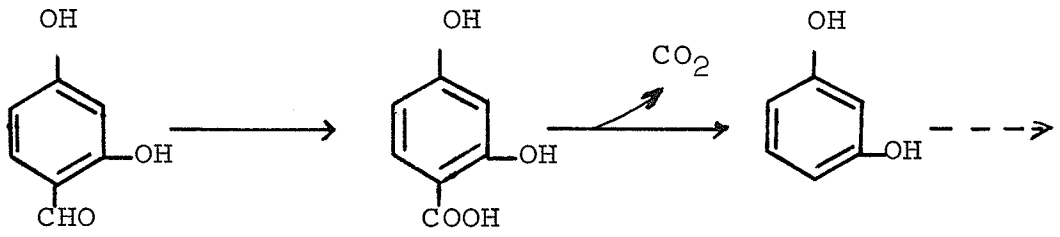
The degradation of 2,3-dihydroxybenzoic acid by Aspergillus niger (4) proceeds through decarboxylation to catechol as follows:



2,3-dihydroxybenzoic acid catechol

A similar decarboxylation mechanism may take place in the formation of catechol from protocatechuic acid in photosynthetic anaerobic bacteria as previously described.

An Aspergillus sp. isolated from soil was found capable of degrading resorcylic acid and its corresponding aldehyde (20). Spectrophotometric and paper chromatographic results established the following sequence of reactions:



2,4-dihydroxybenz-aldehyde 2,4-dihydroxy-benzoic acid resorcinol

Unlike gentisic acid, protocatechuic acid and 2,3-dihydroxybenzoic acid, resorcylic acid is decarboxylated prior to fission. However, the distinctive feature of resorcylic acid and resorcinol is that the two hydroxyl groups are meta to one another. In all other cases previously discussed, where two hydroxyl groups appeared concurrently within the same benzeneoid nucleus, they existed ortho or para to one another.

It is possible that resorcylic aldehyde is metabolized through resorcylic acid and resorcinol in other organisms. This investigation was undertaken in order to determine whether this pathway is widely distributed or whether the metabolism of resorcylic aldehyde through resorcylic acid and resorcinol by the Aspergillus sp. is an unusual case.

ISOLATION AND GROWTH OF ORGANISMS

Organisms used in this study were isolated by the "enrichment technique". The enrichment technique is the isolation of an organism possessing a specific property by successive subculture of a mixed population on a selective medium. A mineral salts solution comprising the following components was used:

K_2HPO_4	1.0 gm
KH_2PO_4	1.0 gm
NH_4NO_3	1.0 gm
$CaCl_2 \cdot 2H_2O$	0.02 gm
$MgSO_4 \cdot 7H_2O$	0.02 gm
$FeCl_3 \cdot 6H_2O$	0.03 gm
Distilled water	1000 ml

Resorcinol, resorcylic aldehyde, resorcylic acid or glucose were added to the mineral salts solution at a final concentration of 0.1%. The pH was adjusted to 6.8-7.0 with NaOH or HCl and the medium was sterilized by heating at 121° C for 20 minutes. Solid media were prepared by adding 2.0%

agar before sterilization. In some cases Brain Heart Infusion (BBL) was used for growth.

About 5.0 gm of cultivated soil from local sources were added to 500 ml of sterile resorcinol medium in a two litre Erlenmeyer flask. The flask was incubated at 28° C on an Eberbach rotary shaker at 120 cycles per minute. After 5 days, 10 ml of the contents were transferred aseptically to an identical flask containing fresh medium. This was incubated as described above for 5 days. The enriched culture was streaked onto resorcinol agar plates and incubated at 28° C for 5 days. After a further selection and purification on resorcinol agar, colonies that developed were streaked onto resorcylic aldehyde, resorcylic acid and resorcinol solid media. No colonies developed on resorcylic aldehyde. On the basis of colony appearance, two apparently different organisms showing the ability to withstand successive transfer on both resorcinol and resorcylic acid media were selected for further study. The organisms were tentatively identified as a Rhodotorula sp. and a Micrococcus sp..

In order to obtain bulk quantities of the Rhodotorula sp. for subsequent investigations, the organism was grown on

glucose, resorcylic acid or resorcinol. An initial inoculum was prepared by incubating the Rhodotorula sp. in 200 ml of sterile medium containing one of the substrates at 28° C on a rotary shaker at 120 cycles per minute. After 24 hours, the inoculum was transferred aseptically to a carboy containing 10 litres of sterile medium of the same composition in which the inoculum was grown. Air, kept sterile by passing it through a cotton filter, was forced into the carboy under positive pressure. When glucose was used as the substrate, good growth was obtained after 24 hours. When resorcinol and resorcylic acid were used as substrates, good growth was obtained after 48 and 72 hours respectively.

A modified program was used to obtain bulk quantities of the Micrococcus sp.. An inoculum was prepared by growing the organism in 500 ml of Brain Heart Infusion (Baltimore Biological Laboratories) in a two litre Erlenmeyer flask. The flask was incubated on a rotary shaker at 120 cycles per minute for 1 day at 28° C. The cells were harvested by centrifugation in a Servall centrifuge at 12,000 x g for 20 minutes and resuspended in sterile 0.05 M potassium phosphate buffer (pH 7.0). This inoculum was transferred aseptically to a carboy which contained 10 litres of sterile Brain Heart

Infusion, resorcylic acid or resorcinol medium. Cells were grown in the same manner as the Rhodotorula sp.. When Brain Heart Infusion medium was used, good growth was obtained after 24 hours. When resorcinol and resorcylic acid were used as substrates, good growth was obtained after 48 and 72 hours respectively.

A detailed study of the growth habits of either isolate was not undertaken.

MANOMETRIC STUDIES

INTRODUCTION

The regulation of biochemical transformations brought about in biological systems is under the direct control of biological catalysts called enzymes. A discussion of enzyme production and regulation is beyond the scope of this work. But one group of enzymes called "induced" enzymes have played a significant role in establishing the mechanism of degradation of aromatic compounds and thus merits some special consideration.

An enzyme formed by an organism and whose production is always concomitant with the growth of that organism is called a "constitutive" enzyme; most enzymes are of this type. An induced enzyme differs from a constitutive enzyme in that it is synthesized "de novo" from intracellular amino acids but whose production is dependent upon the presence of an inducer molecule, usually the substrate which is going to undergo metabolic changes (9, 27, 40). There is no unique feature of these enzymes once they have been formed; constitutivity and inducibility merely reflect

different states of the regulatory apparatus rather than a fundamental difference in the protein synthesizing machinery of the cell.

The ability of an organism to form induced enzymes is under genetic control and is an inherent physiological property of that organism. Induced enzyme formation is referred to as "induction" or "adaptation" and interestingly, the majority of aromatic compounds are metabolized by induced enzymes.

Stanier (43, 44) took advantage of this property and proposed the "sequential induction" technique for establishing catabolic pathways in systems the degradation of which is under induced enzyme control. The technique is based on the principle of Kluyver that every dissimilation is the result of several simple, step-wise, chemical reactions. The theory states that cells adapted to metabolize the primary substrate should be simultaneously adapted to metabolize all the intermediates formed in the oxidation of that substrate. Thus, exposure of the cells to one substrate can initiate a chain reaction requiring catalysis by a series of specific induced enzymes.

Stanier stated the theory in the following three

postulates:

1. "If dissimilation of A proceeds through a series of intermediates, B,C,D,E,F, etc., and if the individual steps in the chain of reactions are under induced enzymic control, then growth of A will produce cells simultaneously induced to A,B,C,D,E,F, etc.

2. If growth on A fails to adapt the cells to a postulated intermediate, x, then x cannot be a member of the reaction chain.

3. Growth on E adapts to F, etc., but not necessarily to A,B,C and D."

Thus, for example, if benzoate is the inducing substrate, and cells are not induced to mandelate and p-hydroxybenzoate, then oxidation of benzoate does not proceed through either of these substances. If mandelate is the inducing substrate and cells are simultaneously induced to benzoate, then mandelate is oxidized through benzoate but not through p-hydroxybenzoate.

Several workers have used the sequential induction technique successfully to investigate catabolic pathways (16, 19). For instance, Blakley and Simpson (5) isolated a soil pseudomonad which metabolized cinnamic acid via phenylpropionic acid, m-hydroxyphenylpropionic acid and 2,3-dihydroxyphenylpropionic acid. Stanier, Hayaishi and Tsuchida (45) studied the bacterial oxidation of tryptophan

and Cohen (8) studied the metabolism of uronic acids by Escherichia coli.

The sequential induction technique assumes that substrates are permeable to the cell. In some instances results obtained with intact cells may exclude true intermediates due to problems of permeability (7). In such cases active preparations of cell free extracts may be used to confirm postulated intermediates. For example, Hayaishi and Stanier (22) used cell free extracts of bacteria to demonstrate the oxidation of tryptophan via kynurenine, anthranilic acid and catechol.

An Aspergillus niger strain isolated from soil was able to metabolize resorcylic aldehyde through resorcylic acid and resorcinol (20). The isolation from soil of two organisms able to utilize both resorcylic acid and resorcinol as the sole carbon source might well proceed by the same route. The aromatic nature of these compounds strongly suggested that they were catabolized by induced enzyme formation. In the study reported here, the sequential induction technique was used to test this hypothesis in an attempt to determine whether the pathway was widely distributed or whether its operation in the Aspergillus sp. was an unusual case.

MATERIALS AND METHODS

Preparation of Resting Cell Suspensions for Manometric Studies

The *Rhodotorula* sp. was grown on glucose, resorcylic acid or resorcinol as described previously. The cells were harvested by means of a steam-driven Sharples Super centrifuge at a speed of 40,000 r.p.m. (20 lbs pressure) and washed twice in 0.05 M potassium phosphate buffer (pH 7.0). The cells were then resuspended in 600 ml of 0.05 M potassium phosphate buffer (pH 7.0) and allowed to respire for 8 hours under forced aeration, in order to reduce the rate of endogenous respiration. The respired cells were reharvested in a Sorval refrigerated centrifuge at 12,000 x *g* for 20 minutes and 1 gm of packed cells was resuspended in 20 ml of phosphate buffer. This preparation is referred to as a "resting cell suspension".

Resting cell suspensions of *Micrococcus* sp. were prepared in the same manner as described for *Rhodotorula* sp. with the slight modification that the cells were not respired prior to use.

Preparation of Cell Free Extracts for Manometric Studies

Cell free extracts were prepared only from the Micrococcus sp. The organism was grown on resorcinol and resorcylic acid and the cells were harvested by means of a steam-driven Sharples Super centrifuge at 40,000 r.p.m. (20 lbs pressure) and washed twice in cold potassium phosphate buffer. The cells were resuspended in cold, modified potassium phosphate buffer which contained 10^{-3} M 2-mercaptoethanol, then sonicated for 7 minutes in a Raytheon 10 Kc oscillator under an atmosphere of nitrogen. This preparation, referred to as "whole sonicate", was used in the determination of enzyme activity.

Manometric Experiments

All manometric experiments were conducted at 30° C with a Braunwill Warburg apparatus using standard manometric methods (48). Each flask contained: 0.2 ml 20% KOH and a fluted filter paper in the centre well; 1.0 ml of resting cell suspension or cell free extract in the main compartment and 4.0 μmoles of substrate in the sidearm. The final volume was brought to 3.0 ml with 0.05 M potassium phosphate

buffer. Flasks were routinely shaken for 20 minutes to equilibrate the system before the substrate was tipped into the cell suspension. Enzyme activity was determined by measuring oxygen uptake.

Effect of Chloramphenicol on Induced Enzyme Synthesis

Chloramphenicol is a broad spectrum antibiotic which inhibits protein synthesis by blocking the incorporation of amino acids into protein (6, 17). Its usefulness in metabolic studies is largely due to the fact that while it inhibits protein synthesis it allows respiration to continue. It can be used, then, to suppress induced enzyme formation.

In the presence of chloramphenicol, therefore, cells grown on glucose should not metabolize resorcylic aldehyde, resorcylic acid or resorcinol if these compounds are degraded by induced enzyme synthesis. Similarly, if resorcinol is in the pathway of resorcylic acid, cells grown on resorcinol should fail to metabolize resorcylic acid in the presence of chloramphenicol.

Resting cell suspensions of Rhodotorula sp. grown on glucose were tested for their ability to metabolize resorcylic aldehyde, resorcylic acid and resorcinol in the

Warburg apparatus. Resorcinol grown cells were also tested for their ability to utilize resorcylic acid and resorcinol. Each flask was set up as described previously with the exception that 4 mgm of chloramphenicol were introduced into the main compartment prior to tipping the flasks.

RESULTS

Oxidation of Several Aromatic Compounds by Resting Cell Suspensions of *Rhodotorula* sp.

The ability of resting cell suspensions of the *Rhodotorula* sp. grown on various substrates to utilize certain compounds was tested in the Warburg apparatus. When the organism was grown on glucose, resorcylic acid and resorcinol were metabolized only after lag periods of 40 and 50 minutes respectively (Fig. 1). The results show that the metabolism of these two compounds is under induced enzyme control.

When the *Rhodotorula* sp. was grown on resorcylic acid, both resorcylic acid and resorcinol were metabolized without any lag periods (Fig. 2). And when the organism was grown on resorcinol, resorcinol was oxidized immediately but resorcylic acid was oxidized after a lag period of 20 minutes (Fig. 3). Thus, application of Stanier's sequential induction technique shows that resorcylic acid is metabolized to resorcinol in the *Rhodotorula* sp.

FIGURE 1. Oxidation of glucose, resorcylic aldehyde, resorcylic acid, and resorcinol by resting cell suspensions of a Rhodotorula sp. grown on glucose. Curves are exogenous plots representative of several trials. Flask contents as described in text.

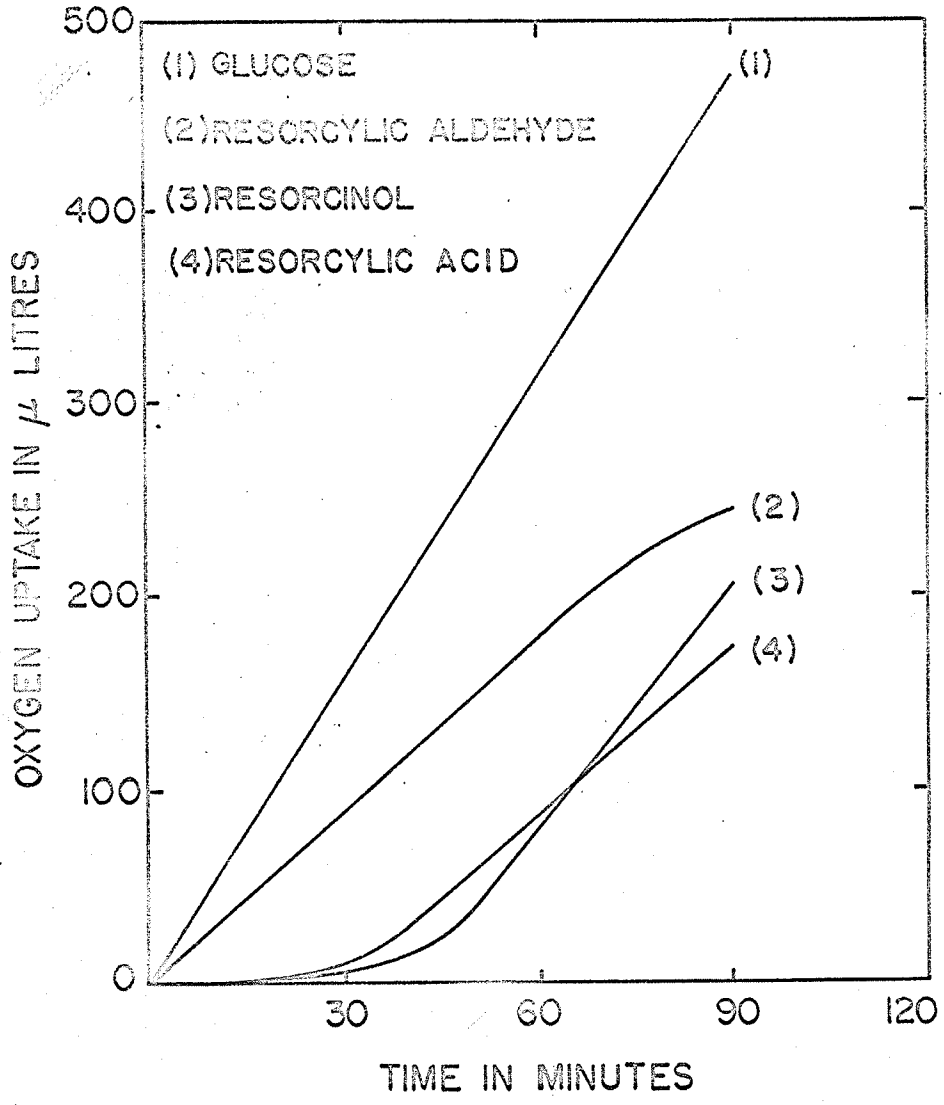

500
400
300
200
100
0
(1) GLUCOSE
(2)RESORCYLIC ALDEHYDE
(3)RESORCINOL
(4)RESORCYLIC ACID
(1)
(2)
(3)
(4)
OXYGEN UPTAKE IN μ LITRES
30
60
90
120
TIME IN MINUTES

FIGURE 2. Oxidation of resorcyclic aldehyde, resorcylic acid and resorcinol by resting cell suspensions of a Rhodotorula sp. grown on resorcylic acid. Curves are exogenous plots representative of several trials. Flask contents as described in text.

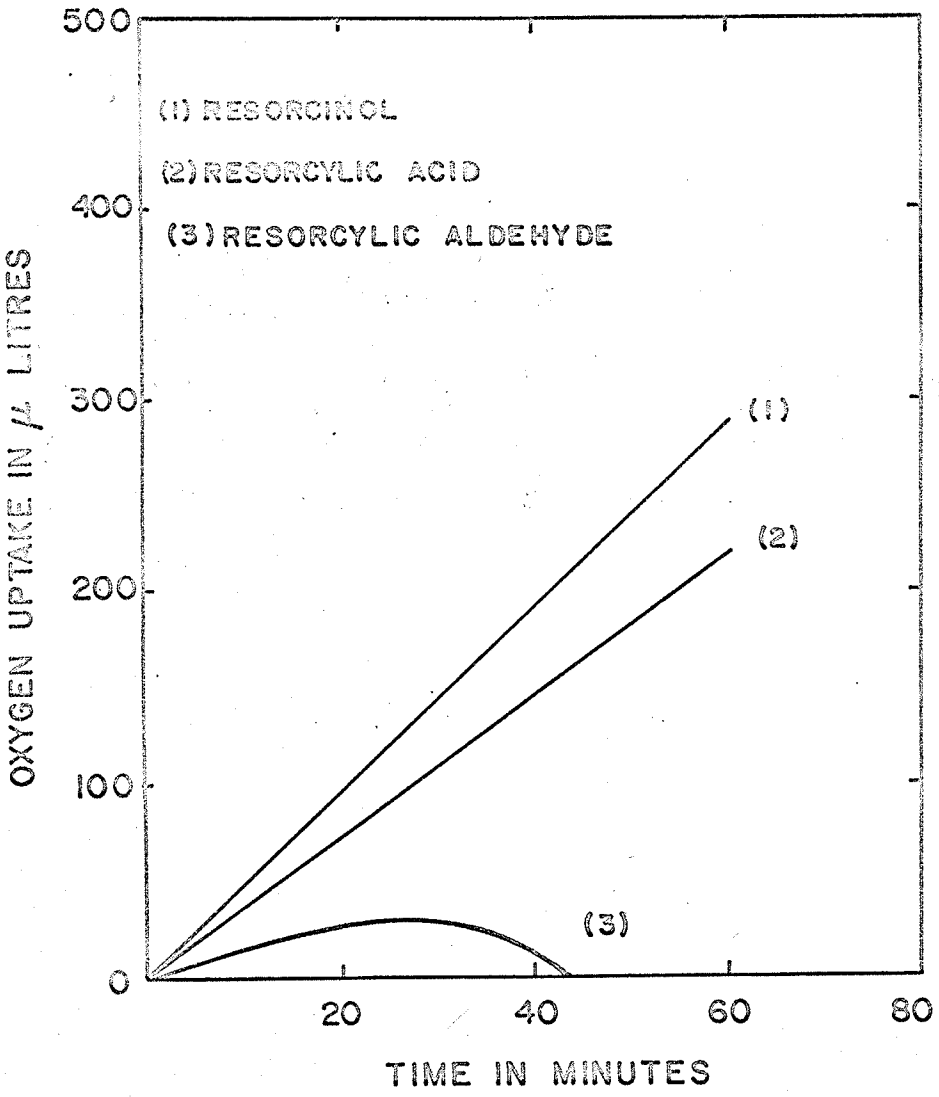
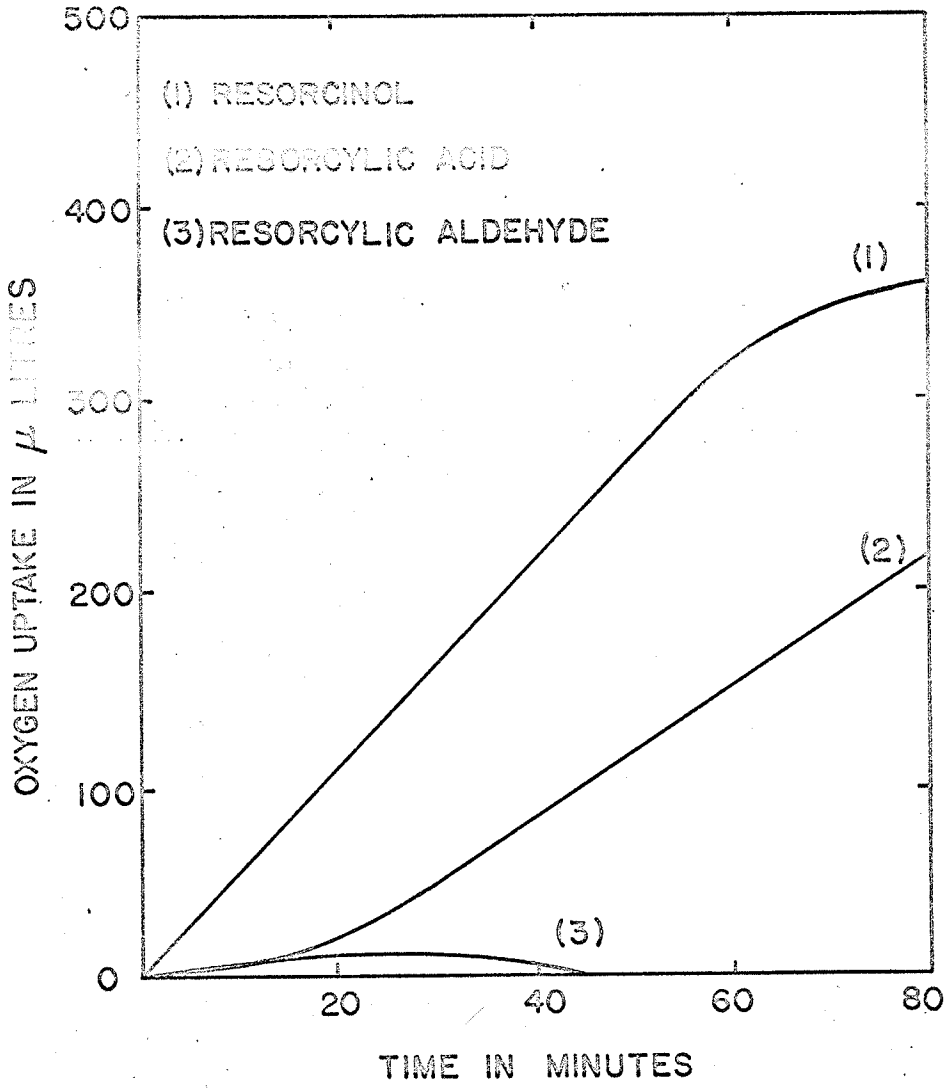

500
400
300
200
100
0
(1) RESORCINOL
(2) RESORCYLIC ACID
(3) RESORCYLIC ALDEHYDE
(1)
(2)
(3)
OXYGEN UPTAKE IN μ LITRES
20
40
60
80
TIME IN MINUTES

FIGURE 3. Oxidation of resorcylic aldehyde, resorcylic acid and resorcinol by resting cell suspensions of Rhodotorula sp. grown on resorcinol. Curves are exogenous plots representative of several trials. Flask contents as described in text.

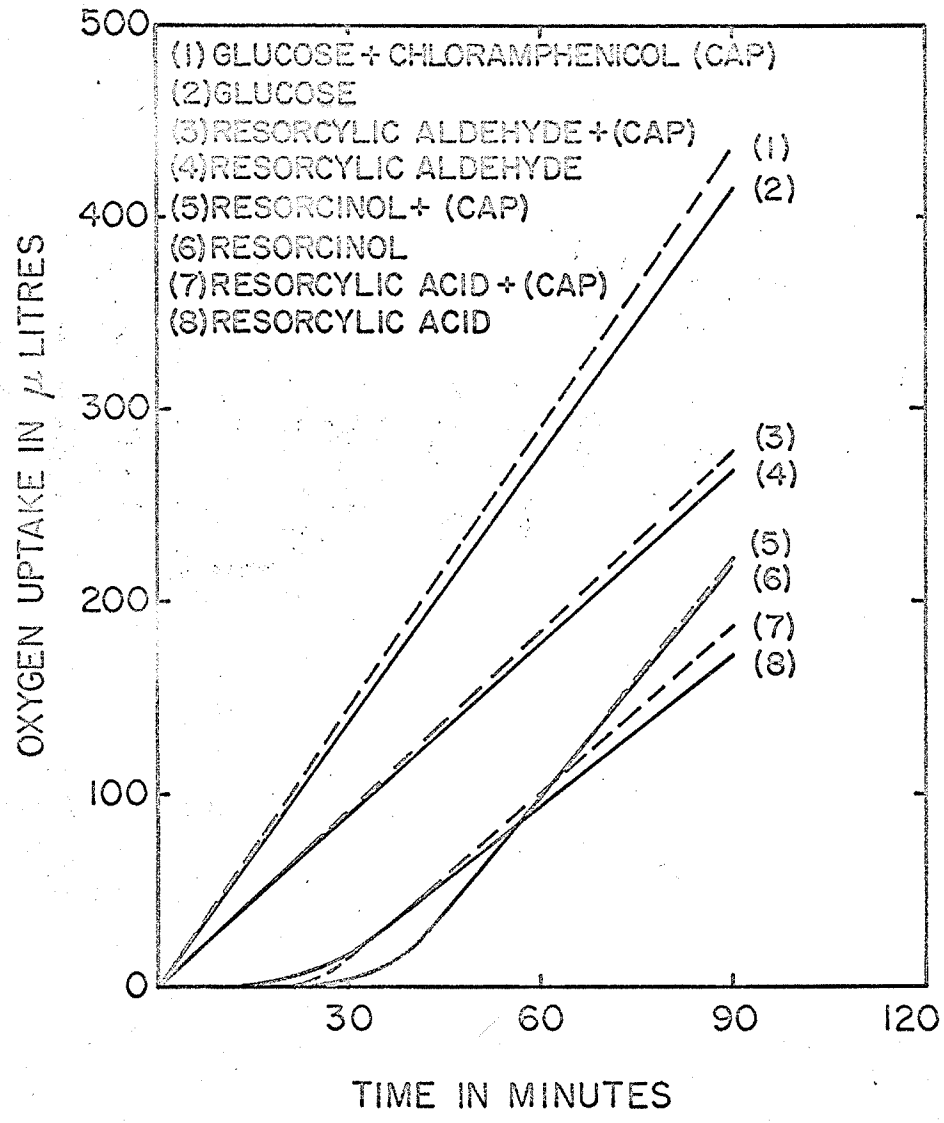

500
400
300
200
100
0
(1) RESORCINOL
(2) RESORCYLIC ACID
(3) RESORCYLIC ALDEHYDE
(1)
(2)
(3)
OXYGEN UPTAKE IN μ LITRES
20
40
60
80
TIME IN MINUTES

The metabolism of resorcylic aldehyde, on the other hand, is not under the influence of induced enzyme control; cells grown on glucose oxidized resorcylic aldehyde immediately (Fig. 1). Interestingly, cells grown on either resorcylic acid or resorcinol failed to oxidize the aldehyde (Figs. 2 and 3). Clearly, the sequential induction technique cannot be used to establish any metabolic relationship between resorcylic aldehyde and resorcylic acid or resorcinol.

Effect of Chloramphenicol on Induced Enzyme Synthesis

Resting cell suspensions of the Rhodotorula sp. grown on glucose were tested for their ability to metabolize several substrates in the presence of chloramphenicol. Figure 4 shows that glucose and resorcylic aldehyde were metabolized immediately in the absence of chloramphenicol. The addition of chloramphenicol did not impair the normal metabolic activity of the organism; the rate of metabolism of glucose and resorcylic aldehyde was identical in both cases. Figure 4 also shows that resorcylic acid and resorcinol were metabolized after lag periods of 30 and 40 minutes respectively. But the addition of chloramphenicol did not prevent the formation of induced enzymes in the Rhodotorula

FIGURE 4. Effect of chloramphenicol on the oxidation of several compounds by resting cell suspensions of Rhodotorula sp. grown on glucose. Substrates tested were glucose, resorcylic aldehyde, resorcylic acid and resorcinol. Curves are exogenous plots representative of several trials. Flask contents as described in text.


(1) GLUCOSE + CHLORAMPHENICOL (CAP)
(2) GLUCOSE
(3) RESORCYLIC ALDEHYDE + (CAP)
(4) RESORCYLIC ALDEHYDE
(5) RESORCINOL + (CAP)
(6) RESORCINOL
(7) RESORCYLIC ACID + (CAP)
(8) RESORCYLIC ACID
OXYGEN UPTAKE IN μ LITRES
TIME IN MINUTES
0
100
200
300
400
500
30
60
90
120

sp.

Identical results were obtained when cells were grown on resorcinol. Figure 5 shows that the addition of chloramphenicol did not prevent the formation of induced enzymes active against resorcylic acid.

Oxidation of Resorcylic Acid and Resorcinol by Resting Cell Suspensions of a Micrococcus sp.

The ability of resting cell suspensions of the Micrococcus sp. grown on various substrates to utilize certain compounds was tested in the Warburg apparatus. Cells grown on resorcylic acid oxidized resorcylic acid immediately but resorcinol was oxidized only after a lag of 20 minutes (Fig. 6). Cells grown on resorcinol utilized resorcinol immediately and resorcylic acid after a lag of 70 minutes (Fig. 7). Clearly no metabolic relationship between resorcylic acid and resorcinol can be established for this organism by application of the sequential induction technique.

Oxidation of Resorcylic Acid and Resorcinol by Cell Free Extracts of Micrococcus sp.

Cell free extracts of the Micrococcus sp. grown on

FIGURE 5. Effect of chloramphenicol on the oxidation of resorcylic acid and resorcinol by resting cell suspensions of Rhodotorula sp. grown on resorcinol. Curves are exogenous plots representative of several trials. Flask contents as described in text.

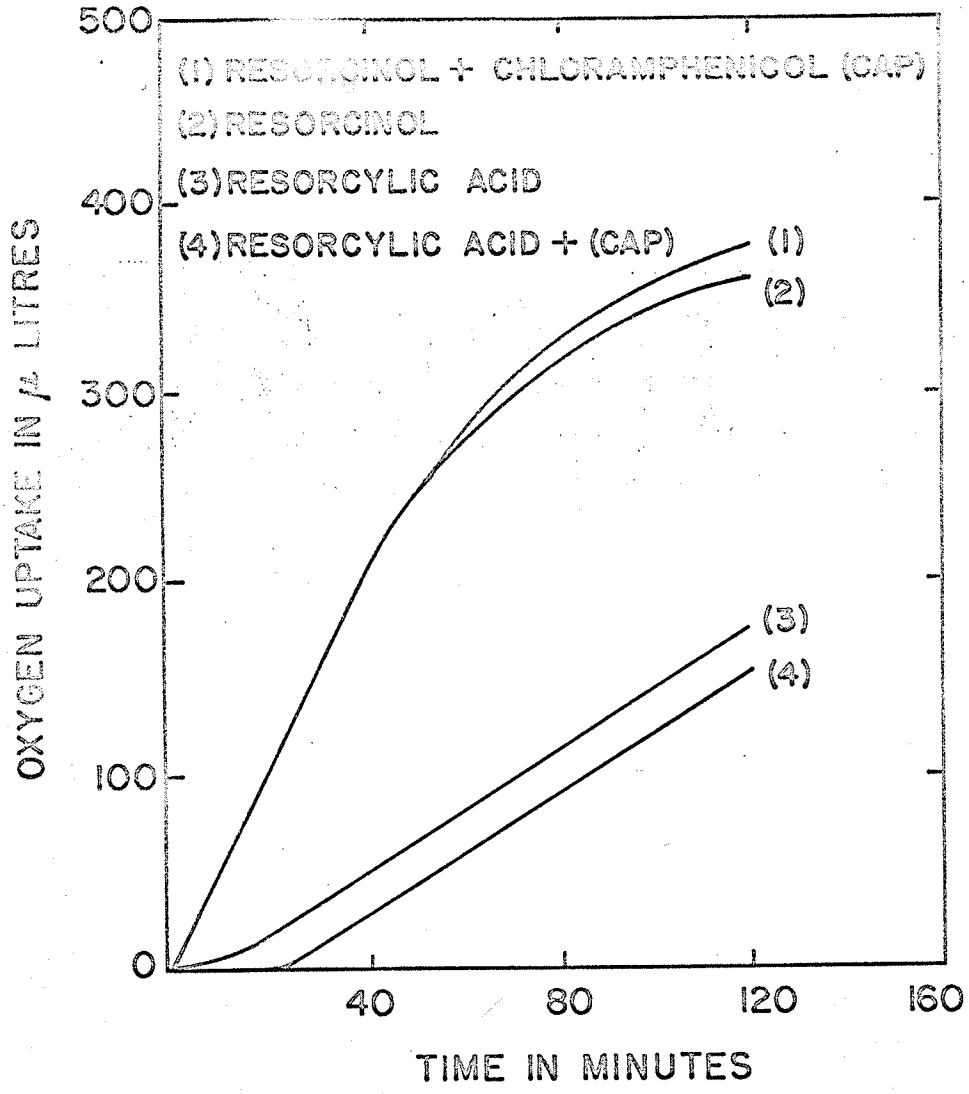

(1) RESORCINOL + CHLORAMPHENICOL (CAP)
(2) RESORCINOL
(3) RESORCYLIC ACID
(4) RESORCYLIC ACID + (CAP)
(1)
(2)
(3)
(4)
OXYGEN UPTAKE IN μ LITRES
500
400
300
200
100
0
40
80
120
160
TIME IN MINUTES

FIGURE 6. Oxidation of resorcylic acid and resorcinol by resting cell suspensions of Micrococcus sp. grown on resorcylic acid. Curves are exogenous plots representative of several trials. Flask contents as described in text.

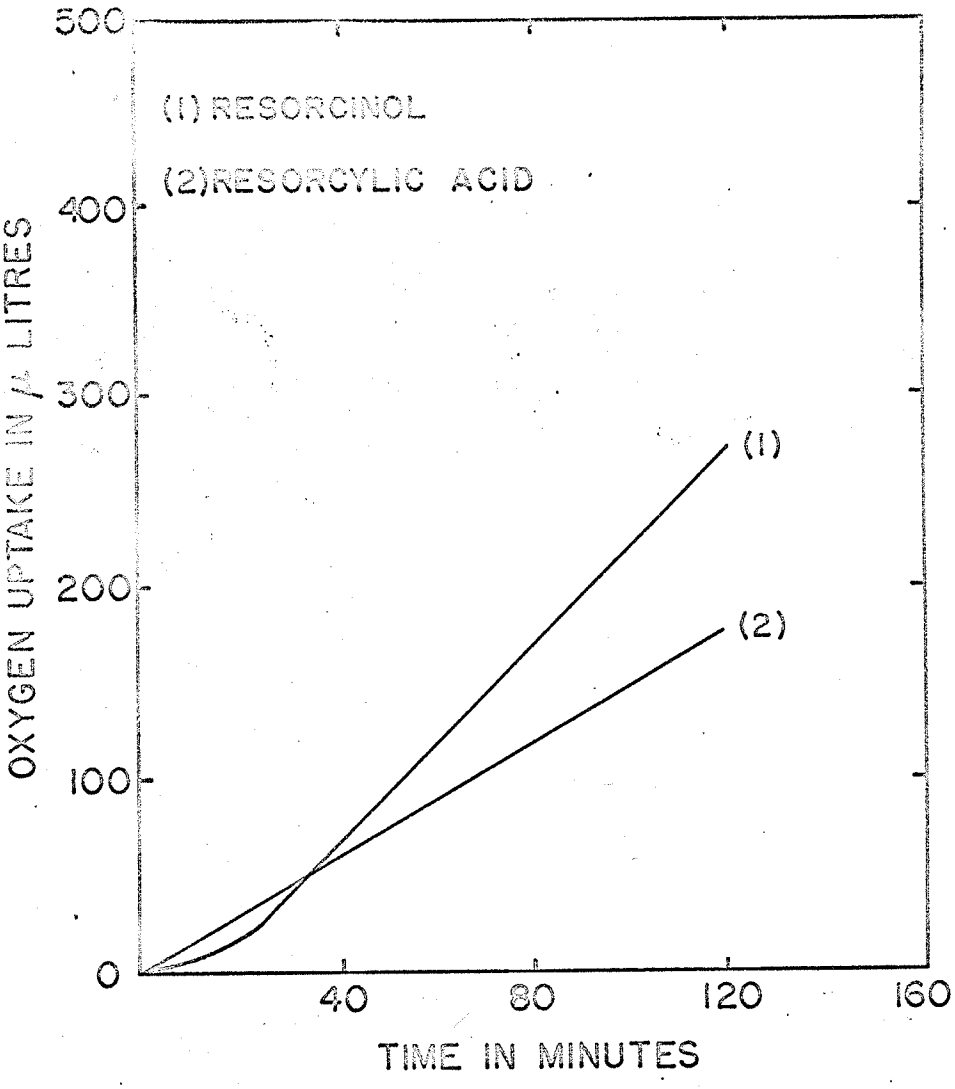
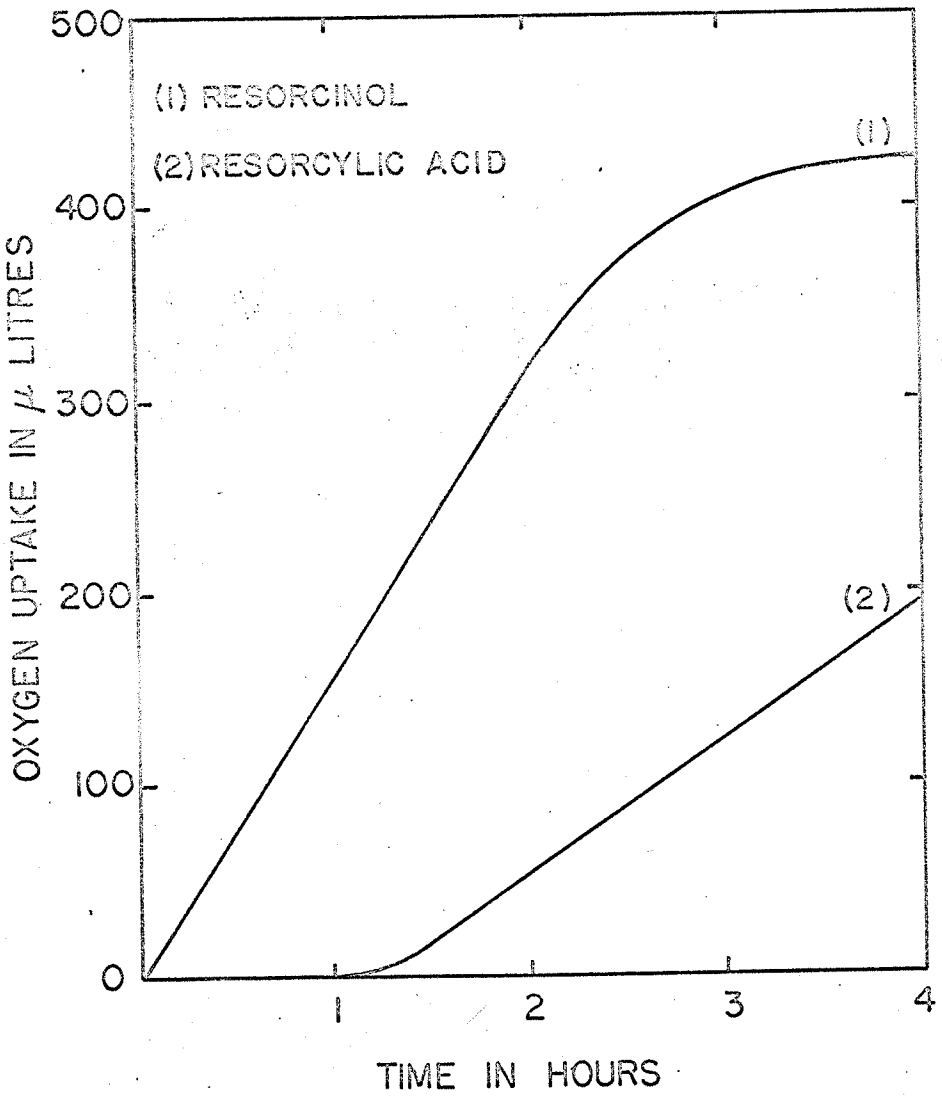

500
400
300
200
100
0
(1) RESORCINOL
(2) RESORCYLIC ACID
OXYGEN UPTAKE IN μ LITRES
(1)
(2)
40
80
120
160
TIME IN MINUTES

FIGURE 7. Oxidation of resorcylic acid and resorcinol by resting cell suspensions of Micrococcus sp. grown on resorcinol. Curves are exogenous plots representative of several trials. Flask contents as described in text.


500
400
300
200
100
0
(1) RESORCINOL
(2) RESORCYLIC ACID
(1)
(2)
OXYGEN UPTAKE IN μ LITRES
1
2
3
4
TIME IN HOURS

resorcylic acid metabolized both resorcylic acid and resorcinol immediately (Fig. 8). Cell free extracts of cells grown on resorcinol metabolized resorcinol immediately but resorcylic acid was not metabolized (Fig. 9).

These results show that the 20 minute lag in the oxidation of resorcinol by whole cells grown on resorcylic acid (Fig. 6) was due to failure of resorcinol to enter the cell rather than to synthesis of a resorcinol degrading enzyme.

Although no metabolic relationship between resorcylic acid and resorcinol could be established by the sequential induction method, the results of experiments using cell free extracts show that growth on resorcylic acid induces the formation of enzymes able to carry out the oxidation of resorcinol, and resorcinol, therefore, is an intermediate in the breakdown of resorcylic acid.

FIGURE 8. Oxidation of resorcylic acid and resorcinol by cell free extracts of Micrococcus sp. grown on resorcylic acid. Curves are exogenous plots representative of several trials. Flask contents as described in text.

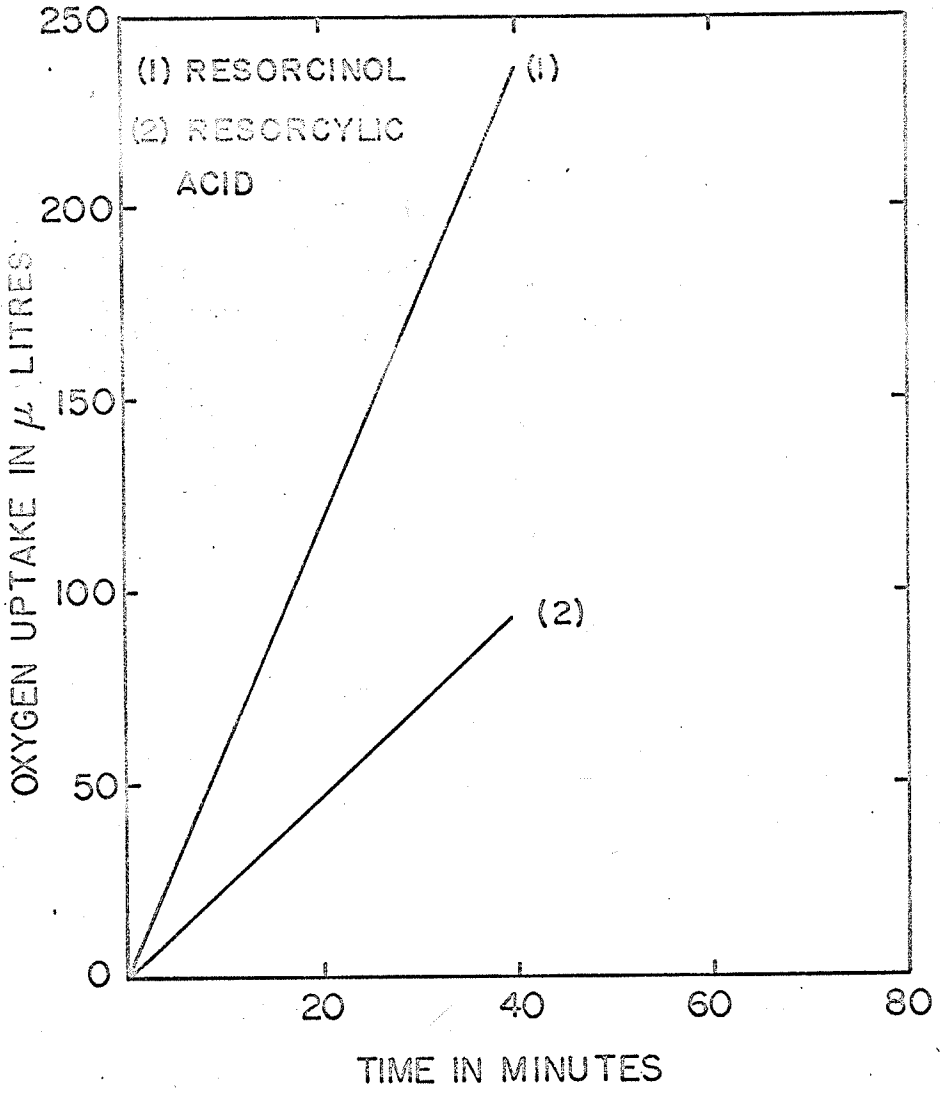
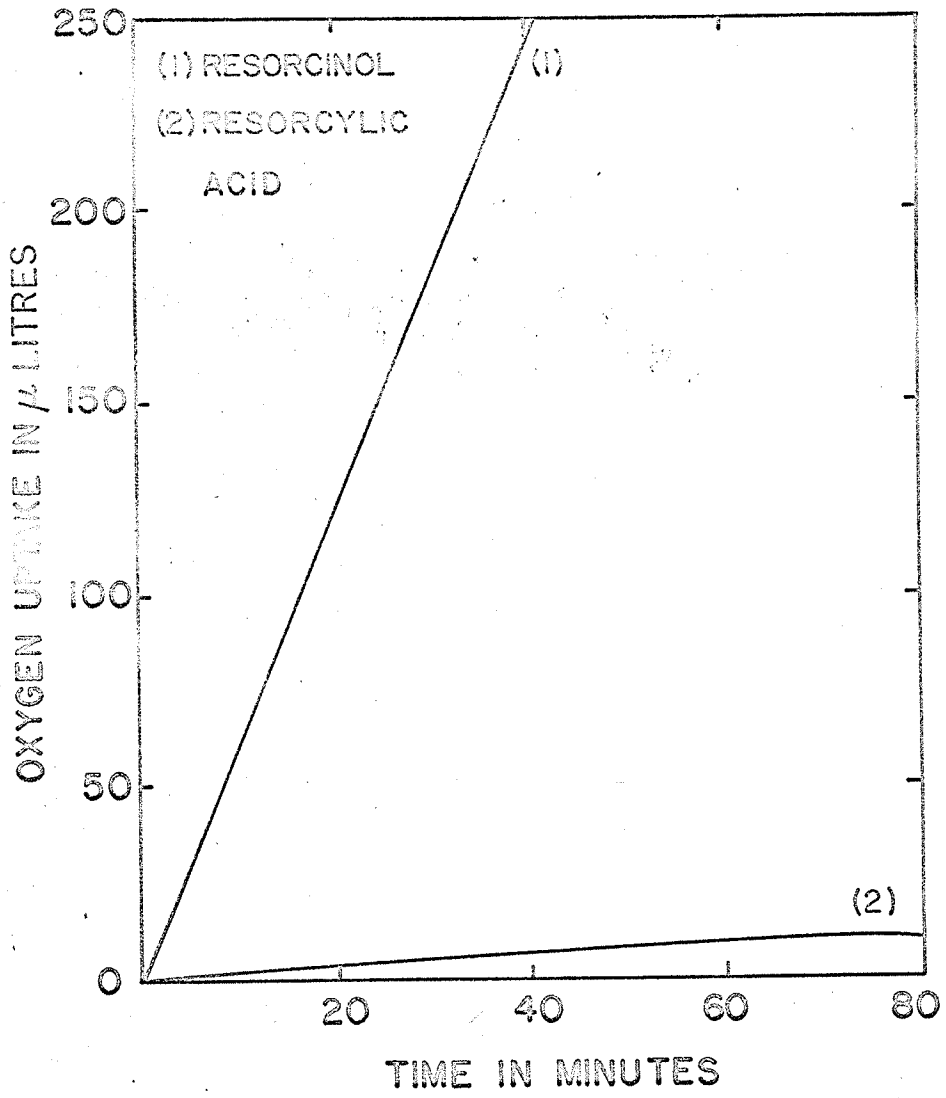

250
200
150
100
50
0
(1) RESORCINOL
(2) RESORCYLIC ACID
(1)
(2)
OXYGEN UPTAKE IN μ LITRES
20
40
60
80
TIME IN MINUTES

FIGURE 9. Oxidation of resorcylic acid and resorcinol by cell free extracts of Micrococcus sp. grown on resorcinol. Curves are exogenous plots representative of several trials. Flask contents as described in text.


250
200
150
100
50
0
(1) RESORCINOL
(2) RESORCYLIC
ACID
(1)
(2)
OXYGEN UPTAKE IN μ LITRES
20
40
60
80
TIME IN MINUTES

PAPER CHROMATOGRAPHIC STUDIES

INTRODUCTION

Gordon and his co-workers (10, 18) first developed the technique of paper chromatography, originally used for the separation of small amounts of amino acids. The technique is based on the fact that organic compounds may have a relatively high solubility in one solvent but dissolve in another solvent miscible with the first to a lesser extent. The most suitable solvents for separation of organic compounds are substances which are partially miscible with water (18). If such a solvent is allowed to flow along a strip of filter paper onto which various organic compounds were spotted, the distance each compound migrated is a function of the rate of flow of the solvent and of the (degree of) solubility of the compound in the given solvent.

The method can be applied successfully to the identification of minute amounts of phenolic compounds containing conjugable groups (1, 2) even in the presence of relatively high concentration of other aromatic compounds.

Quantities of phenolics as low as 1-5 μgm can be identified with confidence (3).

The isolation and identification of phenolic compounds is based principally on the polarity of the hydroxyl groups attached to the aromatic ring and the acidity of the hydroxyl group is often used as a means of separating phenolic compounds (41).

The qualitative identification of metabolites that appear in the fermentation liquor of a growing culture and their subsequent disappearance as growth continues is suitable evidence to include these metabolites as intermediates in the degradation of the growth substrate. Numerous reports have appeared concerning reference systems which have been found suitable for analyzing various mixtures of phenolic compounds. Reio (38) has presented a concise study in this area. Reio's Solvent B was used to follow the metabolism of resorcylic aldehyde through resorcylic acid and resorcinol by an Aspergillus sp. (20), and in this study the same solvent was used to reinvestigate the operation of this pathway in the Rhodotorula sp. and the Micrococcus sp.

MATERIALS AND METHODS

Preparation of Solvent

The solvent used in all chromatographic experiments was solvent B prepared by Reio (38) as follows:

> Stabilized chloroform (chloroform containing 1% EtOH), methanol and 4% formic acid were mixed in the proportion 100:10:10 respectively, shaken for 20 minutes in a separatory funnel and allowed to stand for 1 hour before separation. The organic layer was used as the mobile phase while the water layer was used as the stationary phase. The descending technique was used in all cases.

Preparation of Spray

The spray used in all cases was Diazotized Sulfanilic Acid (DSA) (38) prepared as follows:

Solution A

Dissolve 0.9 gm of sulfanilic acid in 9 ml of conc. HCl and dilute to 100 ml with water.

Solution B

Dissolve 5 gm of $NaNO_2$ in 100 ml of water. Add 25 ml of solution B to 5 ml of solution A in an ice cooled container and store in an ice bath for 1-4 hours before use.

The overspray used consisted of an aqueous solution of Na_2CO_3 (20% W/V).

Preparation of Samples for Chromatography

Resting cell suspensions of both organisms grown on glucose and Brain Heart Infusion were prepared as described previously with the exception that 1 gm of cells was suspended in 5 ml of 0.05 M potassium phosphate buffer pH 7.0. Substrate was added to bring the level to 0.1%. The preparation was incubated on a rotary shaker at 120 cycles per minute at 28° C. At zero time and at hourly intervals thereafter, 2.0 ml samples were withdrawn and acidified with conc. HCl. The acidified liquor was centrifuged at 4,500 x g for 10 minutes to remove the cells and the supernatant was stored under refrigeration until required for chromatography.

The same practice was applied to growing cultures.

Cells were grown on resorcylic acid and resorcinol as previously described and at zero time and at daily intervals thereafter 10 ml samples of the growing culture removed. The sample was prepared for chromatography as described above.

Paper Chromatographic Experiments

Portions of each sample were applied onto Whatman No. 1 filter paper and allowed to air dry. The papers were equilibrated for at least 1 hour in the chromatographic tank containing Reio's Solvent B prior to development of the chromatogram. The descending technique was used in all cases.

When the test substrate was resorcylic aldehyde, chromatograms were run for approximately 4 hours; when the test substrate was resorcylic acid or resorcinol, chromatograms were run for approximately 8 hours. After air drying, the chromatograms were sprayed with the DSA spray and oversprayed with 20% Na_2CO_3 while still slightly moist.

RESULTS

Resting cell suspensions of Rhodotorula sp. and Micrococcus sp. grown on glucose and Brain Heart Infusion respectively were incubated with resorcylic aldehyde. The gradual disappearance of the aldehyde and concomitant appearance of a new product was observed. This new product was identified with confidence as resorcylic acid on the basis of an identical Rf value compared to an authentic sample. With resting cell suspensions of Rhodotorula sp. (Fig. 10), resorcylic acid appeared after one hour. With resting cell suspension of Micrococcus sp. (Fig. 13), the intermediate appeared after two days. This shows that resorcylic aldehyde can be oxidized to the homologous acid in both organisms.

When both organisms were grown on resorcylic acid and samples taken at daily intervals, the substrate was observed to disappear from the Rhodotorula sp. (Fig. 11) and Micrococcus sp. (Fig. 14) systems after 8 and 4 days, respectively, but no products of a phenolic nature could be detected in the fermentation liquor. Both organisms were

FIGURE 10. Chromatogram shows the formation of resorcylic acid from resorcylic aldehyde by resting cell suspensions of Rhodotorula sp. grown on glucose.

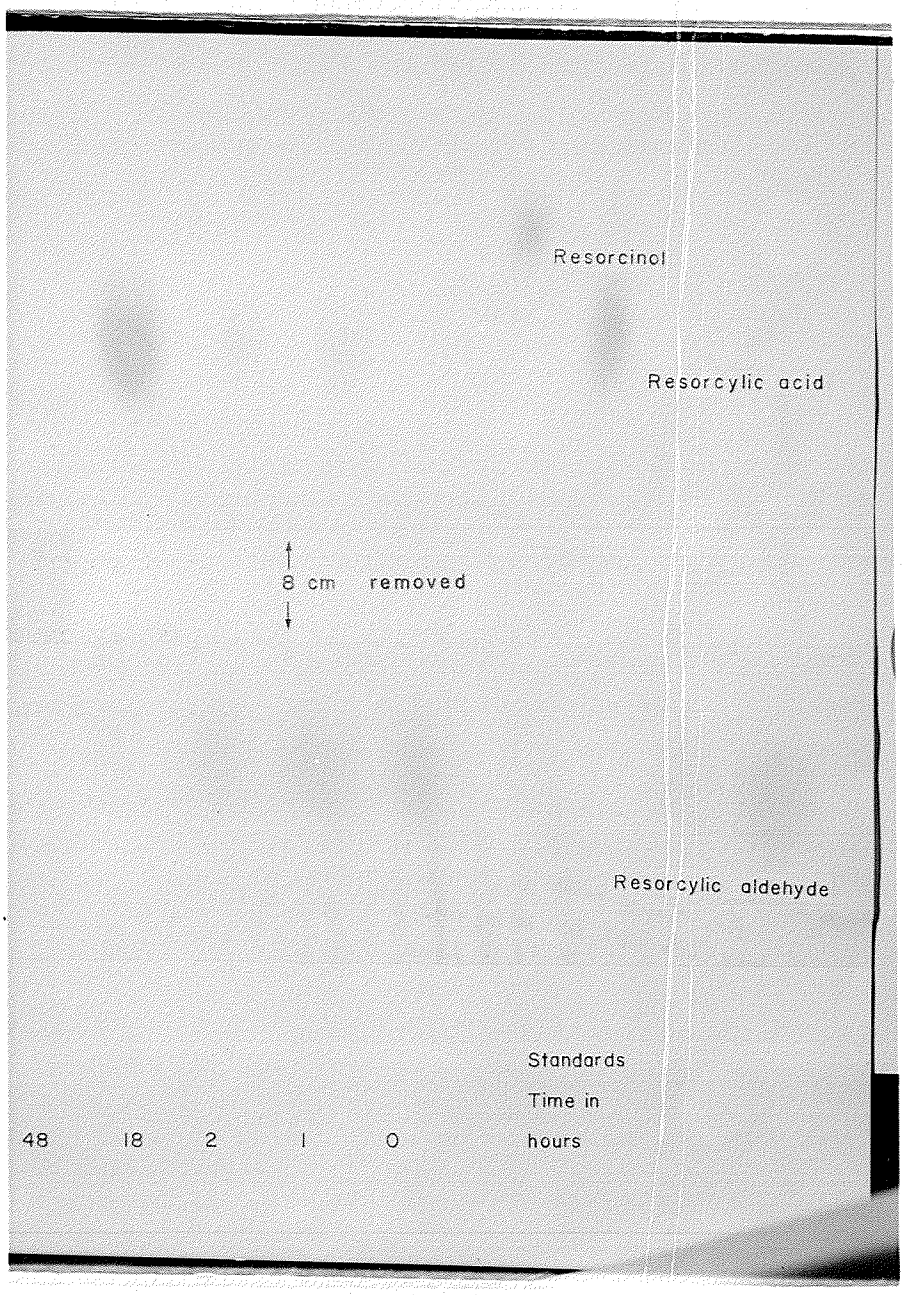

Resorcinol
Resorcylic acid
8 cm removed
Resorcylic aldehyde
Standards
Time in
hours
48
18
2
1
0

FIGURE 11. Chromatogram shows the disappearance of substrate from the medium when Rhodotorula sp. was grown on resorcylic acid.

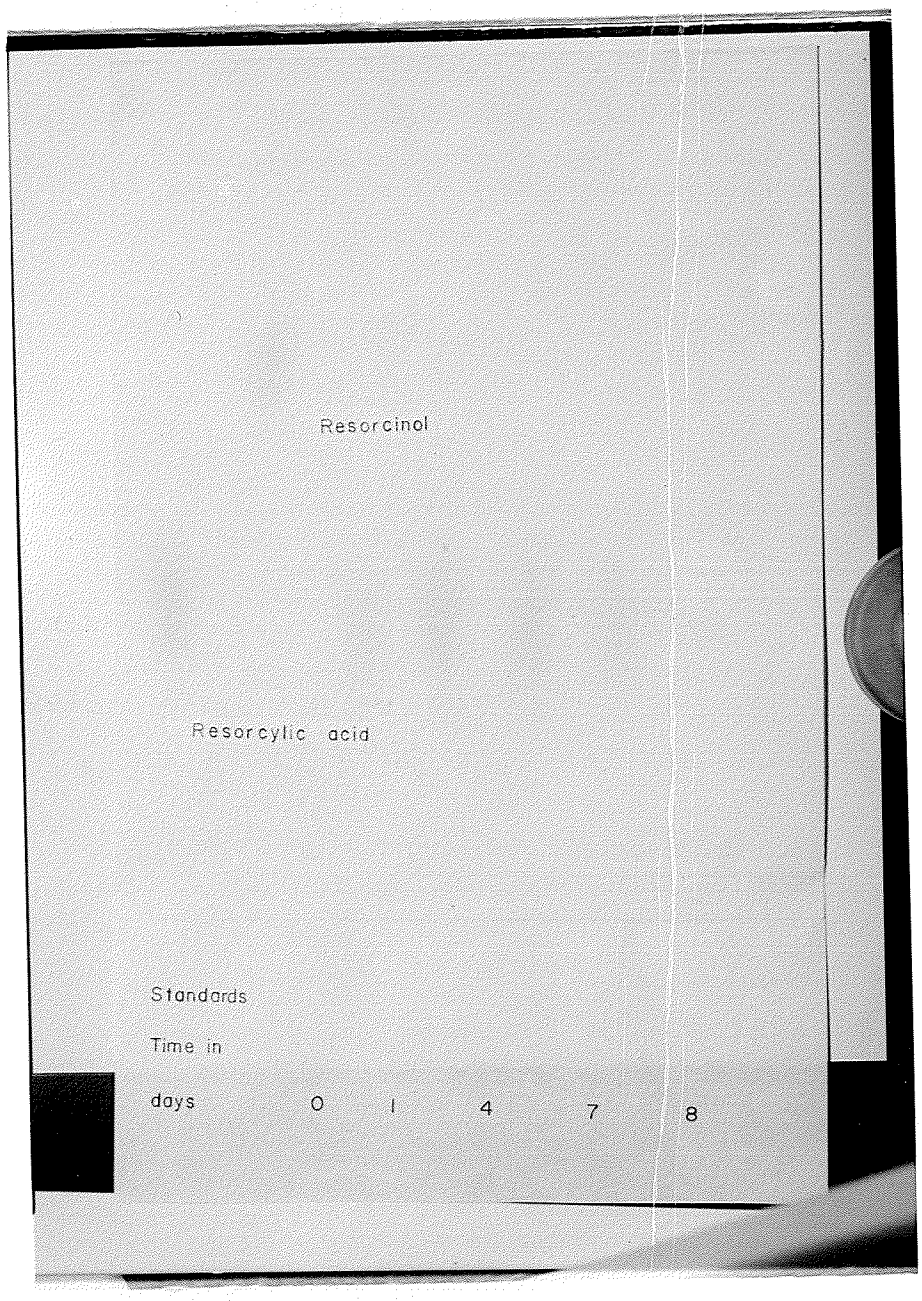

Resorcinol
Resorcylic acid
Standards
Time in
days
0
1
4
7
8

FIGURE 12. Chromatogram shows the disappearance of the substrate from the medium when Rhodotorula sp. was grown on resorcinol.

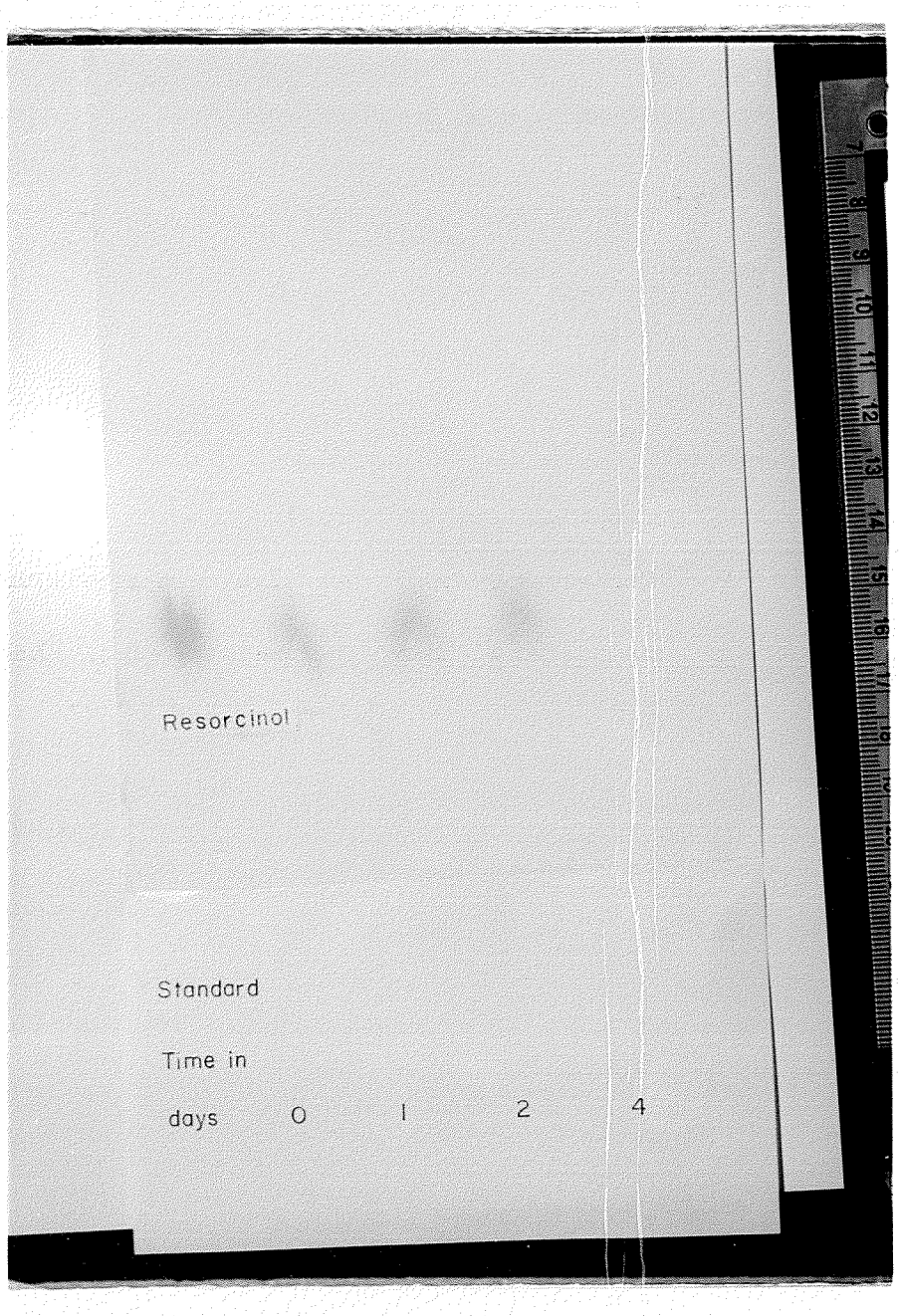

Resorcinol
Standard
Time in
days 0 1 2 4

FIGURE 13. Chromatogram shows the formation of resorcylic acid from resorcylic aldehyde by resting cell suspensions of Micrococcus sp. grown on Brain Heart Infusion.

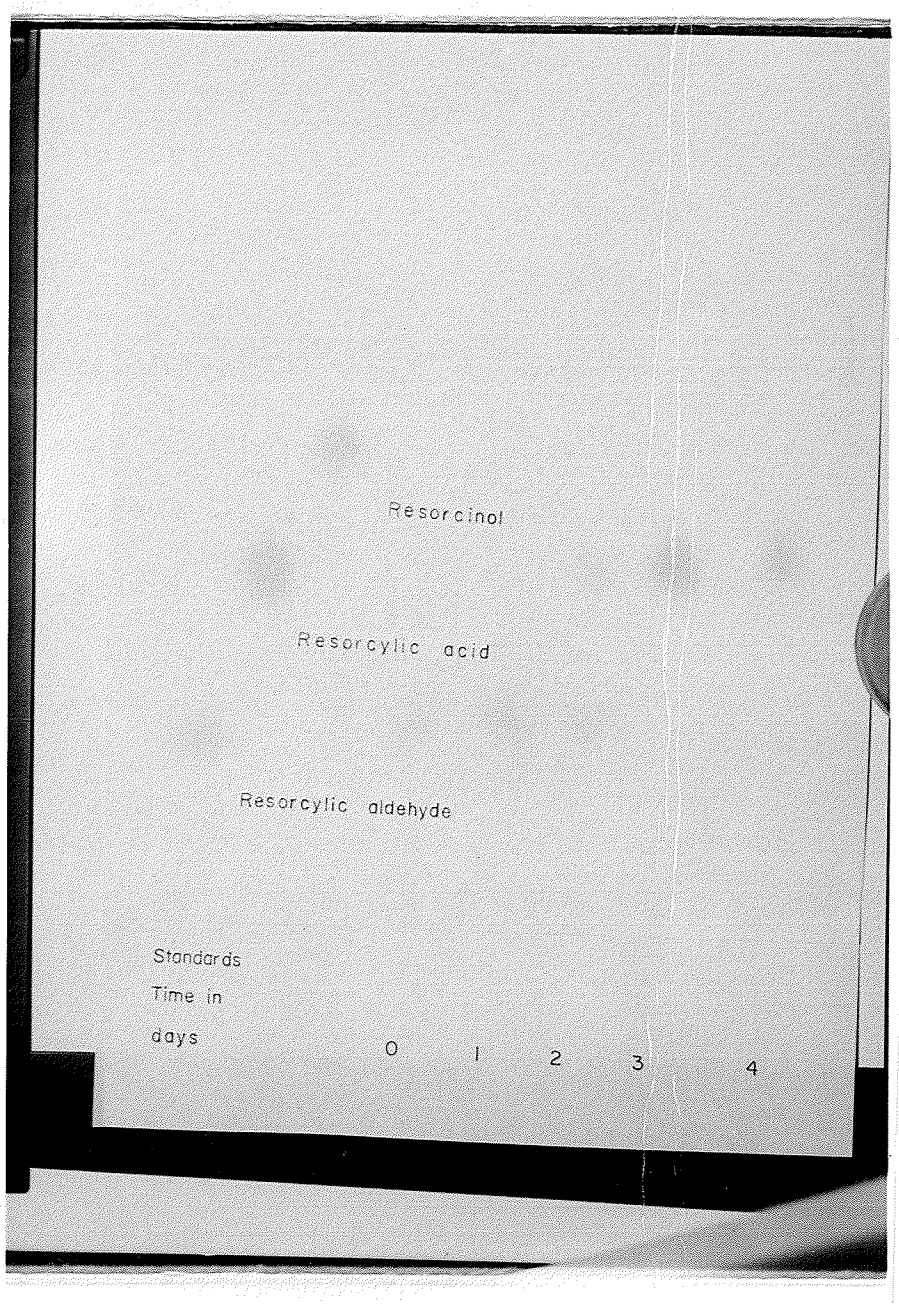
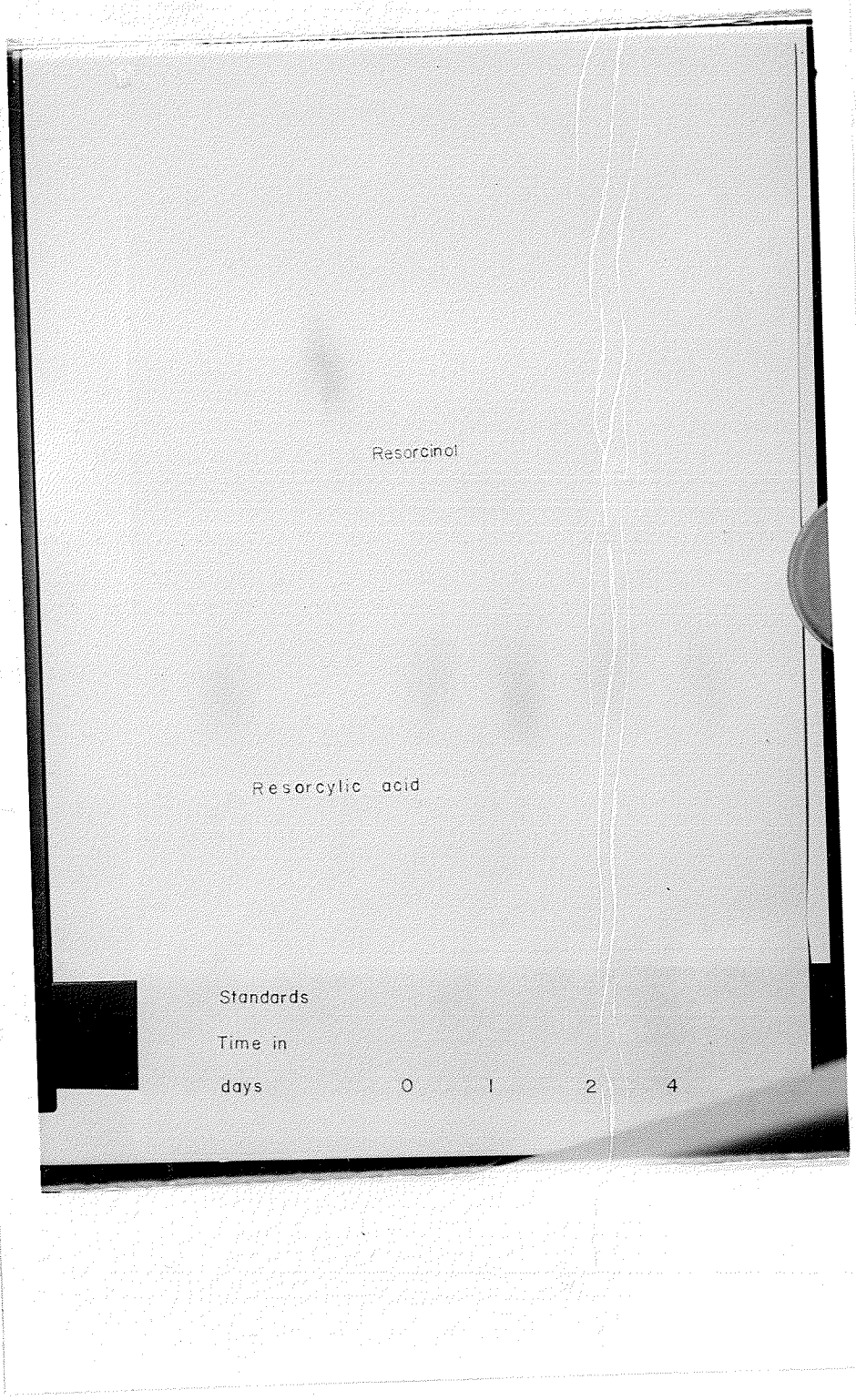
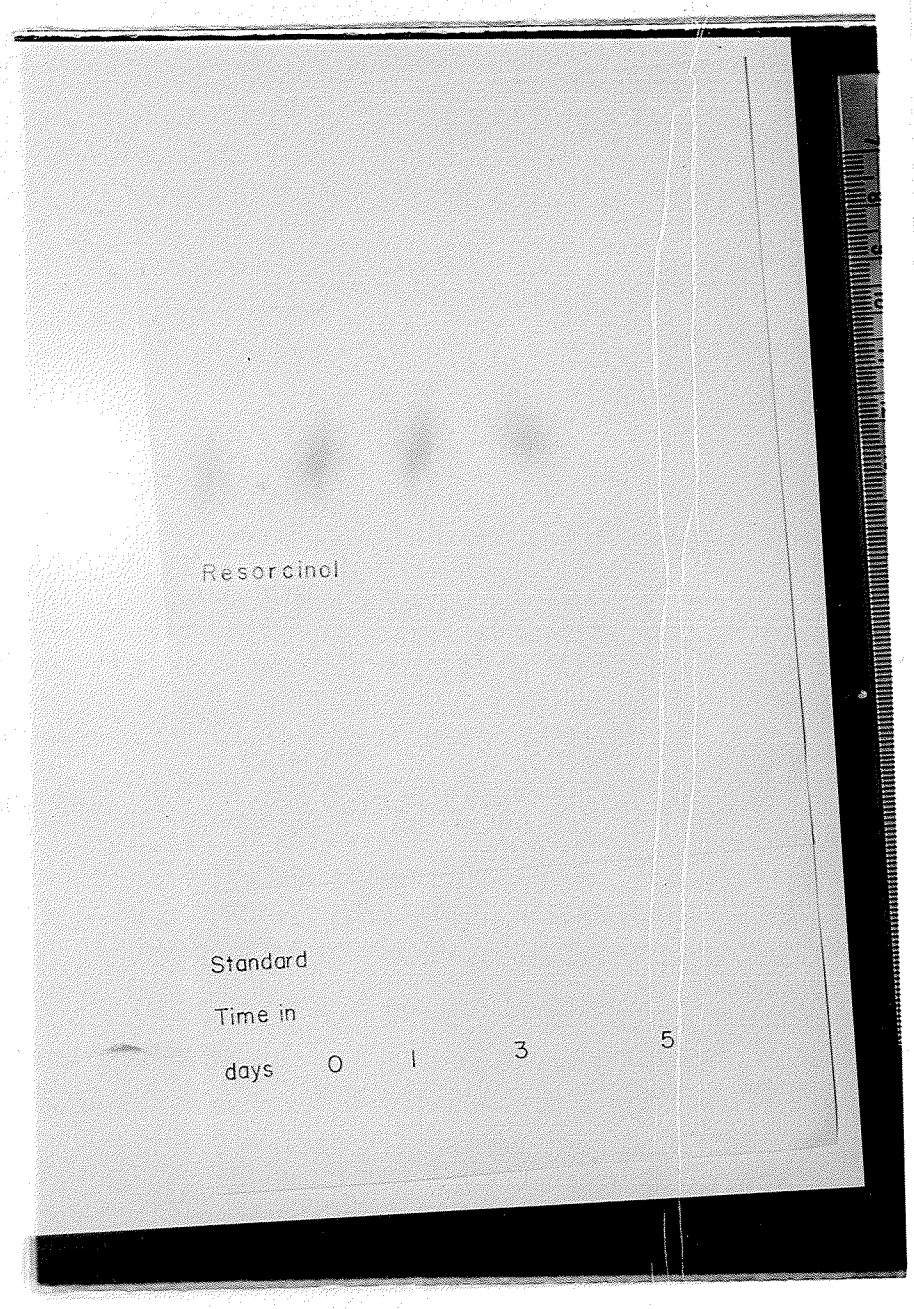

Resorcinol
Resorcylic acid
Resorcylic aldehyde
Standards
Time in
days
0
1
2
3
4

FIGURE 14. Chromatogram shows the disappearance of substrate from the medium when Micrococcus sp. was incubated with resorcylic acid under growth conditions.


Resorcinol
Resorcylic acid
Standards
Time in
days
0
1
2
4

also grown on resorcinol and samples were taken daily and the fermentation liquor was examined chromatographically in order to follow the rate of disappearance of resorcinol. Resorcinol disappeared completely after 4 days in the case of the Rhodotorula sp. (Fig. 12), and after 5 days in the case of the Micrococcus sp. (Fig. 15) and again no products of an aromatic nature were detected in the fermentation liquor. These results confirm that both organisms are able to utilize resorcinol as sole source of carbon and energy.

FIGURE 15. Chromatogram shows the disappearance of substrate from the medium when Micrococcus sp. was incubated with resorcinol under growth conditions.


Resorcinol
Standard
Time in
days 0 1 3 5

DISCUSSION

The evidence obtained from this investigation shows that in both the isolates studied, resorcylic aldehyde is oxidized to resorcylic acid which undergoes decarboxylation to form resorcinol. This mechanism is identical to that shown by Halvorson in Aspergillus niger (20) and consequently appears to be widely distributed in microbial systems.

Resorcylic aldehyde was oxidized to the corresponding acid by resting cell suspensions of the Rhodotorula sp.. The reaction mechanism appears to be brought about by a non-specific enzyme system in the organisms under study because the product of the oxidation, namely resorcylic acid, is metabolized by induced enzymes while the aldehyde is not. No other aliphatic or aromatic aldehydes, however, were used to test this hypothesis. But recently, the same proposal was made by Holms (28) who suggested that the oxidation of benzaldehyde to benzoic acid by a Vibrio sp. was brought about by non-specific enzymes (28).

The oxidation of the aldehyde could be demonstrated only when cells were grown on glucose. No oxidation occurred

when resorcylic acid or resorcinol were used as the growth substrate. Such reactions are dependent upon the presence of pyridine nucleotides in the oxidized form (28) and these components may be limiting in cells grown on the aromatic substrates.

When the Micrococcus sp. was grown on Brain Heart Infusion, resting cell suspensions failed to metabolize either resorcylic acid or resorcinol after 14 hours. But when the organism was incubated with either of these compounds under growth conditions, the disappearance of the substrate was detected in each case by paper chromatography (Figs. 11, 12, 14, 15). Clearly, resorcylic acid and resorcinol are suitable growth substrates but are metabolized by induced enzymes the lag periods of which appear to be of unusually long duration. The possibility cannot be excluded that enzyme induction in the Warburg respirometer flasks is dependent upon some condition not provided to the resting cell suspensions. For example, an inorganic ion or a higher level of free amino acids in the amino acid pool may be a prerequisite for induced enzyme formation. The precise time period required for induced enzyme formation or conditions required for production were

not examined and might well be yet another constructive avenue for study.

The sequential induction technique of Stanier was used in the case of Rhodotorula sp. to show that the resorcylic acid undergoes a direct decarboxylation to form resorcinol. The Micrococcus sp., however, failed to satisfy the criteria of Stanier to show the operation of the same pathway in this organism. Cells grown on resorcylic acid metabolized resorcinol only after a well defined lag period. Clearly, such a lag period was quite unexpected. Results obtained by the sequential induction technique must be interpreted with caution. In doubtful cases, cell free extracts have been used to confirm the presence of expected intracellular enzymes (22). Such was the case here. Active cell free preparations confirmed that resorcinol is indeed an intermediate in resorcylic acid metabolism and that intact cells failed to metabolize the substrate immediately because it could not pass through the cell membrane.

Resorcinol could not be detected on paper chromatograms when either organism was incubated with resorcylic acid under growth conditions or with resting cell suspensions. This differs from the Aspergillus sp. (20) in which resorcinol was

detected in the fermentation liquor using resorcylic acid as the substrate. One explanation is that in the Rhodotorula and Micrococcus sp. resorcinol does not accumulate in detectable quantities because it is oxidized as rapidly as it is formed. Manometric experiments support this view since resorcinol was always metabolized more rapidly than resorcylic acid in the Warburg apparatus. The rate of metabolism was also independent of the substrate on which the cells were grown.

Recently, Larway and Evans (33) isolated soil pseudomonads capable of metabolizing resorcinol through maleylacetic acid by hydroxylation at the C_4 position with subsequent fission between the o-dihydroxy group. Such an intermediate possessing the properties of a dihydroxyphenolic compound was not detected in the fermentation liquor of either organism in this study, but an exhaustive search for such an intermediate was not undertaken. This may be explained on the basis that the intermediate is metabolically very active and does not accumulate in the fermentation and the mechanism reported by Larway and Evans is not incompatable with these studies. A confirmation of the fate of resorcinol in these isolates would be most constructive.

Chloramphenicol proved to be an unsatisfactory inhibitor to prevent the synthesis of induced enzymes in the Rhodotorula sp.. Brock (6) reported that a number of yeasts and fungi were completely resistant to chloramphenicol at a level of 1.0 mg/ml. In the Rhodotorula sp. a somewhat higher level, 1.3 mg/ml, was used. Resistance to protein inhibitors is quite often due to the inability of the antibiotic to enter the cell (17). Since cell free preparations will not synthesize induced enzymes such an explanation is very difficult to prove and no attempt was made to determine whether this explanation is a valid one.

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