AN ABSTRACT OF THE THESIS OF

JAMES PAUL ELDRIDGE (Name of student) for the Doctor of Philosophy (Degree)

in Microbiology (Major) presented on 8-12-71 (Date)

Title: UTILIZATION OF PRODUCTS DERIVED FROM PHOTOLYTICALLY TREATED LIGNINSULFONATE BY Pseudomonas putida

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Abstract approved: Dr. A. W. Anderson

Ligninsulfonate, a water soluble, diverse molecular weight, polymeric by-product of the pulp and paper industry, is continually added to streams as a waste effluent. This material, formed by a random combination of phenylpropanoid subunits, is extremely resistant to microbial degradation. Its modification, to relieve recalcitrance, would eliminate a significant pollution problem and could lead to the formation of a substrate for the propagation of single cell protein. Photolytic alteration would be ideal for such a process, since it does not lead to other unwanted waste products such as would be inherent with chemical treatments.

In this study, a 0.1% W/V sodium ligninsulfonate (Marasperse CB) solution was irradiated, in the presence of oxygen, with a mercury-vapor ultraviolet lamp. A Pseudomonas putida soil isolate

(29-43-1), unable to utilize unirradiated ligninsulfonate as a sole source of carbon and energy, monitored the effect of photolysis on growth. Changes in absorbance at 430 nm, pH, and total carbon were also followed during the photolytic process. The substrate providing the maximum growth occurred with an oxygen flow-rate of 2 ml/min. at an initial pH of 7.6 (unaltered). This substrate was stable and could be reproduced by stopping photolysis at an absorbance between 0.70 and 0.05.

The complex substrate formed by the irradiation of ligninsulfonate exhibited both inhibitory and inducible properties. The inhibitory characteristics primarily resulted from large molecular weight residual material, sulfonate functions, and possibly catechol-like compounds present. The inductive characteristics were probably due to the presence of low concentrations of phenolic monomers.

Gel fractionation studies (G-25 Sephadex) verified that the high phenolic content, large molecular weight (> 5000) ligninsulfonate polymers were degraded to low phenolic content molecules having molecular weights in the range of phenolic monomers. The non-phenolic portion of this low molecular weight material was utilized for growth almost exclusively. However, gas-liquid chromatography (GLC) and thin-layer chromatography (TLC) analyses showed that the phenolic material disappearing after growth resembled vanillin. Also, physiological characterization of P. putida (29-43-1)

demonstrated a correlation between the utilization of irradiated ligninsulfonate and the utilization of phenolic compounds metabolized through catechol. Therefore, even though non-phenolic material was the principal substrate, phenolic monomers were present in sufficient amounts to effect the growth of P. putida (29-43-1).

The irradiation of the lignan, alpha-conidendrin (a diphenyl-propanoid), provided an excellent model system for studying the photolytic treatment of ligninsulfonate. The absence of sulfonate functions in this system made it possible to analyze the inhibitory nature of these groups in irradiated ligninsulfonate indirectly by a comparison of the two systems.

The ability of P. putida (29-43-1) to utilize up to 50% of the irradiated ligninsulfonate substrate suggests that photolytic polymer modification can drastically improve biodegradation. This type of treatment may find applications with other recalcitrant structures as well.

Utilization of Products Derived from Photolytically Treated Ligninsulfonate by *Pseudomonas putida*

by

James Paul Eldridge

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INTRODUCTION

The microbial metabolism of a potential substrate depends on the organism's enzymatic capabilities and on the structure and environment of the substrate. Most organic compounds, under the appropriate conditions, can be metabolized by one or more microorganisms. However, there are certain compounds that are extremely resistant to microbial attack. These recalcitrant compounds are often polymers, with the most prevalent example of a natural material being lignin.

Lignin, next to the structural carbohydrates, is the most abundant naturally recurring material on earth. This large molecular weight polymer functions primarily in plant support, and is essentially non-biodegradable.

The phenylpropane subunits of lignin, along with other related phenolic compounds, are excellent substrates for a number of microorganisms, including the common soil bacterium, *Pseudomonas putida*. However, the complex cross-linking of the phenylpropane subunits occurring in lignin and ligninsulfonate (an industrial by-product) prevents organisms from efficiently metabolizing these

compounds.

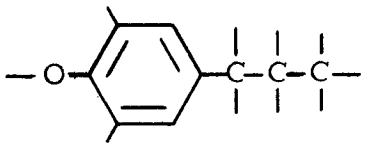
This study has been undertaken to demonstrate that ultraviolet light, in the presence of oxygen, can modify the ligninsulfonate polymer to an extent where it will support the growth of P. putida. Once polymer modification reaches the point where it provides a suitable substrate, the nature of the photolytic alterations and the means by which this organism adapts to the photolytic products can be determined. To do this, a thorough knowledge of the physiological responses of P. putida to irradiated ligninsulfonate is required.

The purpose of this investigation is to establish parameters for the photolytic treatment of a recalcitrant molecule by using ligninsulfonate as a model substance. An understanding of this photolytic process and the means by which P. putida adapts to the altered material may provide information which can be extended to the treatment of other recalcitrant polymers.

LITERATURE REVIEW

Lignin

Lignin is a highly crosslinked, 3-dimensional polymer composed of oxyphenylpropane units.



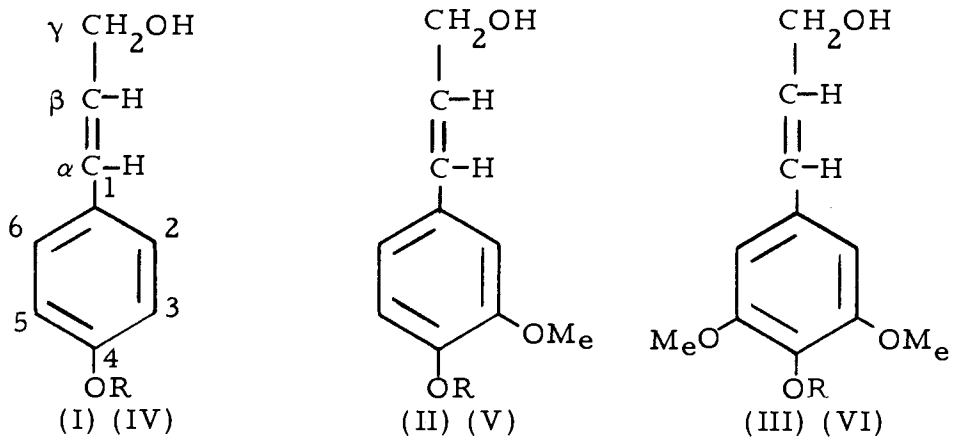
These units are joined together both by ethereal linkages and by carbon-carbon bonds. The carbon-carbon bonds are highly resistant to chemical degradation, making it difficult to analyze monomeric breakdown products (65). Lignin is not a defined chemical compound, and its chemical structure has not been fully elucidated. There is no single "lignin" of uniform composition, and lignin chemists have traditionally worked with preparations that are not standardized (12).

Lignin is isolated from plants as a brown amorphous solid which is insoluble in water and most organic solvents (63). It is not hydrolyzable to monomeric units and does not have the regular structure which characterizes other natural polymers. Degradation of lignin to simple identifiable compounds is usually random and incomplete, and the products obtained often include artifacts formed during the degradation process itself (11). Furthermore, these products have a tendency to undergo secondary condensation

reactions, even under mild treatments. Therefore, structural studies of lignins are extremely difficult, and it is not really known whether lignin is a single substance or a mixture of related polymers.

Lignin is one of the major constituents of wood and accounts for 18 to 38% of mature wood (7, 11, 23). The remainder is made up of cellulose, hemicelluloses, and extractives. Most, if not all, of the lignin is in some sort of chemical combination with plant polysaccharides. It is still unclear, however, whether the binding involves covalent bonds such as ether and ester linkages, or hydrogen bonds, or both. Lignin's biological role in living plants is to give strength and durability to the plant by encrusting the cell walls of its tracheids, vessels, fibers, and sclerids.

The biosynthesis of lignin can be subdivided into three stages (23). The first stage is the formation of C_6-C_3 acids by way of shikimic acid and prephenic acid. The second stage is the transformation of these acids into alcohols (I-III) and their glucosides (IV-VI). Finally, the third stage is the dehydrogenation of the alcohols to form lignin.



(I) (IV) (II) (V) (III) (VI)

R=H $R=C_6H_{11}O_5$

(I) p-coumaryl alcohol (IV) glucocoumaryl alcohol

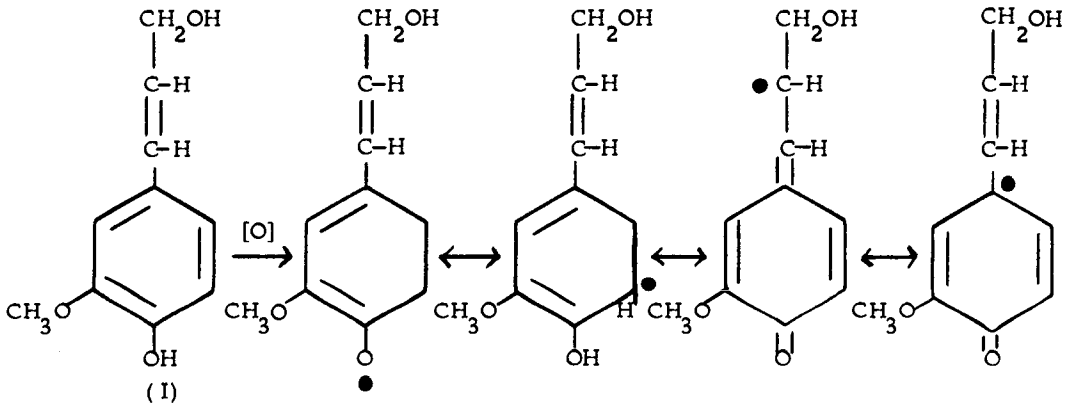
(II) coniferyl alcohol (V) coniferin

(III) sinapyl alcohol (VI) syringin

Freudenberg (23) has suggested that free phenolic group dehydrogenation in these alcohols leads to free-radicals. These radicals undergo non-enzymatic rearrangements and can combine to form quinone methides which become stabilized by intramolecular prototrophy or by condensation with water, other phenols, or carbohydrates. The stabilized products are also phenols and can undergo dehydrogenation reactions to form additional free-radicals and quinone methides. Thus, dimers form trimers which in turn form tetramers, and this eventually leads to the formation of lignin.

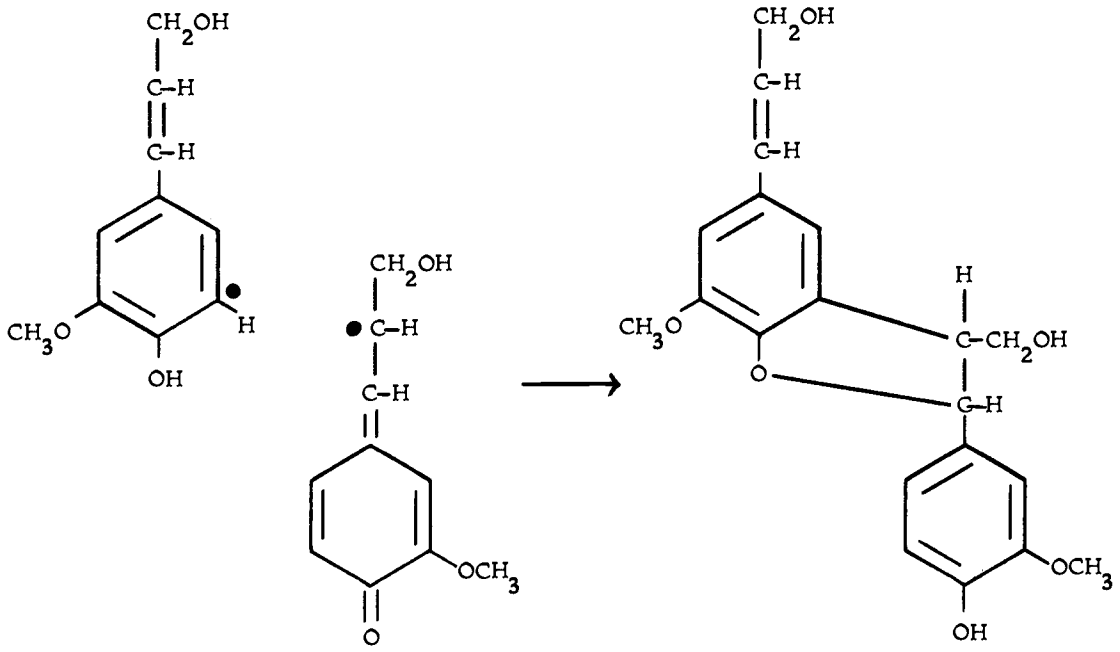
Freudenberg (23) was able to synthetically produce lignin by

oxidatively treating coniferyl alcohol with laccase or peroxidase. These enzymes are known to catalyze phenolic dehydrogenation reactions. By stopping these reactions in their initial stages it was possible to isolate dimers and larger units which would be the intermediates predicted by a free-radical pairing mechanism (57). The free-radical isomeric forms of coniferyl alcohol demonstrate the large number of positions at which a free-radical may occur:



Any combination of these radicals can become neutralized by a free-radical pairing (prototrophy). The formation of a dimer containing one or two unstable quinone methide non-aromatic rings can

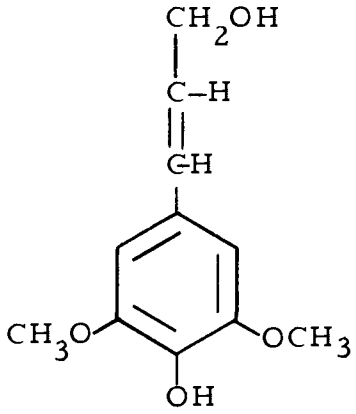
occur. However, these unstable forms are stabilized by ring closure as follows:



(b+c)

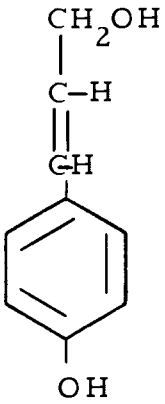
It is evident from the various possibilities of random combinations of these free-radicals that the linking of coniferyl alcohol units is quite involved. In the case of lignification, the situation is further complicated by the fact that in addition to the quaiacyl ring in coniferyl alcohol (I), the corresponding dimethoxylated group (II) in hardwoods, and the non-methoxylated group (III) in annual plants

are involved.



(II)

synapyl alcohol



(III)

p-hydroxycinnamyl alcohol

Ligninsulfonates

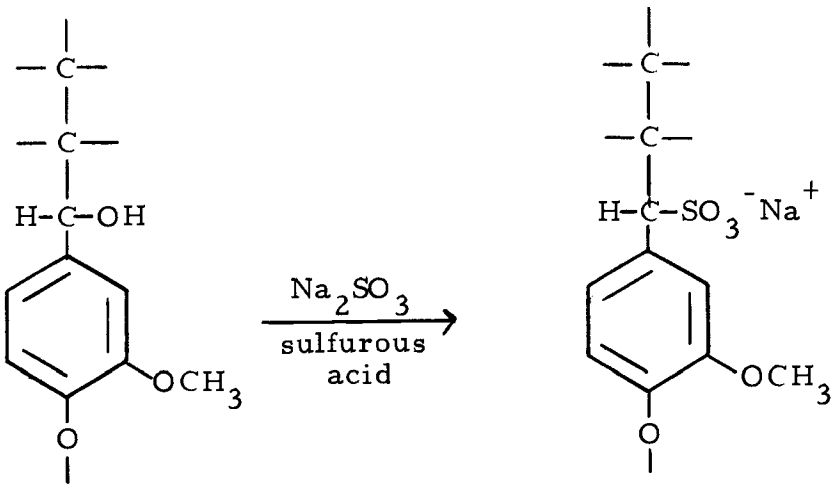
The commercial sulfonation process in the pulp and paper industry results in the separation of cellulose from the plant hemicelluloses and lignin. It involves the treatment of wood chips, at elevated temperatures and pressures, with an aqueous solution of a bisulfite salt and sulfurous acid (11, 45). This process converts the lignin present in the wood chips into soluble lignosulfonic acid.

During the sulfonation of wood, the lignin polymer is considered to undergo three reactions (9, 45). The first reaction is the formation of alkyl sulfonate groups which convert the lignin into a lignosulfonic acid that is still firmly anchored in the wood. The

second reaction is the actual dissolving step and involves the hydrolytic cleavage of acid-sensitive linkages. The final reaction is the condensation of the lignosulfonic acids.

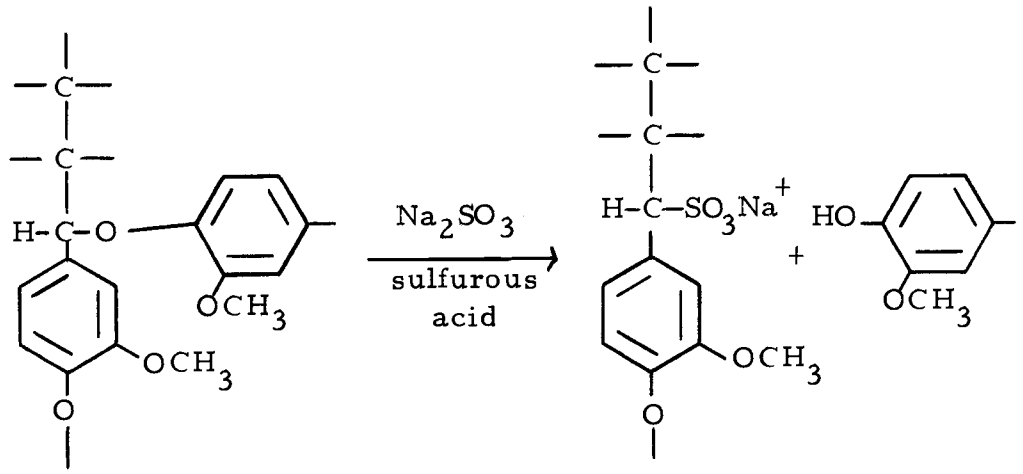
The sulfonation reaction usually occurs at the α-carbon on the propane side chain by one of the following reactions (45):

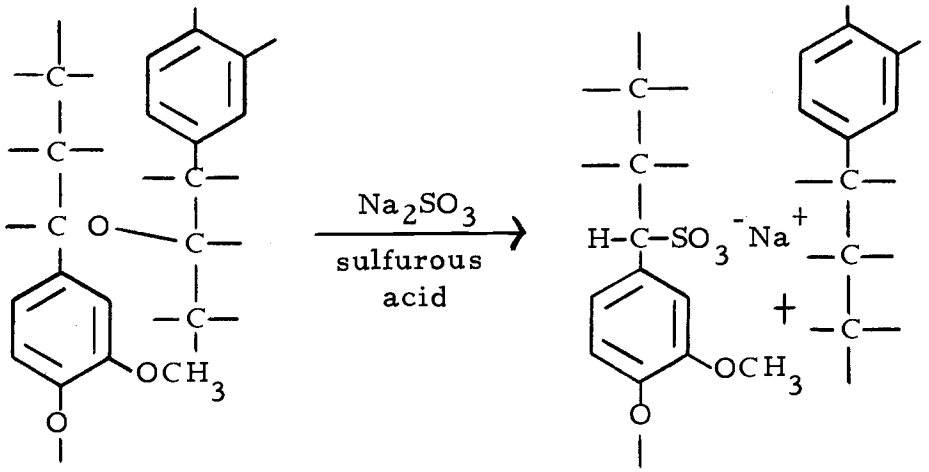
(a) replacement of aliphatic hydroxyl groups,



(usual order of reactivity: α-OH > β - OH > γ-OH)

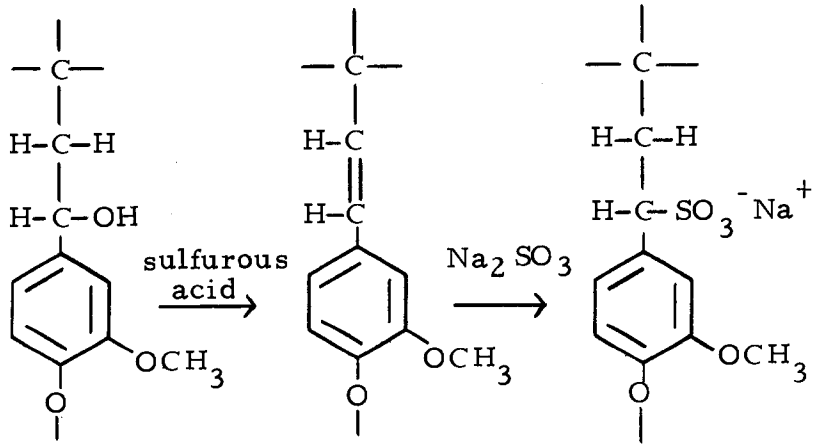
(b) cleavage of aryl-alkyl ether linkages and cleavage of alkly-alkyl ether linkages,





(Usual order of reactivity: α-ether > β - ether > γ-ether)

or (c) addition to a double bond.



Since sulfonation occurs only on the side chain of the phenylpropane units, there is essentially no change in the U. V. absorption spectrum of lignin after sulfonation, indicating that the phenolic content of lignin is the same in ligninsulfonates as in protolignin. The accompanying hydrolysis and condensation reactions cause the lignin-sulfonate to have a wider range of molecular weights and a greater

degree of polymer branching than protolignin. Gel fractionation studies have shown that the molecular weights of ligninsulfonates range from a few hundred to about one hundred thousand and average around ten or twenty thousand (20, 24).

At least one sulfonate group per four phenylpropane units is required for the dissolution of lignin (45). In practice however, ligninsulfonates contain about one sulfonate group for every two or three phenylpropane units. The extent of ligninsulfonate sulfonation increases with decreasing molecular weight, and sulfonation is accompanied by a proportionate increase in the number of reducing groups (22). Also the degree of sulfonation, for any particular molecular weight, is independent of the conditions of the sulfonation process. A study by Johnson (40) demonstrated that 3/4 of the sulfur in the reaction mixture combined only loosely as SO_2 or did not react at all, About 13% reacted to form organic compounds containing firmly-bound sulfur. Of this, approximately 2/3 was bound to the lignin while the rest was bound to a number of lignin-like compounds.

This waste product of the pulp and paper industry has found its way to a limited extent into a number of industrial processes (56, 57). The largest single use is as a dispersant for oil well drilling. Also, essentially all of the flavoring vanillin in the United States and Canada is produced by the alkaline oxidation of ligninsulfonates. The various physical and chemical properties of ligninsulfonates have led to their

use as road binders, and in animal-feed pelletizing, cement products, industrial cleaners, trace element chelation, leather tanning, boiler scale inhibition, plastics, adhesives, and sulfur chemicals.

Microbial Utilizaton of Lignin and Ligninsulfonates

In general the literature indicates the following concerning microbial decomposition of lignin: (a) there are only a limited number of microorganisms capable of degrading lignin; (b) the biological decomposition is extremely slow and incomplete; (c) the susceptibility of lignin to attack varies with its source, age, isolation treatment, and chemical nature; and (d) the higher organisms and fungi are more capable of lignin utilization than are bacteria (60). The fungi capable of degrading lignin fall basically into two groups (57). The first, the white rot fungi, actually metabolize the lignin molecule. The second, the brown rot fungi, do not metabolize the lignin, but are able to utilize the carbohydrates either by breaking down the tri-dimensional lignin network, or by breaking the lignin-carbohydrate bonds. The slow microbial attack of lignin in soil radically changes the structure of the lignin, converting it to humus.

Sorensen (68) was able to enrich for several bacteria belonging to the genera Pseudomonas and Flavobacterium able to decompose native lignin. These bacteria were able to utilize 29% of the lignin carbon in 74 days.

It appears that ligninsulfonates are even more resistant than lignin to microbial attack. This is principally due to two reactions which lignin undergoes during the sulfonation process. The first reaction is the sulfonation of the phenylpropanoid side chains, and the second is the hydrolysis of ether linkages and subsequent formation of new carbon-carbon bonds between aromatic rings. Watkins (72) presented evidence supporting this when he discovered that dimers with aryl-aryl carbon bonds are more resistant to bacterial decomposition than are dimers containing alkyl-alkyl or alkyl-aryl bonds.

Ferm and Nilsson (21) performed enrichments with commercial ligninsulfonates. They isolated 32 yeast fungi, 23 mold fungi and five bacteria from 17 soil and sludge samples. A substantial portion of their study employed a Pseudomonas species. This organism was able to remove over 15% of the total solids from the ligninsulfonate solution tested. Fractionation of ligninsulfonate by gel filtration demonstrated that only the material in the lower half of the molecular weight distribution was utilized for growth. Also, no decrease in the U. V. absorption at 280 nm was observed, which leads to the conclusion that low molecular weight fractions utilized for growth were non-aromatic in nature. It should be noted that this ligninsulfonate contained from 0.6 to 0.8% carbohydrates. They felt, however, that this did not account for the observed growth.

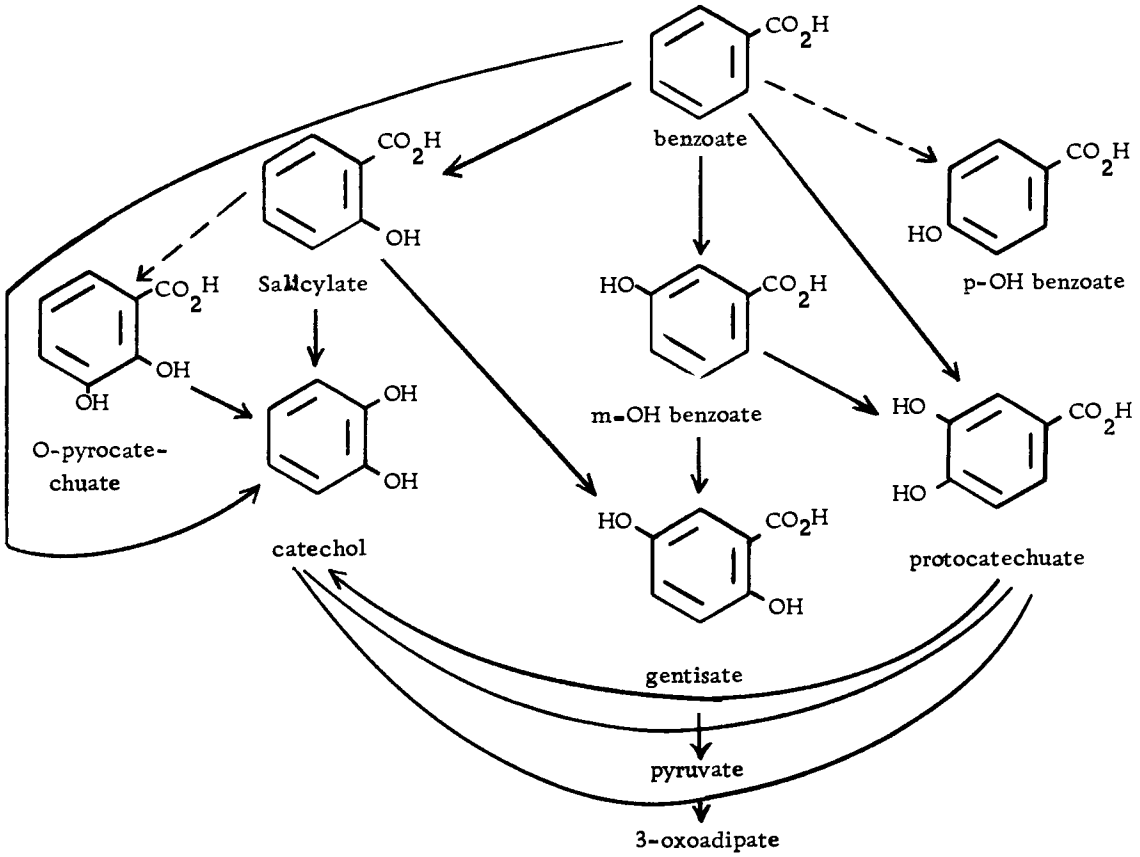
A number of fungi were evaluated by Ledingham and Adams (44) for ligninsulfonate utilization. Maximum utilization of 12 to 18% was observed in 60 days. In subsequent studies (2), with improved methodology, they showed utilization of only 3 to 5%. Also, bacteria evaluated by Abernathy and Watson (1) could only utilize ligninsulfonates to the extent of 10% in 21 days.

The degradation of ligninsulfonate in natural waters was studied by Raabe (60). Decomposition produced a multi-component biochemical oxygen demand (BOD) curve. The first stage of BOD was due to wood sugar oxidation and the second stage was primarily due to the decomposition of ligninsulfonate. It was reported that in 100 days less than 50% of the ligninsulfonate compounds present were oxidized.

Phenolic Model Compound Utilization by *Pseudomonas putida*

Pseudomonas putida is a saprophytic soil bacterium able to degrade many carbon and nitrogen compounds. Among these compounds are the phenolic building blocks and breakdown products of lignin, which are related to benzoates and have a high relative stability due to their large negative resonance energies.

There is a great deal of diversity in the metabolism of such benzoates by microorganisms as shown below (16). The dashed lines represent proposed reactions.



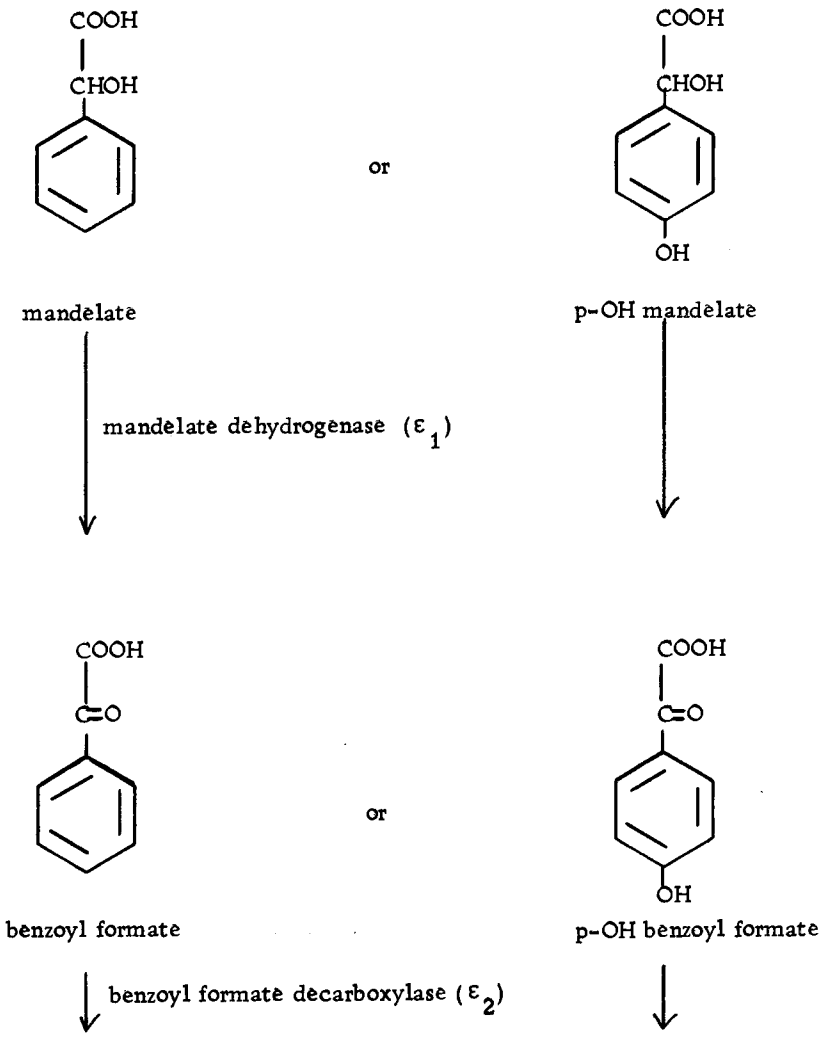
These reactions utilize molecular oxygen as an oxidant. The enzymes are, for the most part, inducible and initially convert the aromatic substrates to their ortho or para dihydroxyphenol derivatives (15, 17, 26). This is followed by ring cleavage with the formation of aliphatic acids which are utilized via the Krebs cycle through a variety of pathways dependent upon the organism and the cultural conditions.

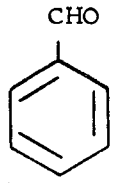
The Pseudomonas organism has a gene arrangement that permits this great metabolic diversity. This can best be demonstrated by a comparison with Escherichia coli. In E. coli the genes controlling sequential steps of many biosynthetic and catabolic pathways tend to be arranged contiguously on the chromosome. These genes are under the control of an operon. In the case of Pseudomonas, the related

markers of biosynthetic and catabolic pathways tend to be scattered throughout the chromosome, either singly or in small clusters (33). These genes are often under the control of a regulon. Ther term regulon was coined by Maas and McFall (50) to denote a system in which the enzymes are regulated as a unit while the genes are not necessarily closely linked. In other words, the mechanism of enzyme control in Pseudomonas is basically the same as in E. coli, but the pattern of the mechanism is considerably different. This difference in control patterns can be understood when the ecology of the organisms is observed. E. coli is a normal inhabitant of the animal gut. This environment contains a large and rather constant food supply that enters at regular intervals and must be disposed of in a limited time. Therefore, the simultaneous synthesis of complete enzyme systems, as with the operon, would be an effective means of coping with this type of environment. In other words, the expenditure of large amounts of energy for the synthesis of enzymes would be justified with a constant food supply. Pseudomonas, on the other hand, is commonly found in soil or water where the food supply is irregular, varied and often in low concentration. A pattern of control mechanisms permitting a more diverse metabolic system to function would be needed in this particular environment. Therefore, the control by a regulon, which induces few and only immediately required enzumes, would be the most efficient means of control.

In this case, only a minimum amount of energy is funnelled into the synthesis of enzymes in response to a limited food supply

Since many of the phenolic breakdown products of lignin are metabolized through the mandelate pathway, a knowledge of the physiological controls of this pathway is desirable. This pathway is shown below (28, 70). The portion of this pathway beginning with catechol and protocatechuate is known as the β-ketodipate pathway. The induction patterns of P. putida for both branches of this pathway, as described by Ornston (54), also are shown below:



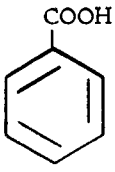


benzaldehyde

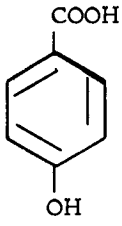
or

p-OH benzaldehyde

dehydrogenase (ε_3)



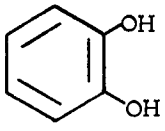
benzoate



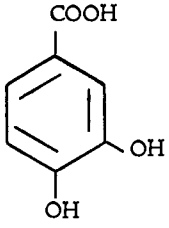
p-OH benzoate

benzoate
oxidase (ε_4)

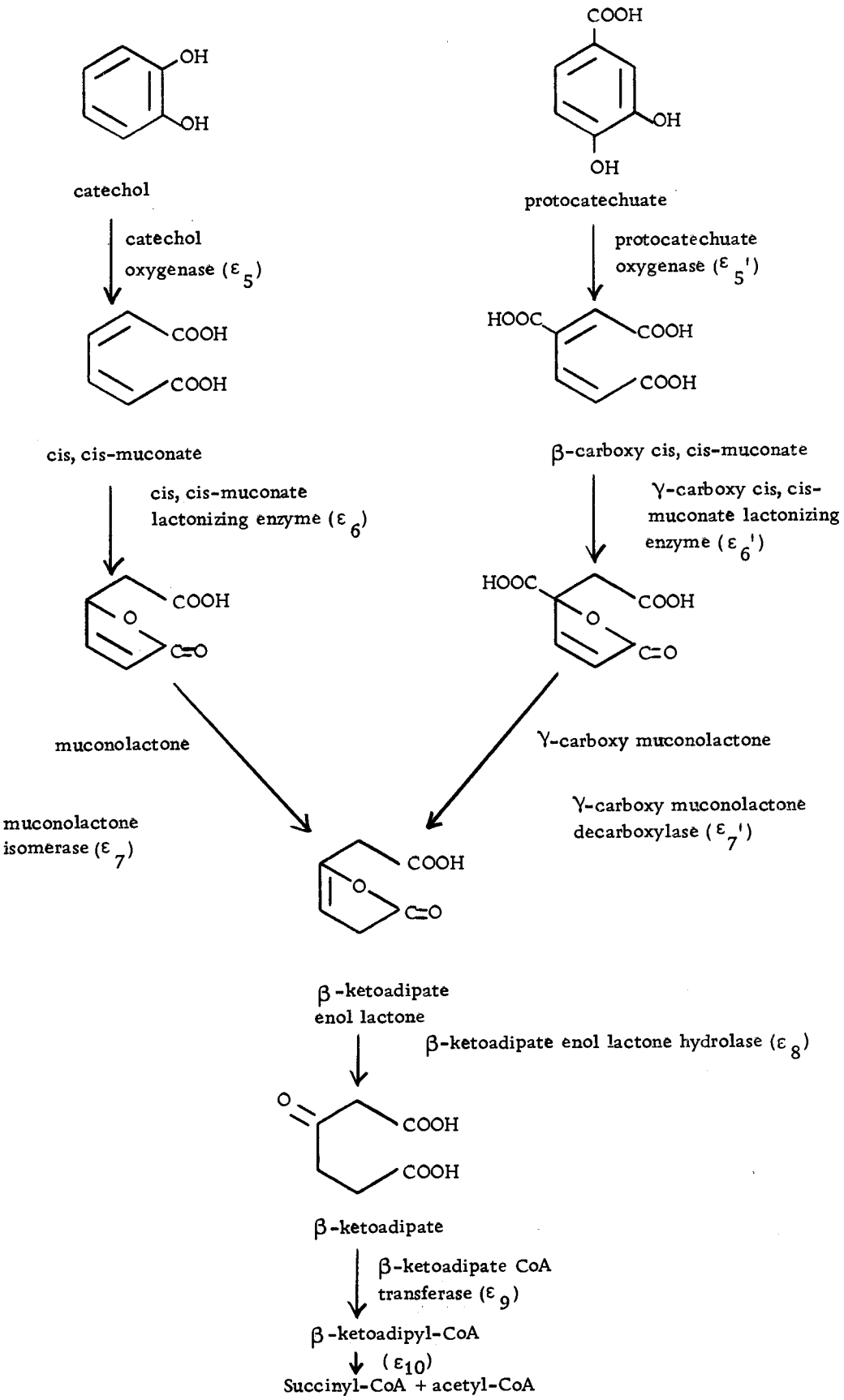
p-OH benzoate
hydroxylase

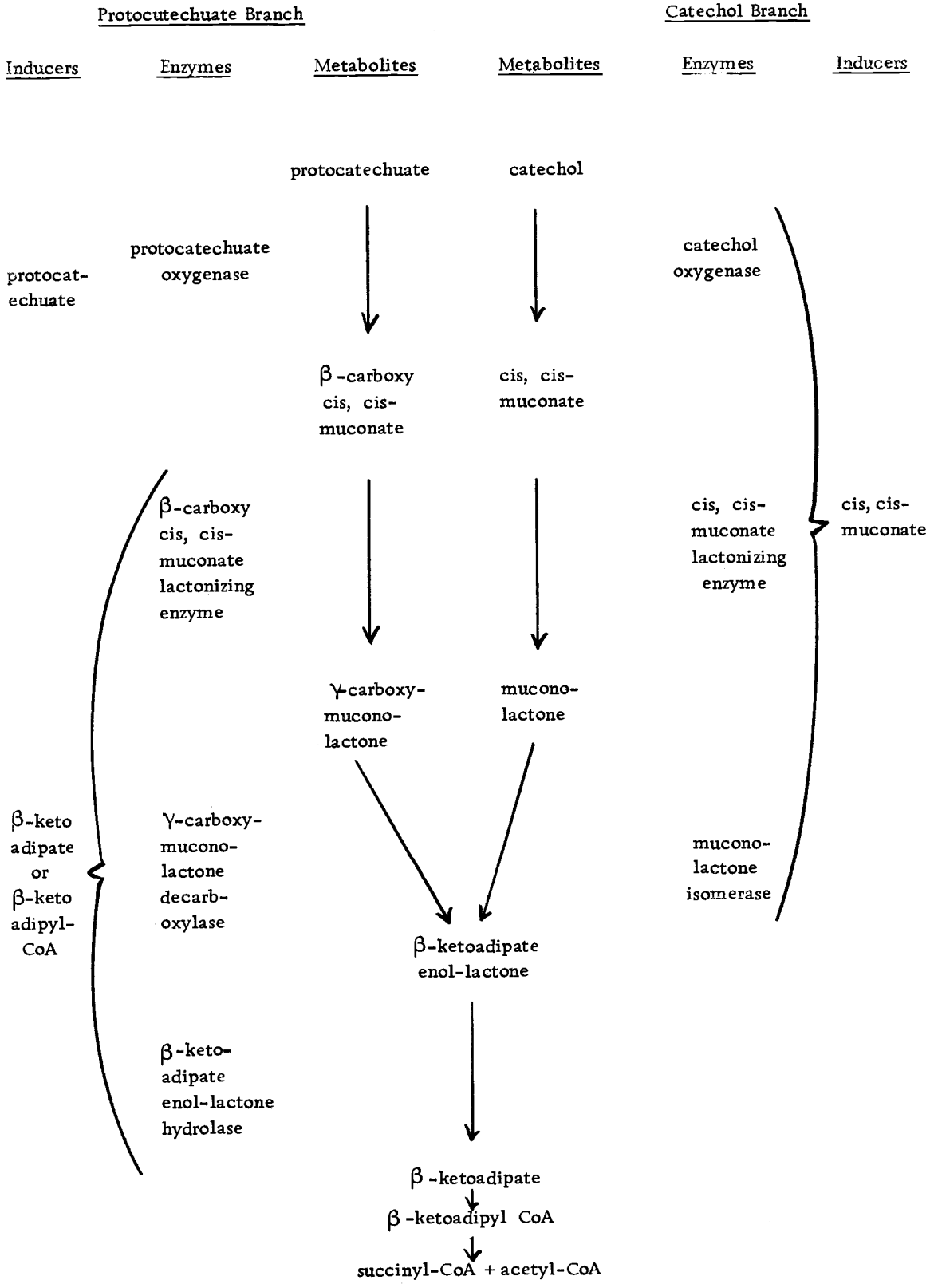


Catechol



protocatechuate


OH
OH
catechol
catechol
oxygenase (ε_5)
COOH
COOH
cis, cis-muconate
cis, cis-muconate
lactonizing enzyme (ε_6)
COOH
O
C=O
muconolactone
muconolactone
isomerase (ε_7)
COOH
COOH
OH
protocatechuate
protocatechuate
oxygenase (ε_5')
HOOC
COOH
COOH
β-carboxy cis, cis-muconate
γ-carboxy cis, cis-
muconate lactonizing
enzyme (ε_6')
HOOC
COOH
O
C=O
γ-carboxy muconolactone
γ-carboxy muconolactone
decarboxylase (ε_7')
COOH
O
C=O
β-ketoadipate
enol lactone
β-ketoadipate enol lactone hydrolase (ε_8)
O
COOH
COOH
β-ketoadipate
β-ketoadipate CoA
transferase (ε_9)
β-ketoadipyl-CoA
(ε_{10})
Succinyl-CoA + acetyl-CoA


Protocutechuate Branch
Catechol Branch
Inducers
Enzymes
Metabolites
Metabolites
Enzymes
Inducers
protocatechuate
catechol
protocat-echuate
protocatechuate oxygenase
catechol oxygenase
β-carboxy cis, cis-muconate
cis, cis-muconate
β-carboxy cis, cis-muconate lactonizing enzyme
cis, cis-muconate lactonizing enzyme
cis, cis-muconate
γ-carboxy-mucono-lactone
mucono-lactone
β-keto adipate or β-keto adipyl-CoA
γ-carboxy-mucono-lactone decarb-oxylase
mucono-lactone isomerase
β-ketoadipate enol-lactone
β-keto-adipate enol-lactone hydrolase
β-ketoadipate
β-ketoadipyl CoA
succinyl-CoA + acetyl-CoA

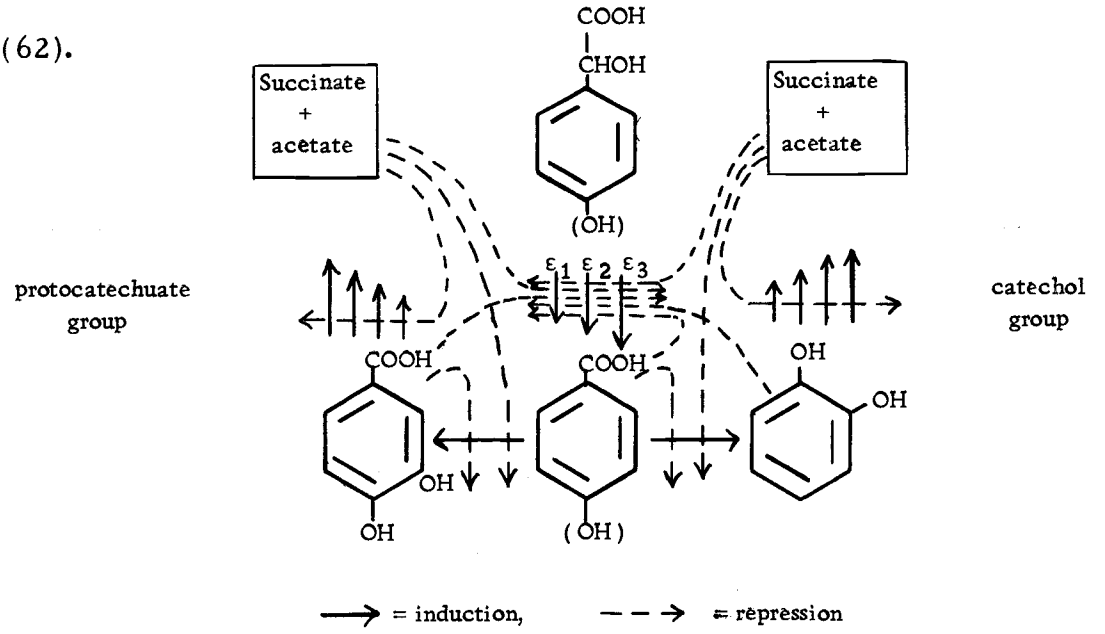
A consequence of this regulation pattern is the gratuitous induction of two enzymes which function only in the protocatechuate branch by compounds metabolized through the catechol branch. This is a result of the coordinate induction of three enzymes in the catechol branch by cis, cis-muconate. Two of these enzymes decompose cis, cis-muconate and the third produces it. Therefore, in the uninduced cell, sufficient levels of catechol oxygenase must be present to permit endogenous inducer generation. One of the explanations (54) for this unique regulatory mechanism is that β-carboxy cis, cis-muconate, γ-carboxymuconolactone and β-ketoadipate enol lactone do not possess chemical structures which permit them to function as inducers. Hence, the enzymes that metabolize them would have to be induced by a metabolic precursor or by a metabolic product of these compounds.

The induction and multi-sensitive end-product repression of the enzymes degrading mandelate and p-hydroxymandelate through these pathways has been studied with P. putida (62). It was noted that these pathways are excellent examples of sequential induction, since the addition of any intermediate causes the induction of all subsequent enzymes in the pathway.

The first three enzymes, E_1-E_3, are common to both pathways, and are co-ordinately induced and repressed. Since they are related in function, and in induction and repression patterns, they

could possibly be under the control of a single operon. However, with an operon, the genes have to be closely linked genetically. There is no evidence for this, thus these genes have been described as belonging to a regulon.

Repression is independently exerted on this first regulon by the end-products of enzymes contolled by succedding regulons. In other words, catechol, protocatechuate, acetate and succinate can all repress the first regulon. This pattern is repeated further along the pathway, so benzoate oxidase, which is controlled by the second regulon, is repressed by its immediate end-product (catechol) and also by acetate and succinate. There is a parallel system of multi-sensitive repression mechanisms controlling the enzymes that degrade the hydroxy-compounds. The induction and repression characteristics of these partially duplicate pathways is shown below (62).



Repression appears to be exerted by compounds that are likely to occur in the external environment or at converging points of degradative pathways. For example, benzoate is commonly found under natural conditions, and catechol is at the point where the tryptophan and mandelate degradative pathways converge. The net effect of this control system is the cell can not form inducible degradative enzymes if the end-products are already being supplied from without, or are being produced by the degradation of some other carbon and energy source.

The ability of a vareity of substrate-inducers to initiate the synthesis of a multienzyme system catalyzing their degradation to common metabolites is very economical for the cell. Due to this low specificity, the cell does not have to contain or replicate extra genetic information for each of these compounds. This not only conserves DNA, but also conserves the synthesis of RNA and protein.

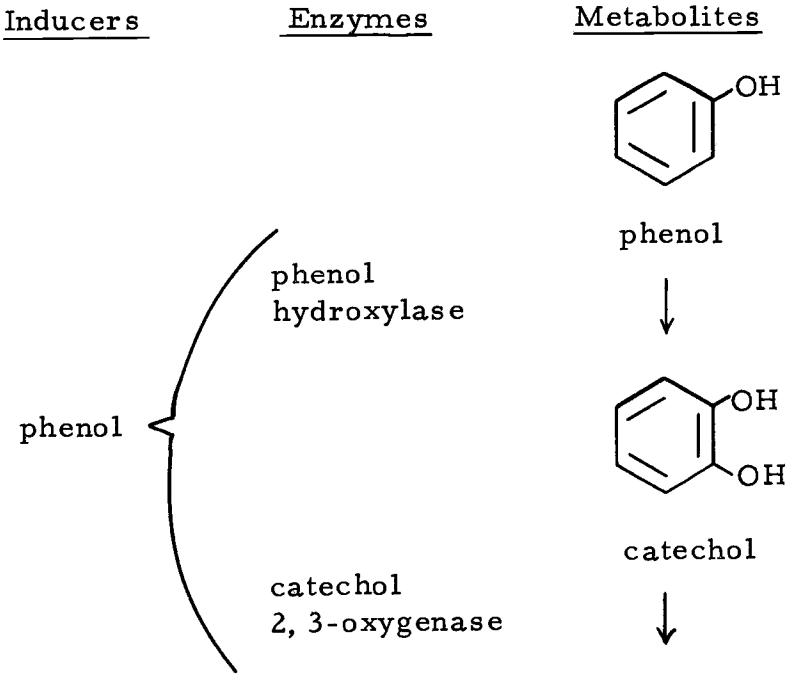
This complex system apparently has evolved to deal with the intricate system of converging pathways used by the pseudomonads to degrade a large variety of aromatic compounds to components of the TCA cycle. The evolutionary evolvement of the β-ketoadipate pathway was discussed by Ornston and Stanier (55). They felt that it would be difficult to conceive of the evolution of the reaction chains by successive development of enzymes starting at the primary substrate. Their reasoning was based on the fact that the oxidative

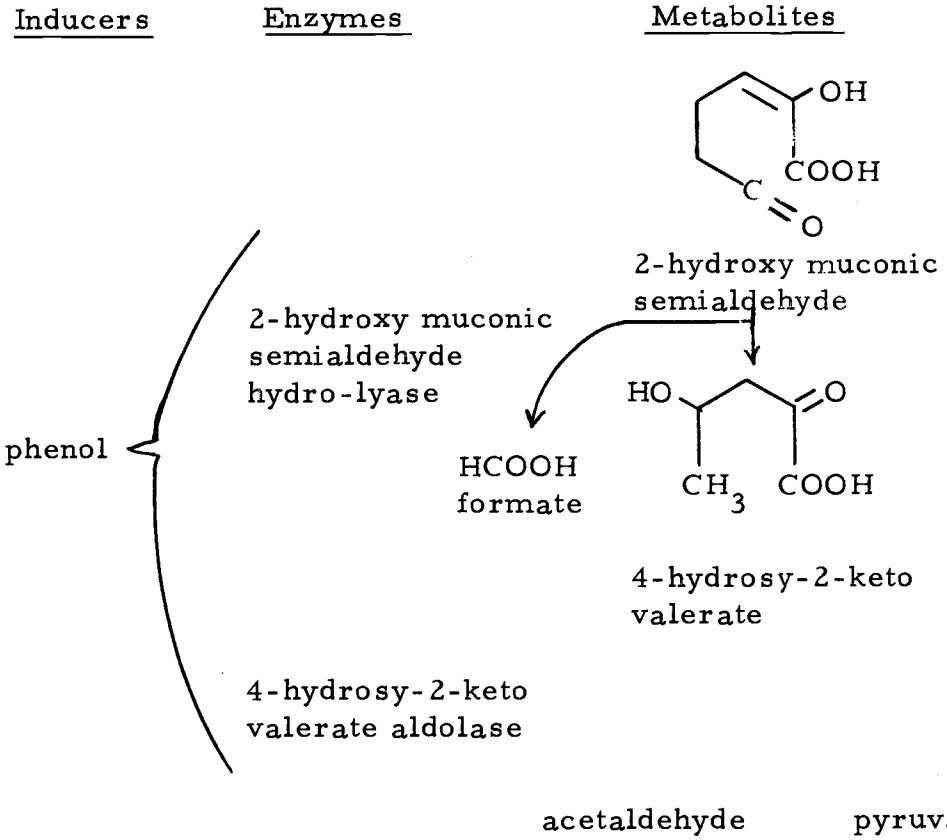
reactions were mediated by oxygenases and therefore, could not serve for the generation of ATP. Hence, these reactions possess no physiologically selective value. It was equally difficult to conceive of the evolution of these pathways by the reverse process, since many of the intermediates are chemically unstable. The selective value of these pathways could only be realized when they were completely functional. Therefore, they proposed that the pathways originated from arrays of relatively non-specific enzymes which already existed in the cell. This array of enzymes fulfilled other physiological functions, but somehow could work together inefficiently to catalyze one of the reaction sequences. Natural selection could then improve the efficiency of this pathway. Another evolutionary problem was the parallelism between the catechol and protocatechuate branches. Ornston and Stanier (55) felt that once the enzymes of one pathway became operative within the cell, they would become excellent raw materials for the enzyme set of the other, homologous pathway. There was also the possibility that both pathways may have developed independently from this pre-existing array of enzymes. However, the former possibility appeared to be more plausible since the homology of these enzyme systems had been verified by noting strong resemblances in their primary structures and in their physical and chemical properties.

One notable difference between the lac operon of E. coli and

the mandelate regulon of P. putida is that, in the former, metabolic repression can not be reversed by increasing the concentration of the inducer. In the mandelate system, on the other hand, the repressors act additively and their effect can be counteracted by increasing the substrate concentration.

The type of ring scission exhibited in the β-ketoadipate pathway is referred to as ortho cleavage. However, certain compounds induce a ring cleavage adjacent to one of the two hydroxyl groups instead of between them. This is referred to as meta cleavage. Feist and Hegeman (18, 19) have compared these types of cleavage mechanisms by inducing P. putida with phenol and benzoate. The metabolism of benzoate through the ortho cleavage (β-ketoadipate) pathway has already been discussed. The meta pathway degradation of phenol by P. putida is described below (18).





The simultaneous existence of both pathways can best be understood when their means of regulation are considered. The four enzymes of the meta cleavage pathway are induced by the primary substrate. On the other hand, in the ortho cleavage pathway, the ring cleavage product of catechol is the inducer. This difference in regulation explains why enzymes of only one pathway are synthesized during the growth on a particular substrate even though a common intermediate is formed. In the case of P. putida, growth on benzoate does not induce enzymes of the meta cleavage pathway since these are only induced by compounds such as phenols and cresols. When this organism is grown on phenol, the ortho cleavage pathway

enzymes are not induced because the high level of phenol-induced catechol 2, 3-oxygenase prevents catechol and therefore, cis, cis-muconate accumulation.

The specificity difference of induction and function in these pathways suggests that they are neither tangentail nor redundant. The meta cleavage pathway has a low specificity of induction and has evolved to catabolize a large variety of aromatic compounds which presumably arise during the degradation of natural products such as lignin. Therefore, catechol is only one of a number of possible intermediates metabolized through this pathway. The ortho pathway is more specific and primarily functions in the catabolism of precursors of catechol and of catechol itself.

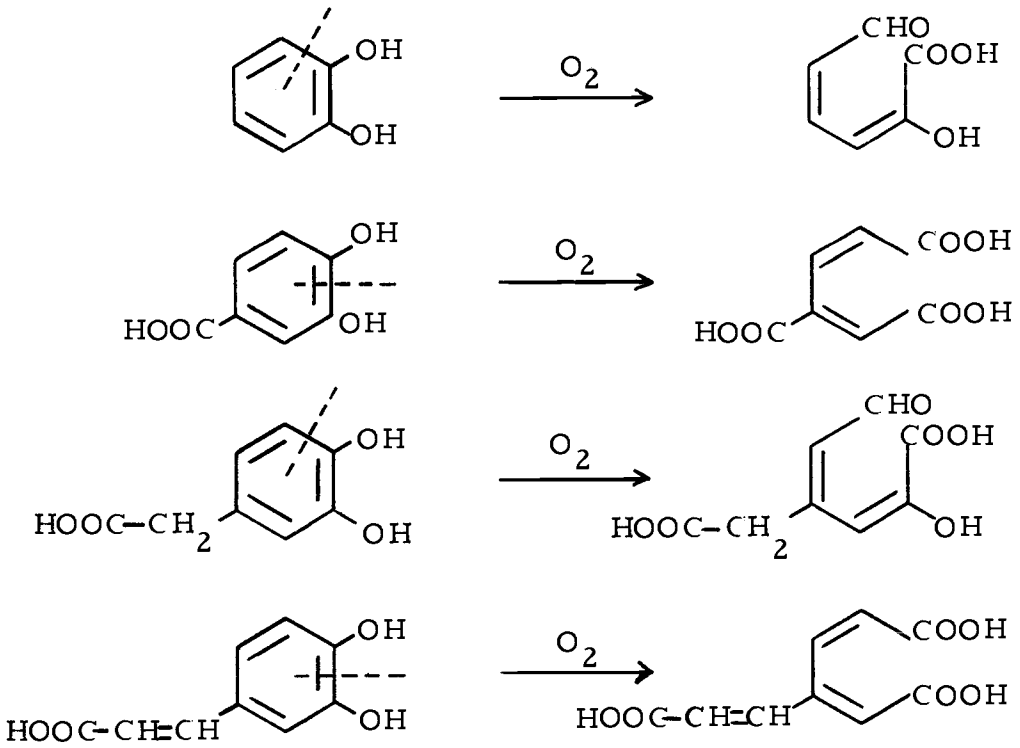
A taxonomic analysis of the fluorescent pseudomonads (32) has revealed that strains capable of degrading catechol by means of the meta cleavage pathway predominantly belong to P. putida. However, these strains are relatively rare within this species.

The substitution on the aromatic ring determines whether that compound can be metabolized, and also, the type of ring cleavage it undergoes. Hegeman (31) found that, before an aromatic molecule could be metabolized, an electro-negative function on the ring or on the side chain would be necessary. This may be a carbonyl, a hydroxyl, a thioether, or an oxygen ether function. The steric configuration of an oxygen-bearing function was not found to be

important (32). Also the length and proximity of these groups on the side chain was not found to be critical.

Studies on the metabolism of toluene and isopropyl benzene (26) have shown that these compounds are converted to ortho dihydorxy compounds in which the side chains are left intact. These results substantiated the observation that many aromatic compounds undergo enzymatic aromatic ring hydroxylation in preference to degradation of the aliphatic side chain.

Seidman et al. (66) grew P. fluorescens with phenol, p-hydroxybenzoate, p-hydroxyphenylacetate, and p-hydroxytranscinnamate as sole carbon and energy sources. Each of these compounds was first hydroxylated ortho to the hydroxyl group on the benzene ring. The rings were then cleaved in the following ways.



The electron-donating or electron-withdrawing capacity of the side chain substituents apparently determined whether ortho- or meta-cleavage enzymes were derepressed.

Watkins (72) utilized monomeric lignin model compounds in an effort to show a relationship between their structure and availability. He found that compounds which supported good soil bacterial growth had side chains with primary alcohol, aldehyde or carboxylic acid end groups. The sulfonation of the side chains tended to reduce the availability of these compounds. The reason for this reduced availability can best be understood when the natural source of the sulfonated compounds is considered. They are present in spent sulfite liquors in concentrations that are probably too low to support growth. In addition, these liquors contain low molecular weight carbohydrates which are excellent sources of carbon and energy. Therefore, spent sulfite liquor acts as a selective substrate which favors bacteria capable of rapid growth on carbohydrates over those able to utilize low molecular weight sulfonates.

Studies with various substituents on the benzene ring have demonstrated that they have a marked effect on biodegradation (3, 71). The effect of monosubstituted benzenes on soil bacteria is shown below.

Compound	Substituent	Decomposition Period, Days
Benzoate	COOH	1
Phenol	OH	1
Nitrobenzene	NO_2	64
Aniline	NH_2	4
Anisole	OCH_3	8
Benzenesulfonate	SO_3H	16

The key steps in aromatic compound metabolism are ring hydroxylation and oxidative ring cleavage. The enzymes catalyzing the hydroxylation reactions have been termed mixed-function oxidases (49) or monooxygenases (30). In these reactions, one atom of oxygen is incorporated into the substrate molecule. In the presence of a suitable electron donor, such as NADH, the other atom of oxygen is reduced to water. This type of reaction is represented by the following equation.

$$R\text{-}H + O_2 + XH_2 \longrightarrow ROH + H_2O + X$$

R-H represents the substrate molecule

XH_2 represents the electron donor

The oxygenases responsible for ring cleavage are referred to as dioxygenases. They mediate the incorporation of both atoms of the oxygen molecule into one molecule of substrate. Evans (17) postulated a ring-cleavage mechanism in which the first step was

considered to be the formation of an oxyferrooxygenase. In this step electrons are donated to the oxygen molecule from the iron and this results in the weakening of the O-O bond. The association of this perferryl complex with the o-dihydroxyphenol group then leads to a redistribution of electrons and the eventual cleavage of the C-C bond. This mechanism is described below.

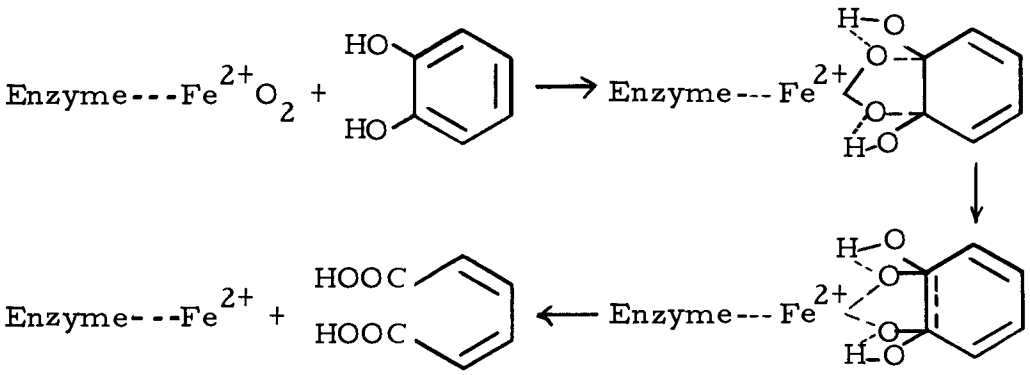


Photo-oxidation

Light is an electromagnetic radiation and therefore exhibits both wave and particle properties. The wave properties of frequency and wave length are related as follows:

$$c = \nu\lambda$$

c = velocity of radiation

ν = frequency (sec^{-1})

λ = wave length

When describing the particulate properties of light, the energy is said to be contained in packets called quanta. The energy content of these

quanta is dependent on the frequency of the radiation as seen in the following equation:

$$E = h\nu$$

E = energy of a single quanta

h = Planck's constant

With the aid of these equations, it has been noted that the quanta of ultra-violet radiations (λ = 400 to 200 nm) contain energy in the range of interatomic bond strengths (13). For example, if an "Einstein" (mole or Avogadro's number of quanta) of 313 nm radiation is absorbed in a system, there is an energy increase of 91 Kcal (58). This is comparable to the dissociation energy of a carbon-carbon bond (71 Kcal $mole^{-1}$).

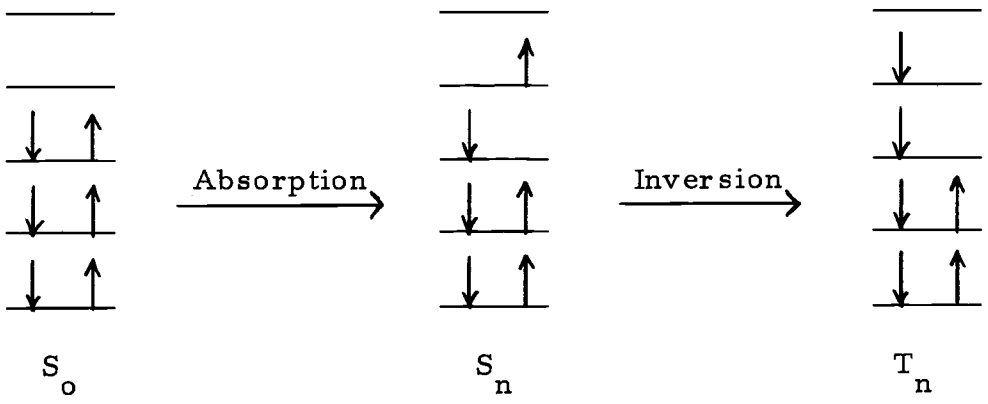
Upon absorption of a quantum of electromagnetic radiation, the energy of the electromagnetic wave is transferred to the absorbing molecule. This is accomplished by a mechanism which involves the excitation of the overall absorbing species to a higher frequency and/or electronic energy state (53). The excitation energy is made up of an electronic, a vibrational, and a rotational term.

$$\Delta E = \Delta E \text{ elect.} + \Delta E \text{ vib.} + \Delta E \text{ rot.}$$

Most polyatomic molecules have a number of metastable excited electronic states. In all of the transitions to these excited states, an electron from some orbital which is occupied in the ground state is

elevated to an orbital which is vacant in the ground state (29). The first excited state formed may undergo radiationless transitions to other excited states before returning to the ground state, or it can undergo a chemical reaction. Also, the excited state may be transferred to other molecules.

Organic molecules usually contain an even number of electrons which are spin-paired in the ground state (29). In other words, there are n electrons in n/2 orbitals. In this state the molecule has no net spin angular momentum and is said to be in the singlet state. When light is absorbed, an electron is promoted to a vacant orbital. This causes two electrons to become orbitally unpaired. Practically all electron promotions are the result of singlet-singlet transitions ($S_o \rightarrow S_n$). Since the spins of the electrons do not have to remain paired in most excited configurations, spin inversion can take place and form a second excited state called the triplet state (T_n).



S_o = ground singlet state

Usually light emission occurs from the lowest excited singlet (S_1) or triplet (T_1) state. The former process is known as fluorescence and the latter is called phosphorescence.

One type of electronic transition is referred to as π-π^* excitation (53). Here light absorption by a molecule results in the promotion of one electron from the bonding π level to the antibonding π^* level. Another type of transition is termed n-π^* excitation. This occurs in molecules containing heteroatoms such as sulfur, oxygen, or nitrogen which contain unshared electron pairs. The π-π^* transition is highly probable and requires higher energies than does the n-π^* transition. However, the n-π^* transition has a low probability of occurring. It should be noted that, in highly conjugated molecules, there is a lower antibonding π^* level, and so the excitation energies are correspondingly less.

There are two important laws of photochemistry that should be mentioned here (67). The first of these laws, the Grotthus-Draper law, states that only radiations which are absorbed by a reacting system can produce a chemical change. The second law, the Stark-Einstein law of the photochemical equivalent, states that each molecule taking part in a chemical reaction induced by light exposure absorbs one quantum of radiation.

A photochemical reaction involves one or more primary processes. A primary process is considered to involve the

absorption of a quantum of radiation by the reacting molecule and the subsequent formation of an excited state (58). In addition, the primary process includes the reactions of the molecule while still in the excited state. The deactivation pathway followed by a particular molecule is determined by its structural configuration and by the frequency of the absorbed radiation (13). An example of this is observed when unsaturated linkages, conjugate to the absorbing chromophore, cause substantial spectroscopic shifts and increases in extinction coefficients (53).

A secondary process is one which involves the fragments from the primary process or spectroscopic transpositions of the excited molecule, and ultimately leads to ground state products. It can occur by way of three basic pathways (27, 53). First, the molecule can undergo a chemical reaction which causes the excited molecule to form ground state products. These reactions usually involve free radicals and include decompositions, additions, rearrangements, substitutions, and chain reactions. Second, the excited molecule can lose its energy by a radiative process such as fluorescence or phosphorescence. And finally, the molecule can simply transfer its excess energy to another molecule. The only conditions required for this energy transfer is that the energy state of the acceptor molecule must be lower than that of the donor molecule. The important result of this energy transfer is that the donor molecule

is degraded to its ground state.

The utilization of photochemical processes for the decomposition of refractory organic compounds has been the subject of a great many investigations. For example, the organic matter has been successfully removed from fresh water (6) and salt water (4) by this type of process. Also, the pesticides DDT (52, 59), aldrin, dieldrin, and endrin (13) have been broken down by photolysis. Furthermore, synthetic polymers such as polymethylmethacrylate (38), polymethacrylic acid (14), polyacrylnitrile (34, 35, 37), and isotactic polymethacrylic acid (36) have been degraded in this manner.

Photolysis can also cause polymerization to occur, with or without accompanying degradation, as demonstrated in the photochemical cross-linking of polypropylene (42) and polyacrylonitrile (34).

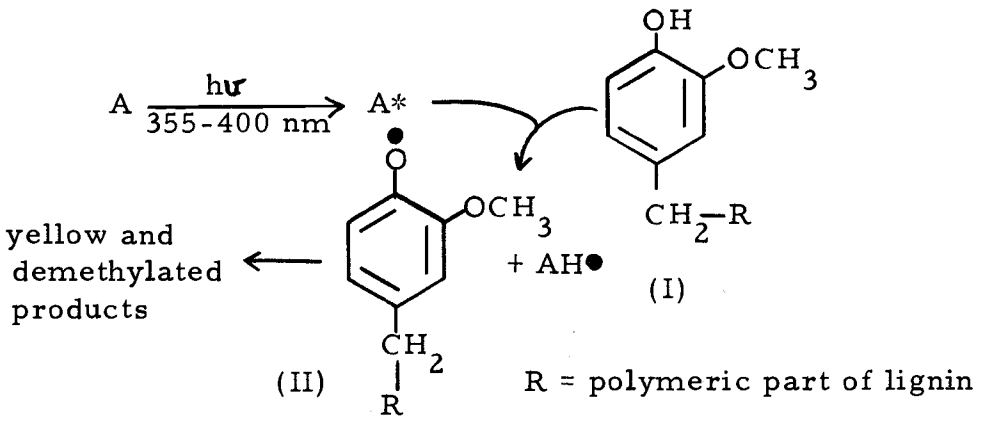
These reports demonstrate that the photolytic mechanism is oxidative in nature, and that it operates through free-radical generation. Also polymerization, as well as decomposition of a polymeric substance can occur simultaneously. In addition, the polymer structure determines, to a large extent, how the photolytic reaction proceeds, while the reaction rate itself is linearly dependent on the light intensity.

Even though the lignin molecule is considerably more complex than the above mentioned polymers, the photo-oxidative reaction

mechanisms are essentially the same. First, the absorption of light below 400 nm causes the lignin molecules to become excited, and this light absorption is found to be a characteristic of the absorbing molecule's structure. The excitation energy is then dissipated by emissions, radiationless conversions, or chemical reactions. The reactions involve free radical intermediates and include photochemical eliminations, decompositions, polymerizations, substitutions, molecular rearrangements, atomic abstractions, and chain reactions.

Lin and Kringstad (46) have recently described photosensitive groups in lignin and lignin model compounds. They found that the portions of lignin containing saturated propanoid side chains were not sensitive to photoexcitation by light in the 300 to 390 nm range. However, α-carbonyl, biphenyl, and ring conjugated double bonds did become excited when exposed to these wavelengths. They concluded that the α-carbonyl compounds are primarily responsible for photochemical yellowing in lignin.

Leary (43) showed that the yellowing of lignin does not occur in the absence of air. Therefore, he felt yellowing was probably caused by an oxidation process in the presence of light. The yellowing was described as beginning with the oxidation of phenolic hydroxyl groups in the lignin. This oxidation was initiated by hydrogen abstraction from the phenols, following the excitation of an unknown substance which absorbs light near 365 nm.



The products derived from phenoxy radical (II) oxidation are quinones, quinone methides, and cyclohexadienones. These products probably resulted from the decomposition of peroxy-radicals formed by the reaction of oxygen with the phenoxy-radicals (II).

Another theory has been proposed by Kringstad (41) to explain this phenomenon. He felt that the first step in the mechanism was the creation of free-radicals, and these radicals then combined with oxygen as follows:

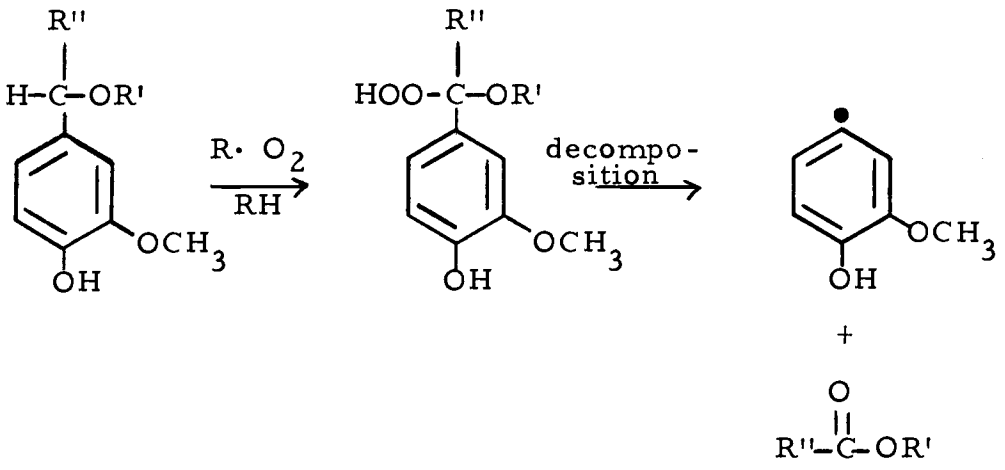
1. $\xrightarrow{h\nu}$ R·
2. R· + O_2 ⟶ ROO·
3. ROO· + RH ⟶ ROOH + R·
4. 2R· ⟶

   ROO· + R· ⟶ Non-radical products

   2ROO· ⟶

This mechanism for the fragmentation of the lignin molecule, and the subsequent formation of quinone derivatives, was in agreement with a previous study by Luner (47). Luner felt that a free radical and

oxygen attack the lignin molecule at the α-position of the aliphatic side chain of the phenylpropane units containing hydroxyl groups. The side chain is then split off resulting in a fragmentation of the molecule. Luner's suggested reaction mechanism is shown below:



According to these reaction mechanisms, a controlled modification of ligninsulfonate by ultra-violet irradiation in the presence of oxygen, should yield materials that are suitable for microbial utilization.

MATERIALS AND METHODS

Photolysis

A type "A" medium pressure 500 watt mercury-vapor lamp (model #673A-36, Engelhard-Hanovia Company, Newark, N. J.), powered by a step-up transformer (model #20651-1, Hanovia Company), was used as the irradiation source. The lamp emitted approximately 29 percent of its radiation between 184.9 and 404.5 nm, 16 percent between 435.8 and 623.4 nm and 55 percent in the infrared region (58).

A double wall quartz immersion well with a 60/50 male ground glass joint (model #19434 Engelhard-Hanovia Company, Newark, N. J.) was used to contain the lamp. Coolant water was circulated through the double wall at a rate sufficient to hold the irradiated solution temperature near 37° C.

The irradiated solution was circulated through a pyrex reaction vessel designed to fit externally to, and integrally with, the immersion well. The reaction vessel was attached by a 60/50 female ground glass joint, and had an inlet tube at the bottom and an outlet tube at the top.

The assembled photolysis vessel (350 ml capacity) was connected to a 4 liter reservoir by Tygon tubing. A centrifugal

polyethylene-housing in-line pump (Chemical Rubber Co., Cleveland, Ohio) circulated the irradiated solution through the photolysis vessel at 8 liters/min. Oxygenation was achieved by passing compressed oxygen gas through a flowmeter (#10, Gilmon Instruments, Inc., Great Neck, New York) and then through a fine pore glass sparger situated at the reservoir outlet.

Irradiated Substrate Preparation

Irradiation studies were carried out with Marasperse CB, a desugared, partially desulfonated, sodium-exchanged lignin-sulfonate (American Can Company, Nennah, Wisconsin). The following analysis of this material was given by the company:

pH-3% solution	8.5-9.0
Total sulfur as S (%)	1.70
Sulfate Sulfur as S (%)	trace
Sulfite Sulfur as S (%)	none
CaO (%)	0.20
MgO (%)	0.02
Na_2O (%)	10.00
R_2O_3 (%)	trace
Reducing Sugars (%)	none
OCH_3 (%)	12.64

Sodium ligninsulfonate concentrations of 0.1% W/V were utilized throughout this study.

The lignan, alpha-conidendrin (K and K Laboratories, Inc., Plainview, N. Y.) was irradiated at a 0.1% W/V concentration. Solubilization was achieved by adjusting the pH to 10 with 1N NaOH to form the sodium salt of alpha-conidendric acid.

Unless otherwise noted, two liter volumes were added to the external reservoir and circulated through the system. The desired oxygen flow-rate (usually 2 ml/min.) was allowed to equilibrate with the solution to be irradiated at least five minutes prior to firing the lamp. Foam accumulation in the reservoir was prevented by directing the incoming solution parallel to and below the surface of the solution where rapid swirling action broke up the foam.

Each irradiation was taken to the desired endpoint, the solution collected in a chemically clean dry flask, and stored at 4° C until further use. The entire system was then washed thoroughly with distilled water and air dried. Periodically the system was dismantled, cleaned in chromic acid, washed with detergent, rinsed in distilled water and dried.

Spectrophotometry

Chromophoric changes in irradiated ligninsulfonate and alpha-conidendrin were monitored on a spectrophotometer

(spectronic 20, Bausch and Lomb, Rochester, N. Y.) set at 430 nm.

Determination of the phenolic content of fractions from a Sephadex G-25 column was accomplished with a Zeiss Spectra Photometer PMQ II (Carl Zeis, West Germany).

pH

The pH of irradiated samples was monitored on a Corning Model 5 pH meter (Scientific Instruments, Medfield, Mass.) with a 8 mm combination electrode #9007 (Broadley-James-Corp., Fullerton, Calif.).

Total Carbon Analysis

Total organic carbon (TC) analysis was used to follow the fate of carbon during photolysis and bacterial growth. No significant TC differences were noted between samples acidified to pH 2 and purged with N_2 (atmospheric CO_2 elimination) and samples combusted to CO_2 directly, as long as a pH of 10 or less was maintained.

A total carbon analyzer was constructed by Robert C. Rockhill (64) and the parts list, in order of their integration into the TC unit, consisted of the following:

1. Oxygen flowmeter and needle valve (0-2.5 liters/min.) (FEP Co. #91-150-70, LECO, St. Joseph, Mich.).
2. Injection port, consisting of a 75 mm, 18 gauge needle fastened

with epoxy cement to an 8 mm OD inlet tube connected to a quartz combusion tube of 15 mm diameter and 420 mm length. The combustion tube contained sequentially, asbestos and platinum mesh catalysts (custom made) (64).

3. Pyrometer controller model 293 (Wheelco Instruments Division, Barber-Coleman Co.).
4. Electric furnace-type 77 (Hevi-Duty Electric Co., Milwaukee, Wis.).
5. U-tube moisture condenser (custom made).
6. High efficiency small volume filter, particles filtered down to 0.3 microns (#DZ-81740, Mine Safety Appliance Co., Pittsburgh, Pa.).
7. LIRA infrared analyzer, model 300 (Mine Safety Appliance Co., Pittsburgh, Pa.) standardized at 0-600 μg/ml CO_2.
8. Servo-rite recorder, model PWD (Texas Instruments Co., Inc., Houston, Texas) 0-5 MV input.

Twenty micro-liter samples were injected through the injection port using a 3-inch long, 22 gauge needle on a #705 50 μl Hamilton syringe (Hamilton Co., Whittier, Calif.). Immediately upon injection, carbon in the samples was oxidized to CO_2 in the furnace held at 900° C. An oxygen flow-rate of 500 ml/min. was maintained, and samples could be injected every 60 seconds. Three injections were made per sample and their values were averaged.

A warm-up period of at least two hours was required for the LIRA unit and recorder to stabilize.

Standards, prepared from glacial acetic acid, ranged from 10 to 1000 μg/ml of carbon. A straight line standard curve was obtained with these standards by plotting carbon concentration (μg/ml) versus LIRA meter readings.

Gas-Liquid Chromatography (GLC)

GLC analyses were performed on a Varian Aerograph Model 1200, equipped with a hydrogen flame ionization detector (Varian Aerograph, Walnut Creek, Calif.). A Barber-Coleman strip-chart recorder Model 8000-2600 (Wheelco Instruments Div., Barber-Coleman Co., Rockford, Ill.) monitored the results. The standard chromatographic conditions were as follows:

Injector temperature = 220° C

Detector temperature = 225° C

Isothermal column temperature = 180° C

H_2 = 55 ml/min.

He = 50 ml/min.

Air = 190 ml/min.

sample size = 2 μl

A 4 foot, 1/8 inch stainless steel column was prepared as described in a personal communication from Patricia Maglothin (48).

The column material was prepared by dissolving 0.2% W/V ethylene glycol succinate (EGS) and 0.2% W/V ethylene glycol adipate (EGA) in chloroform and mixing this with an equal portion of 0.4% W/V GE XF1150 dissolved in acetone. This mixture was added to the Gas-Chrom P (100/120 mesh) solid support (Applied Science Laboratories, Inc., State College, Pa.).

Derivatives were prepared by an alditol-acetate procedure (8) which was modified and is shown below:

Modified alditol-acetate procedure--

1. Dilute sample to 5 ml.
2. Adjust pH to 5.5.
3. Add 0.015 gm $NaBH_4$ + 0.1 ml myoinositol (10 mg/ml) internal standard.
4. Let stand for 30 minutes at room temperature.
5. Remove excess $NaBH_4$ by adding glacial acetic acid dropwise until evolution of H_2 ceases.
6. Evaporate to dryness or near dryness on rotary evaporator (custom made).
7. Add 10 ml methanol, stir, and evaporate to dryness. Repeat twice.
8. Dry in oven at 100-110° C for 10 minutes (Precision Thelco, Model 17, Precision Scientific Co., Chicago, Ill.).
9. Add 10 ml acetic anhydride and about 0.5 gm of sodium acetate.

Reflux at 140° C for 20 minutes.

10. Cool swiftly.
11. Evaporate to dryness on rotary evaporator.
12. Add about 5 ml dichloromethane ($MeCl_2$) and stir. Centrifuge 30 minutes at 13,000 × G (Servall Superspeed Centrifuge, Type 553, Ivan Sorvall, Inc., Norwalk, Conn.). Repeat twice.
13. Pool the $MeCl_2$ solutions and evaporate to 1 ml.

Samples taken from the Sephadex G-25 column were derivatized as such while small amounts of model phenolic compounds (Aldrich Chemical Co., Inc., Milwaukee, Wis.) were diluted to 5 ml with distilled water. This derivatization procedure and column were primarily designed for carbohydrate identification. However, they were successfully utilized in this study for phenolic identification.

Thin-Layer Chromatography (TLC)

TLC determinations were performed from ether extracts of irradiated and unirradiated ligninsulfonate solutions. The ether preparations were obtained by extracting 100 ml of ligninsulfonate with three volumes of diethyl ether after acidification to pH 2 with 1N HCl. Each extraction was performed by vigorously mixing the two phases in a separatory funnel for one hour. After separation, the ether was evaporated off at 30° C in vacuo and the remaining dry residue was dissolved in 2 ml of 95 percent ethanol. This was

then used for TLC application.

Thin layer plates were prepared by spreading 50 percent W/V silica gel G (American Optical Corp., #08075) in water suspension on 8 inch by 8 inch glass plates to a thickness of 0.250 nm. The plates were allowed to air dry for two hours and then activated at 105° C for 30 minutes (61). About 5 mm of the silica gel was trimmed off each edge of the plate in order to obtain a more uniform solvent front and better Rf reproducibility.

Solvent systems employed in this study were benzene/ethanol (150:22), benzene/acetone (3:2) and methanol/chloroform (3:7) (5). One-hundred ml volumes of each system were added to 4.5 × 9 × 9 inch rectangular glass tanks closed with glass lids (American Optical Corp., Model 02041, Richmond, Calif.). Tank saturation was maintained by immersing two 7 × 7 inch and two 4 × 7 inch pieces of Whatman #1 filter paper in the solvent, and placing them against the sides of the tank with the lower edge of the papers touching the bottom of the tank.

Sample volumes of 20 μl were applied 5 mm above the edge of the adsorbent and dried with forced air. The ascending method of development was utilized, and the solvent front was allowed to travel approximately ten cm above the point of application. The TLC plates were taken from the tank and air dried for at least five hours in a fume hood before spraying.

The tetrazotized benzidine spray reagent used to characterize phenolic compounds was prepared as follows (8):

Solution I: 5 gm benzidine dissolved in 14 ml concentrated HCl and diluted with H_2O to 1 liter.

Solution II: Aqueous 10% sodium nitrite.

Equal volumes of the two solutions were mixed immediately before use. This mixture could be kept for 2 to 3 hours. After spraying, the plates were placed in the drying cabinet at 105° C for a few minutes until the spots were clearly visible.

Gel Filtration

Molecular weight fractionation of ligninsulfonate can successfully be accomplished with a Sephadex G-25 gel (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) (21, 39, 51). This gel has an exclusion molecular weight of 5,000 and a fractionation range of 5,000 to 100. It has been reported that certain compounds, especially low molecular weight aromatics, are adsorbed to Sephadex gels (25). Also, acids tend to be prematurely eluted from Sephadex columns, but this can be avoided when they are eluted with a salt solution (25).

In this study, irradiated ligninsulfonate solutions were concentrated by lyophization (Virtis Research Equipment, No. 10-145

MR-BA, Gardner, N. Y.) and distilled water was added to give a 50% W/V concentration. The column characteristics and fractionation conditions were as follows:

Characteristics--

K 15 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.)

G-25 Sephadex

30 cm long

48 ml bed volume

Automatic fraction collector (LKB Ultrovac Fraction Collector, Type 7000, LKB-Producter AB, Stockholm-Bromma-1, Sweden).

Conditions--

Flow rate = 0.5 ml/min.

Sample size = 0.5 ml (50% W/V concentration)

Eluent = 0.03 M NaCl, pH 11

Phenolic binding prevented fractionation of a 50% W/V concentration of unirradiated ligninsulfonate, so a 1% W/V concentration was fractionated. The results were then multiplied by 50 in order to compare them with irradiated ligninsulfonate values.

Growth Characterization

The mineral salts medium utilized in this study is shown below:

Compound	Concentration (gm/liter)
$MgSO_4$	0.2
$CaCl_2$	0.02
KH_2PO_4	1.5
K_2HPO_4	3.0
NH_4NO_3	1.0
$FeCl_3$	0.008

Enrichments and routine transfer of isolated bacteria were performed on 2 percent agar plates containing equal portions of mineral salts and irradiated ligninsulfonate (or model phenolic compounds).

Unless otherwise noted, all growth responses were monitored by nephelometry (Nepho-colorimeter, Model 9, Coleman Instruments Corp., Maywood, Ill.). Culture tubes were prepared by adding 5 ml of carbon source and 2 ml of mineral salts to 18 × 150 mm disposable culture tubes (Pyrex Laboratory Glassware, Corning Glass Works, Corning, N. Y.) which were placed on a modified Eberbach reciprocal shaker (156 cycles/min.). After growth, the cells were removed by centrifugation at 12,000 × G for 20 minutes (Servall Superspeed Centrifuge, Type 553, Ivan Sorvall, Inc., Norwalk, Conn.).

The Pseudomonas putida isolate utilized in this study has the

American Type Culture Collection (ATTC) number of 21487 assigned to it. The laboratory identification number 29-43-1 will be used throughout this study to identify this organism.

The culture system was evaluated to insure optimal growth responses and valid carbon utilization data. The pH optimum for P. putida (29-43-1) growth was between pH 6 and 7, and no growth was observed below pH 5 or above pH 10. The culture tube aeration was not found to effect growth. The inoculation procedure, unless otherwise noted, consisted of washing cells from a 48-hour-old irradiated ligninsulfonate plate 3 times with a sterile mineral salts solution. One drop from a sterile 1 ml pipette, at a 50 to 100 nephelo unit concentration of cells, was used to inoculate each growth tube. Neither the washing steps or the inoculum size affected the final amount of growth.

Pseudomonas putida (29-43-1) was not found to adsorb irradiated ligninsulfonate. This was determined by adding 200 nephelo unit concentrations of cells (in mineral salts) to an equal volume of irradiated ligninsulfonate, shaking for 1 minute, and then centrifuging out the cells. The resulting total carbon values were compared to uninoculated controls and found to be identical.

RESULTS AND DISCUSSION

Enrichment Studies with Irradiated Ligninsulfonate

The first step in this study was to isolate bacteria capable of utilizing irradiated ligninsulfonate as a sole source of carbon and energy. Since the unirradiated ligninsulfonate (Marasperse CB) failed to support bacterial growth, the isolation of these organisms confirmed the feasibility of this particular photolytic process.

Initially a large number of organisms were isolated from enrichment cultures containing phenolic monomers. These monomers (vanillin, vanillyl alcohol, protocatechuic acid, cinnamyl alcohol, and syringic acid) were related to the structural sub-units of lignin. However, the isolates enriched for in this way could not be transferred on irradiated ligninsulfonate media. Therefore, a direct relationship between monomeric lignin sub-units and irradiated ligninsulfonate could not be demonstrated. Next, enrichments with irradiated ligninsulfonate were undertaken. This time a number of bacteria were successfully isolated which could utilize irradiated ligninsulfonate as a sole carbon and energy source.

Organism #29-43-1 adapted particularly well to this substrate and was utilized throughout this study. This organism was an exceptionally large colony picked from an irradiated ligninsulfonate agar

transfer of organism #29-35-2. Organism #29-35-2 was obtained from an Agricultural Hall (O.S.U.) soil sample. The bacterium #29-43-1 was subsequently identified as belonging to the species Pseudomonas putida (10).

Substrate Optimization

To evaluate this irradiation system, a constant and reproducible substrate was necessary. However, before this substrate could be obtained, certain aspects of the system had to be considered. It was noted that the photolytic process was dependent only on the number of molecules to be irradiated, so increasing concentration or volume variables simply increased the length of the irradiation time. A convenient volume of 2 liters at an initial 0.1% W/V lignin-sulfonate concentration was found to produce a desirable substrate in approximately 24 hours. Consequently, the two parameters which affect the photolysis process tested in this study were the oxygen flow-rate and the initial pH of the ligninsulfonate solution.

Since the photolytic process analyzed here is an oxidative one, the amount of oxygen purged through the system is critical. Also, the photolysis process is highly pH dependent, due to its reliance on hydrogen atom abstraction in its free-radical mechanism of action. In this study it was assumed that the optimal O_2 flow-rate for any given pH would be the optimal flow-rate for all other pHs.

It was also assumed that the optimal pH for one O_2 flow-rate would be the optimal pH for all O_2 flow-rates.

The photolytic process was monitored by periodically testing samples for absorbance at 430 nm, total carbon and pH. At first, the effect of the oxygen flow-rate on photolysis was determined. Then the flow-rate producing the best growth-supporting substrate was established. This flow-rate was used to determine the effect of alterations in the pH of ligninsulfonate prior to irradiation. The pH producing the best growth response was then determined. Finally, the ability of the photolysis system to produce a constant reproducible substrate was evaluated.

The dependence of photolysis on the oxygen flow-rate is shown in Figures 1 through 4. The changes in parameters used to monitor the photolytic process was severely retarded when the system was not purged with oxygen. Figure 1 shows that there is an early increase in chromophore content at the intermediate flow-rates. However, 2 ml/min. or 12 ml/min. flow-rates produce a much earlier decrease in abosrobance with little or no early increase in chromophores. Figure 2 indicates that there is a similar decrease in pH at all flow-rates. However, the 2 ml/min. flow-rate does not show the increase in pH characterized by the other flow-rates. Possibly this pH stability is due to the inability of low flow-rates to oxidize the acids formed during the photolytic process. The total

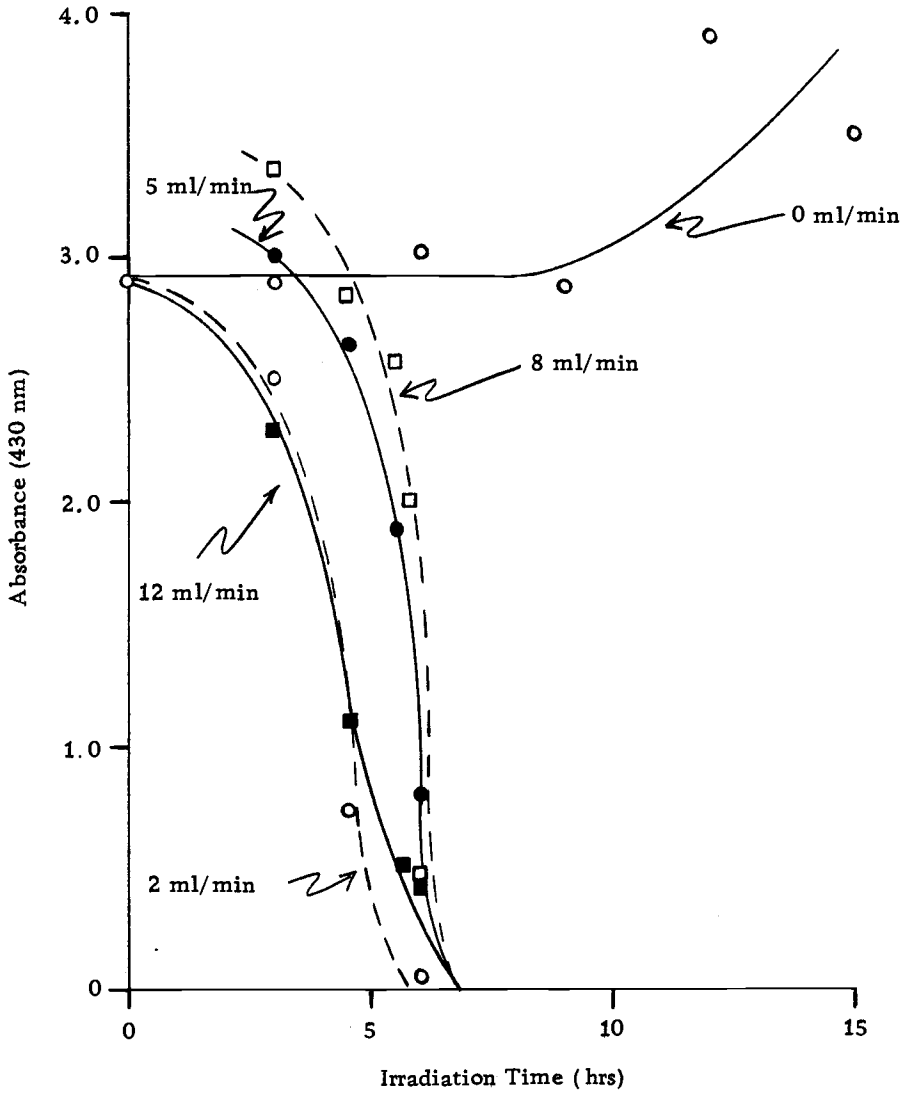


Figure 1. Effect of oxygen flow-rate on the irradiation of ligninsulfonate as monitored by the absorbance at 430 nm

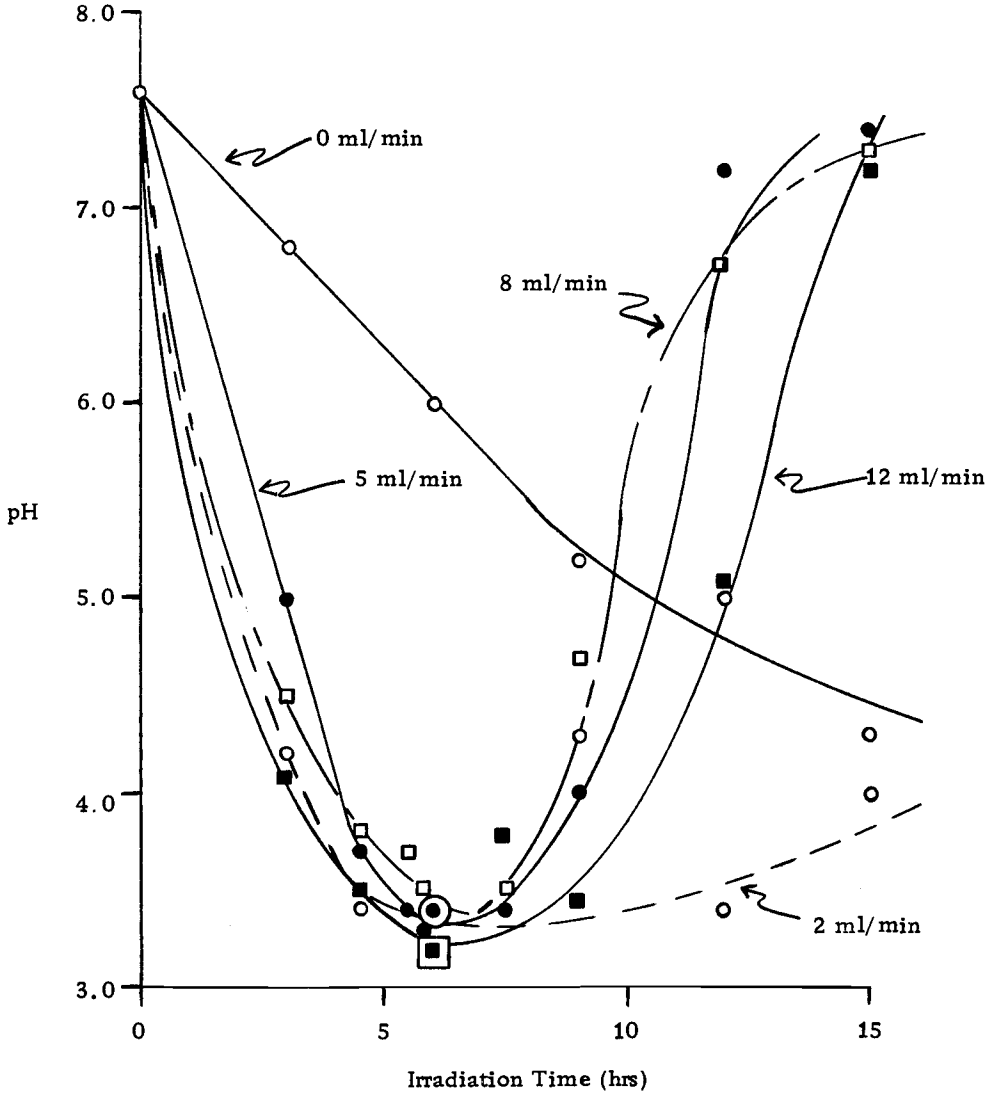


Figure 2. Effect of oxygen flow-rate on the irradiation of ligninsulfonate as monitored by pH

carbon decrease as seen in Figure 3 is independent of the oxygen flow-rate. When this is considered along with the dependence of absorption and pH on oxygen flow-rates, it can be concluded that the various flow-rates produce different species of breakdown products rather than different amounts of breakdown products. This is substantiated in Figure 4 which shows that the 2 and 12 ml/min. flow-rates produce substrates which support a larger amount of growth. As has been noted, these are the flow-rates that cause the most rapid decrease in absorbance. Since the chromophore content increases shortly after the initiation of the irradiation process at the intermediate flow-rates, the possibility exists that a net polymerization took place here. This increase in polymerization could increase the molecular weight and amount of branching of lignin-sulfonate. Further photolytic treatment might then produce products that differ somewhat from the products obtained without this initial polymerization. Apparently the low flow-rate does not provide sufficient oxygen to initiate this early polymerization. On the other hand, the high flow-rate possibly provides so much oxygen that the polymerization process is more than counteracted by the degradation process.

The 2 ml/min. flow-rate was utilized in all subsequent experiments since it provided a substrate comparable to that obtained with the 12 ml/min. rate, and also produced a much more stable final pH.

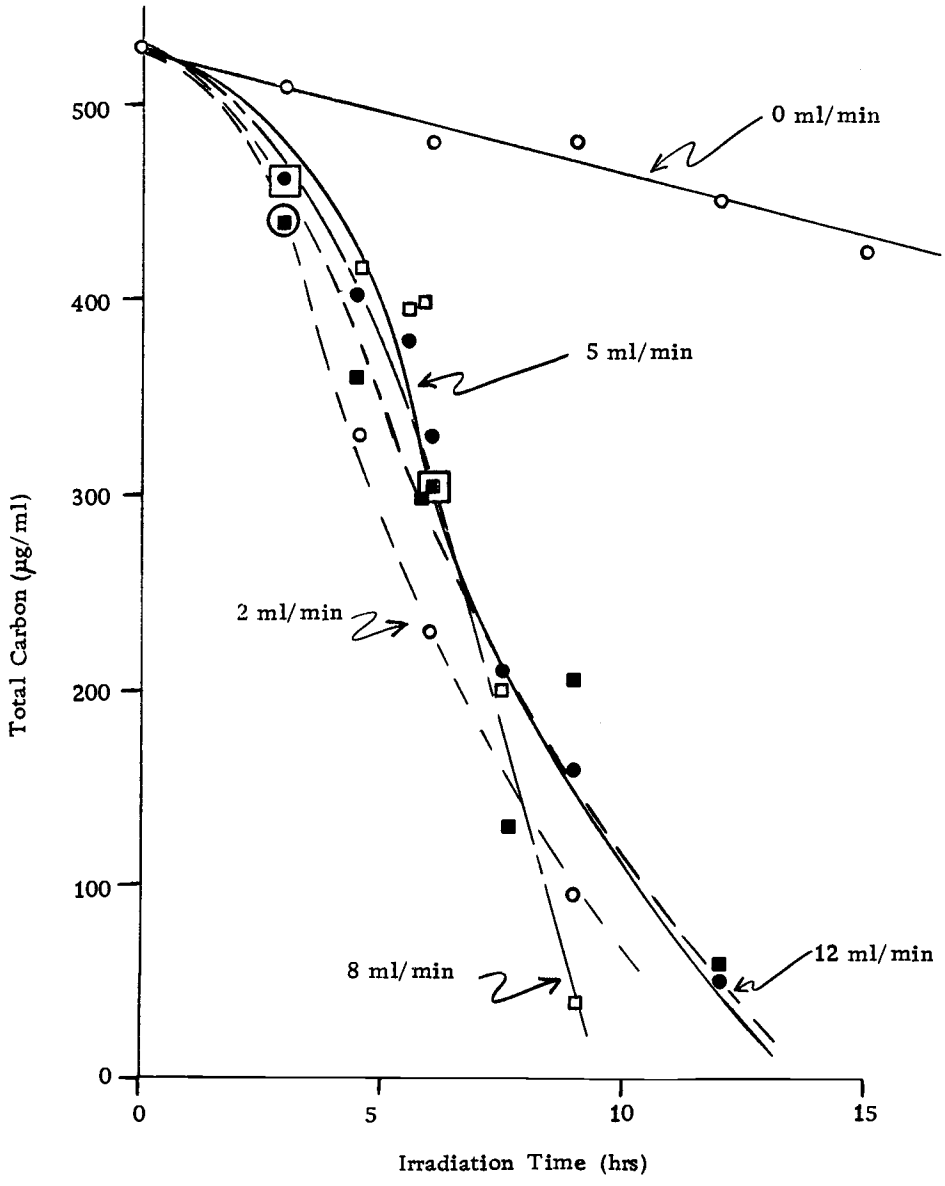


Figure 3. Effect of oxygen flow-rate on the irradiation of ligninsulfonate as monitored by total carbon

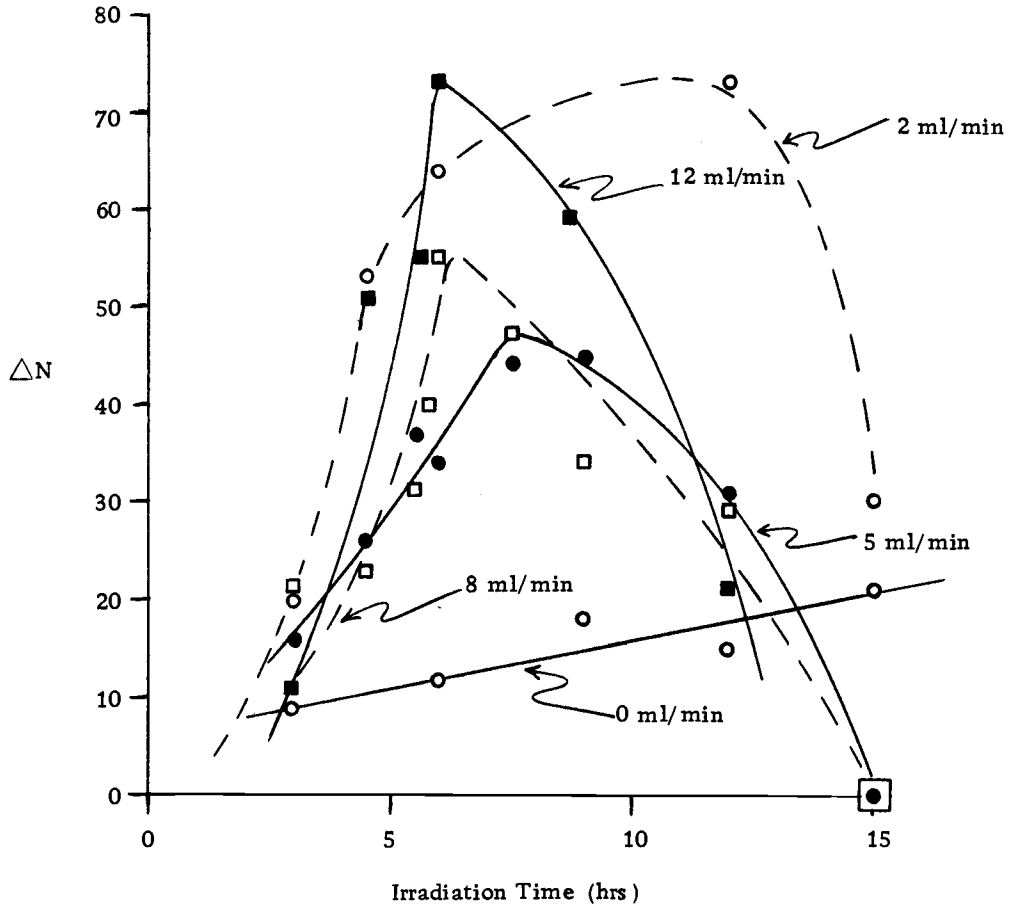


Figure 4. Effect of oxygen flow-rate on the amount of growth of P. putida (29-43-1) as monitored by nephelometry

Next, the optimal initial pH of the ligninsulfonate solution prior to irradiation was determined. As shown in Figure 5, the lower the pH the more rapid is the loss in absorbance. This is accompanied by a corresponding loss of carbon as shown in Figure 7. The suggested reaction mechanism for this phenomenon involves the phenolic hydroxyl groups which must be protonated in order for hydrogen abstraction to initiate the free-radical degradative process. An obvious excpetion to the pH effect is at pH 12. Here the high pH itself causes phenoxy radicals to be formed. This then aids in the degradation process by further increasing the number of free radicals. Figure 6 indicates that the initial ligninsulfonate pH has little effect on the pH change during the irradiation process. The pH starts dropping immediately upon irradiation and decreases at about the same rate in all cases. The higher pH values are seen to drop 4 to 5 pH units.

The growth responses on the various irradiated samples of differing initial pH values are shown in Figure 8. In addition to the adjusted pH values used in other portions of this experiment, an unadjusted solution (pH 7.6) was tested. This unadjusted solution, after irradiation, produced as high a level of growth as the best adjusted solution (pH 7). The lack of growth on the irradiated pH 2 ligninsulfonate solution was due to a pH effect. The reason for this was that the buffer capacity of the mineral salts in the growth tubes

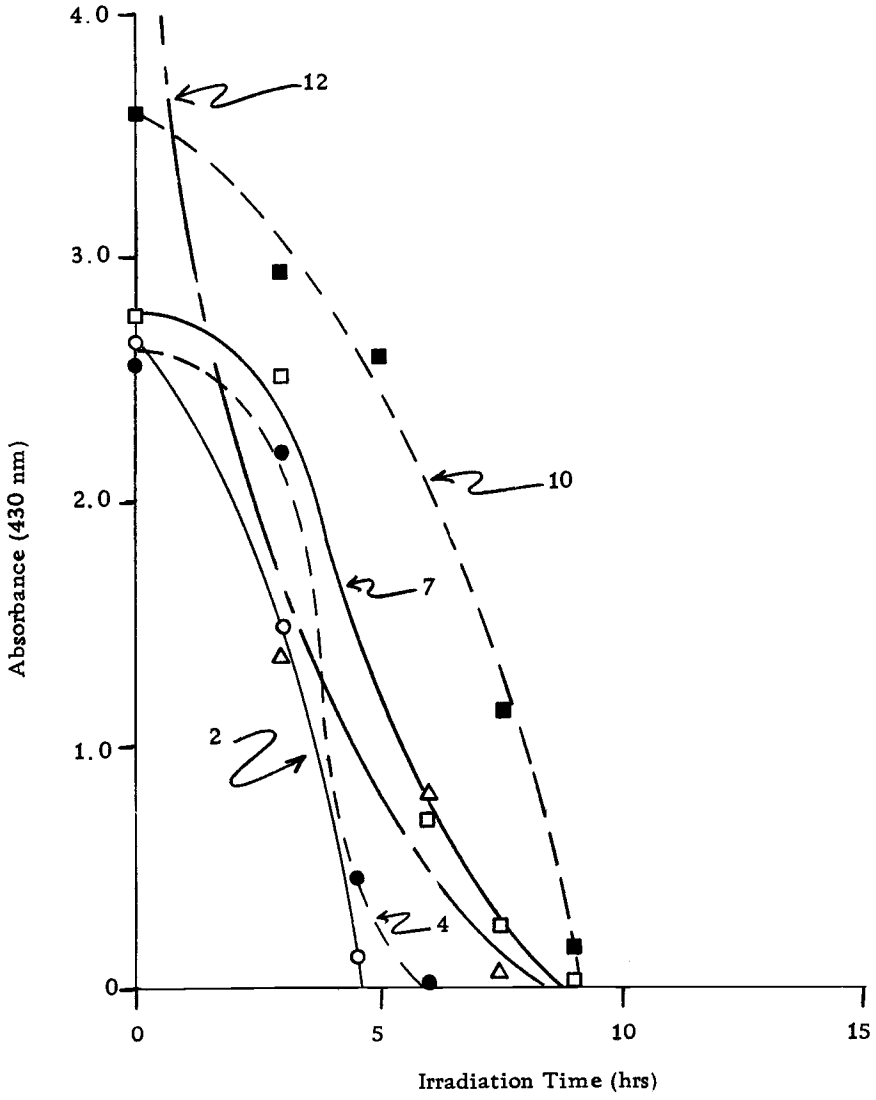


Figure 5. Effect of initial pH on the irradiation of ligninsulfonate as monitored by absorbance at 430 nm

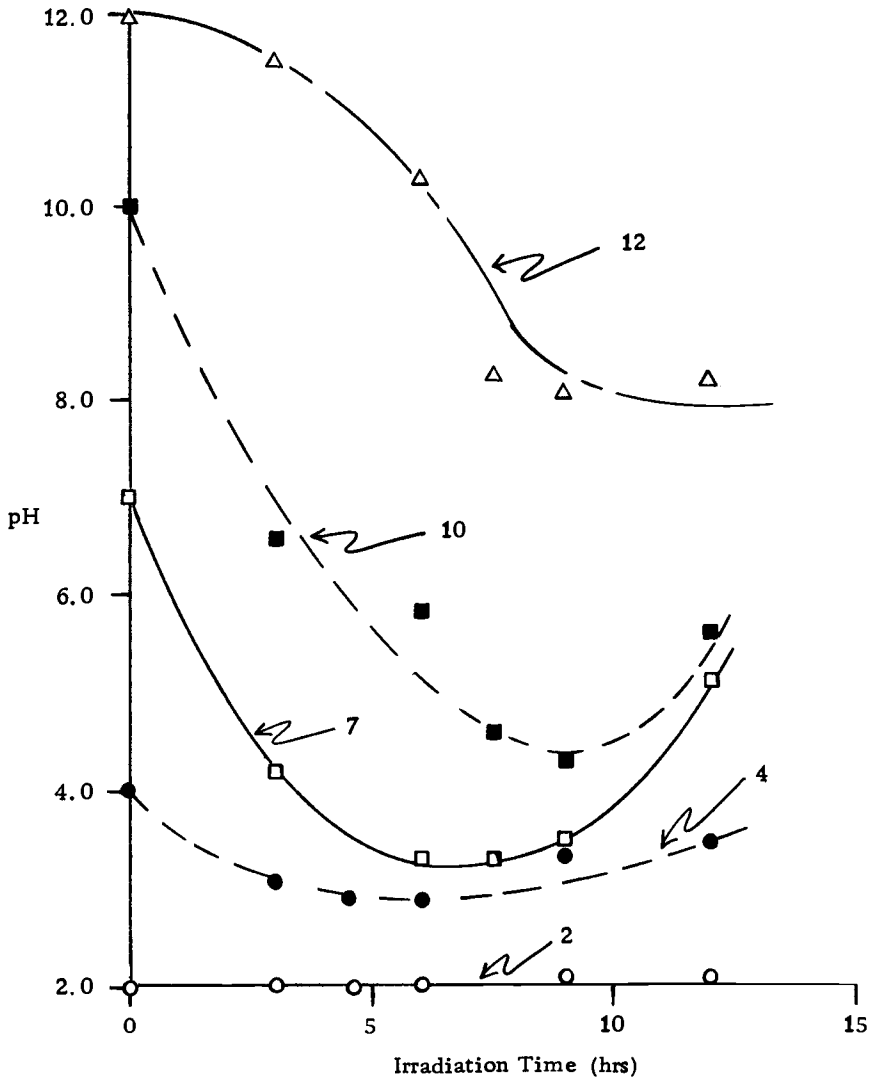


Figure 6. Effect of initial pH on the irradiation of ligninsulfonate as monitored by pH

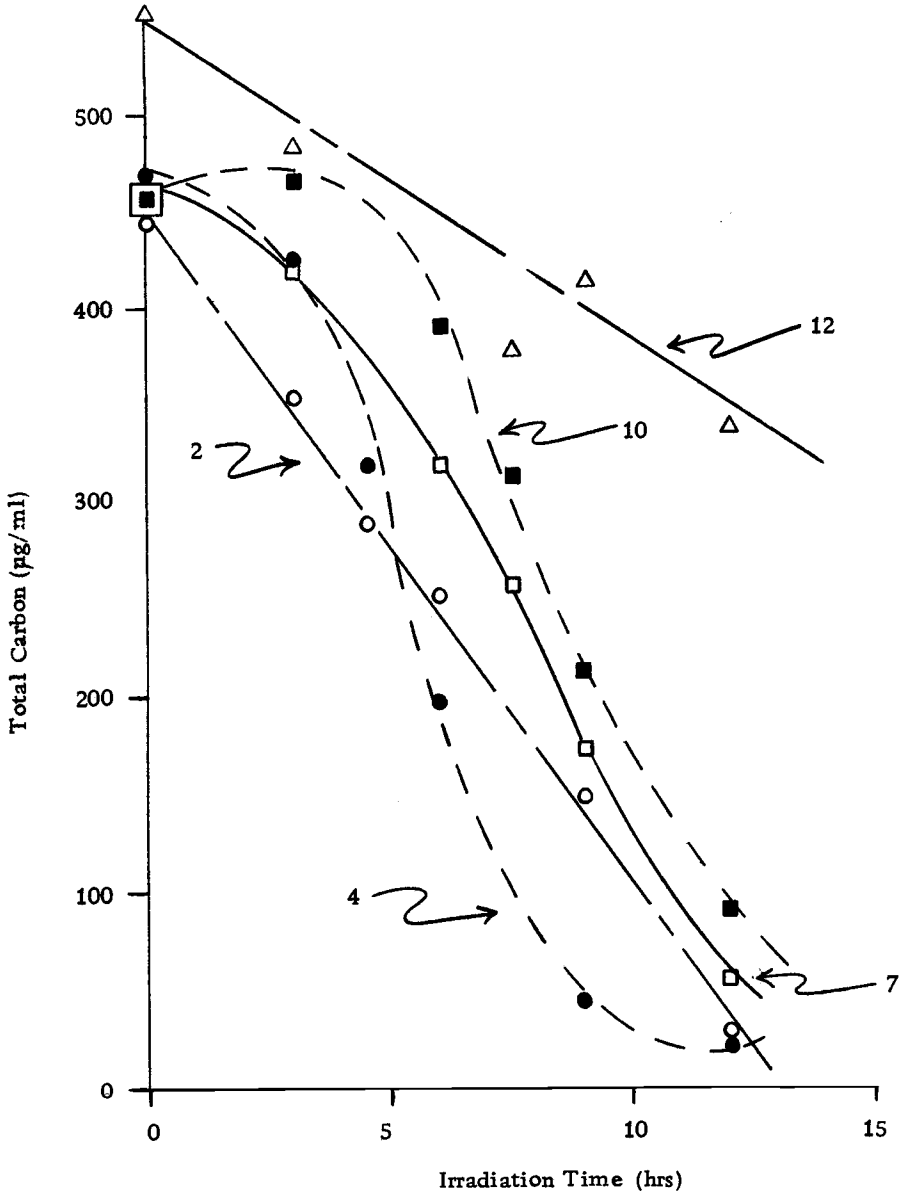


Figure 7. Effect of initial pH on the irradiation of ligninsulfonate as monitored by total carbon

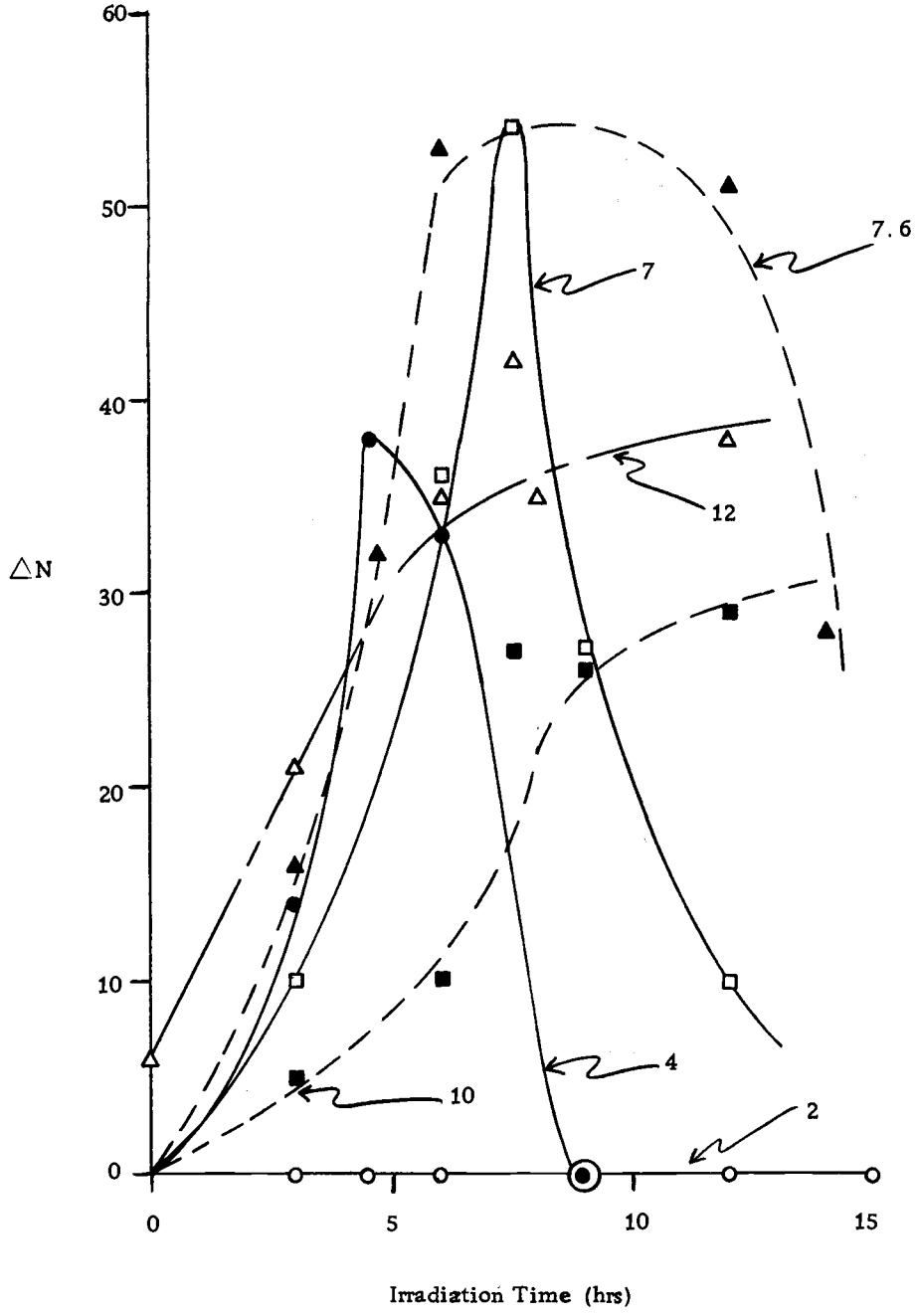


Figure 8. Effect of initial pH on amount of growth of P. putida (29-43-1) as monitored by nephelometry

was too low to properly adjust the medium's pH.

As a result of these experiments the optimum irradiation conditions for a 0.1% ligninsulfonate solution were an oxygen flow-rate of 2 ml/min. along with no pH adjustment. The ability of these standard conditions to give a reproducible substrate is shown in Figure 9. The most efficient method of determining the completion of the irradiation process is to monitor the irradiated ligninsulfonate absorbance at 430 nm. Figure 9 shows that there is a wide range of optical density values which give a rather constant substrate. Any values between 0.05 and 0.70 contain similar carbon levels and have similar pH values. Also, the amount of growth and carbon utilized over this range are constant.

Characterization of Irradiated Ligninsulfonate as a Microbial Substrate

Due to the extensive control mechanisms operative in P. putida grown on phenolic compounds, it was felt necessary to determine the effects of various concentrations of irradiated ligninsulfonate on growth. In order to determine this, the amount of growth (ΔN), the decrease in carbon after growth, the % of carbon utilized, and the length of the lag period were monitored at various concentrations of the irradiated ligninsulfonate substrate (Figure 10).

The growth increase (ΔN) is linear up to the 50% (V/V) concentration. At this point something becomes limiting. This increase

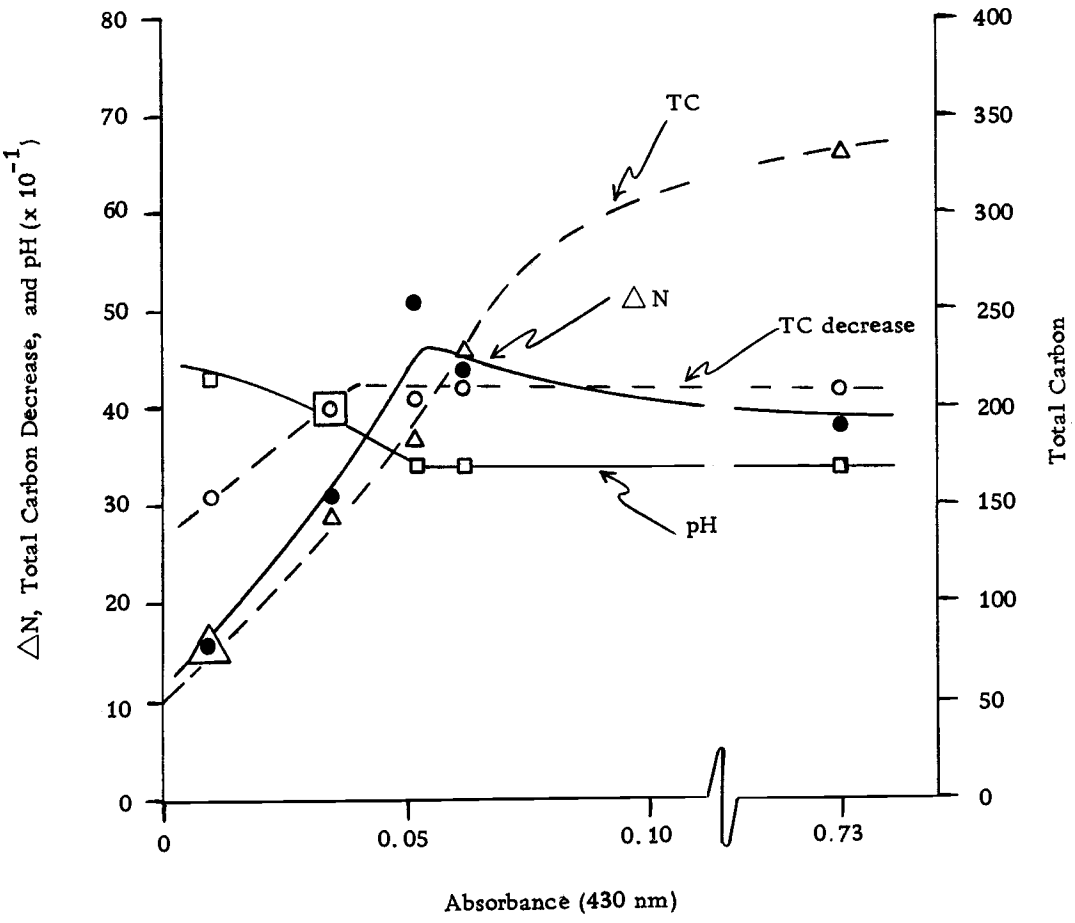


Figure 9. Characterization of optimal conditions
Total carbon = carbon remaining after irradiation
△N = amount of growth (nephelometry)
Total carbon decrease = amount of carbon utilized during growth

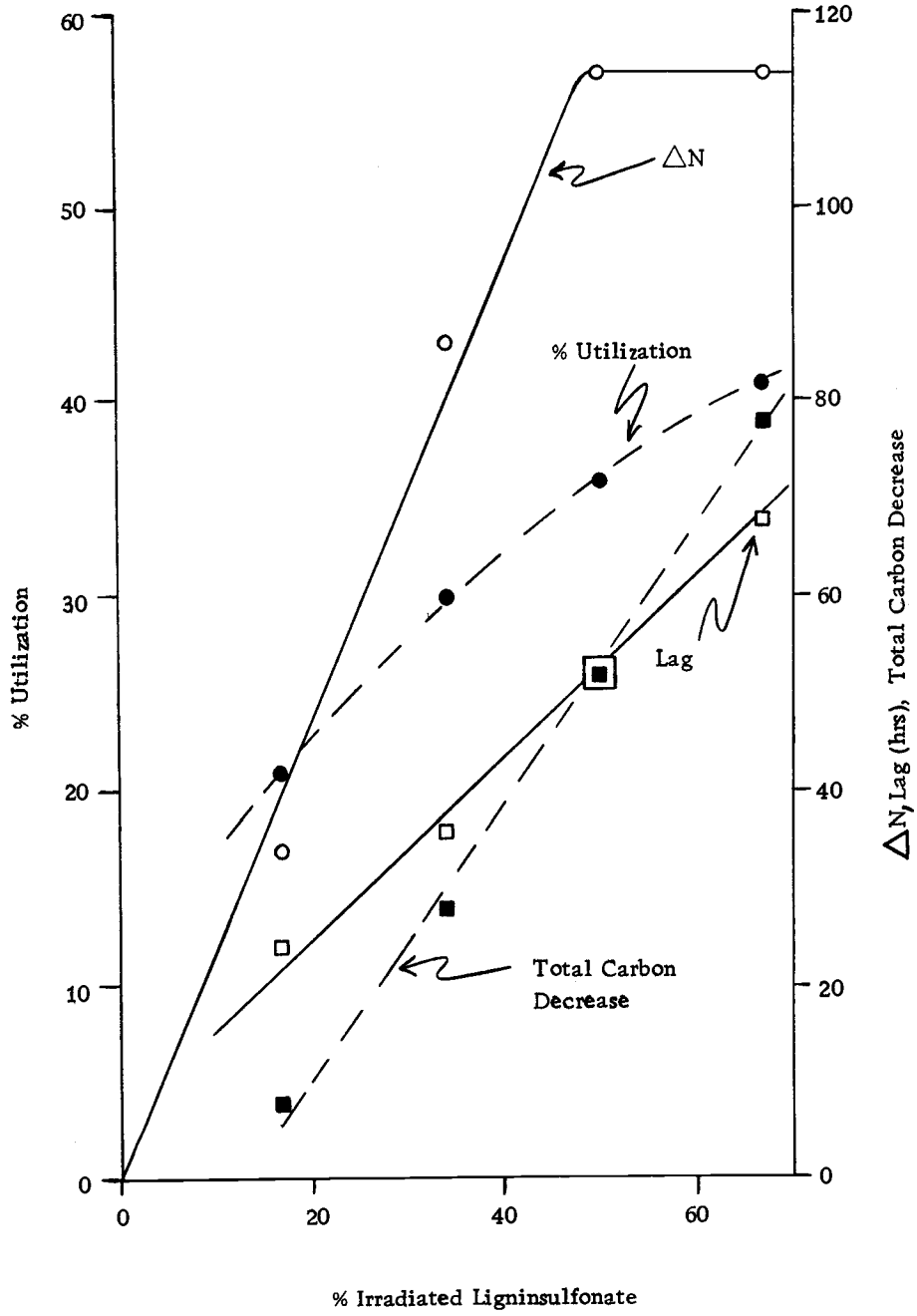


Figure 10. Effect of substrate concentration on growth of P. putida (29-43-1) (irradiated ligninsulfonate)

in growth is found to be more rapid than the increase in substrate concentration. In addition, there is a linear increase in the amount of carbon utilized during growth, and this increase is also more rapid than the increase in substrate concentration. These relationships seem to indicate an induction of enzymes capable of degrading components in irradiated ligninsulfonate. This statement is based on the fact that as the substrate concentration is increased, the extent of induction also increases. This increased formation of enzymes then improves the efficiency of substrate utilization and hence, a greater than proportional amount of growth ensues.

Although the utilization efficiency improves with increasing substrate concentration, an increase in lag time also occurs. This increased lag time is directly proportional to the increase in substrate concentration. Certain phenolic monomers will be shown later to cause similar increases in lag time with increases in concentration. Also, the large molecular weight irradiated ligninsulfonate molecules may be exerting some type of inhibitory effect. This latter possibility will be discussed next.

The effect of large molecular weight ligninsulfonate molecules on the utilizable components in irradiated ligninsulfonate can be demonstrated by adding unirradiated ligninsulfonate to the irradiated material. By varying the proportions of irradiated to unirradiated ligninsulfonate, the effect on growth of the large molecular weight

components in the unirradiated material can be shown. Figure 11 demonstrates that the effect of unirradiated ligninsulfonate is definitely an inhibitory one. This experiment does not necessarily prove that inhibitory components in the irradiated ligninsulfonate solution are large molecular weight material, but it suggests this possibility exists. The significant point in Figure 11 is that unirradiated ligninsulfonate is able to inhibit growth of P. putida by its mere presence. Therefore, the recalcitrance of ligninsulfonate is not solely due to the inability of P. putida to degrade it, but is contributed to by its action as a growth inhibitor. Thus, even though degradable molecular weight ranges are present in the unirradiated material, P. putida is not able to utilize them. It can therefore be concluded that the photolytic process not only produces a suitable substrate for P. putida, but also relieves a great deal of the inhibitory effects of the original material.

In an attempt to relieve the inhibition caused by the unirradiated ligninsulfonate, 500 ml volumes were irradiated for very short periods of time before additions were made to the irradiated ligninsulfonate substrate. It was felt that this procedure could possibly open up the large molecular weight polymers thereby permitting greater utilization of the degradable material present. However, these short periods of irradiation turned out to have no effect on the inhibitory properties of the ligninsulfonate.

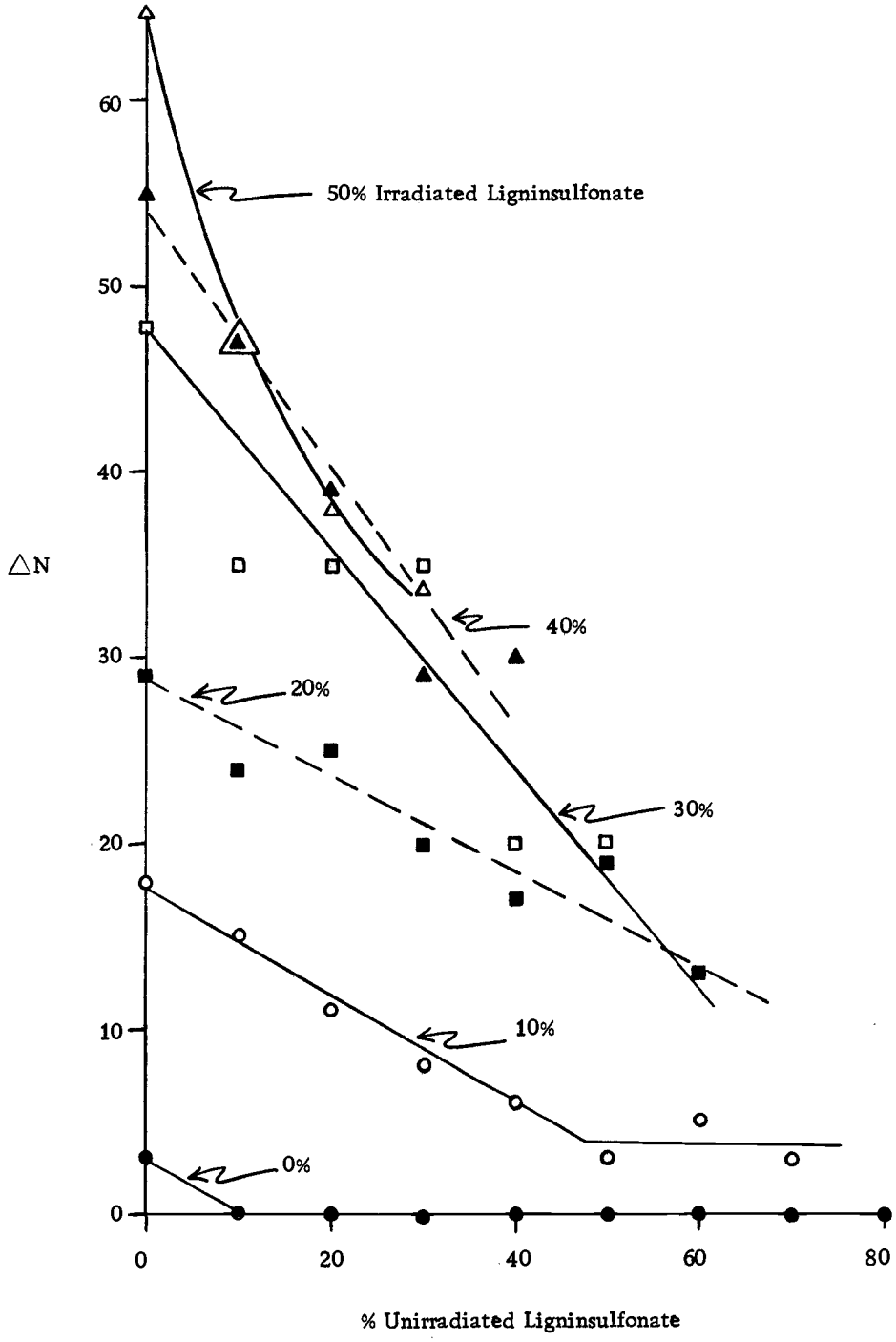


Figure 11. Growth responses to varying combinations of irradiated and unirradiated ligninsulfonate

One benefit derived from this study on short periods of irradiation was additional evidence of the importance of protonation in the free-radical photo-oxidation process. Figure 12 shows that at the beginning of the photolytic process the pH is slightly alkaline. Then during the first hour of irradiation the pH is seen to drop slightly and the absorbance steadily decreases. The pH drops below 7 after one hour and the absorbance rapidly increases. The peak of absorbance at 1 1/2 hours is probably due to free-radical polymerization which soon becomes compensated for by a free-radical degradation process. The photo-oxidation process lowers the pH, creating an increased availability of protons that are required for free-radical formation by hydrogen abstraction. The rapid drop in pH upon irradiation is probably due to the splitting off of sulfonate groups and the subsequent formation of sulfuric acid.

To determine which components in the irradiated ligninsulfonate solution are inhibitory and which are utilizable, a molecular weight fractionation was performed. Two liters of 0.1% W/V ligninsulfonate were irradiated and then freeze-dried by lyophilization. The lyophilized sample was concentrated to 50% W/V and then a portion of this was fractionated on a Sephadex G-25 column. Sephadex G-25 is known to exclude all molecular weights greater than 5000. Figure 13 compares the fractionation profiles of unirradiated and irradiated ligninsulfonate. When a 50% W/V concentration of unirradiated

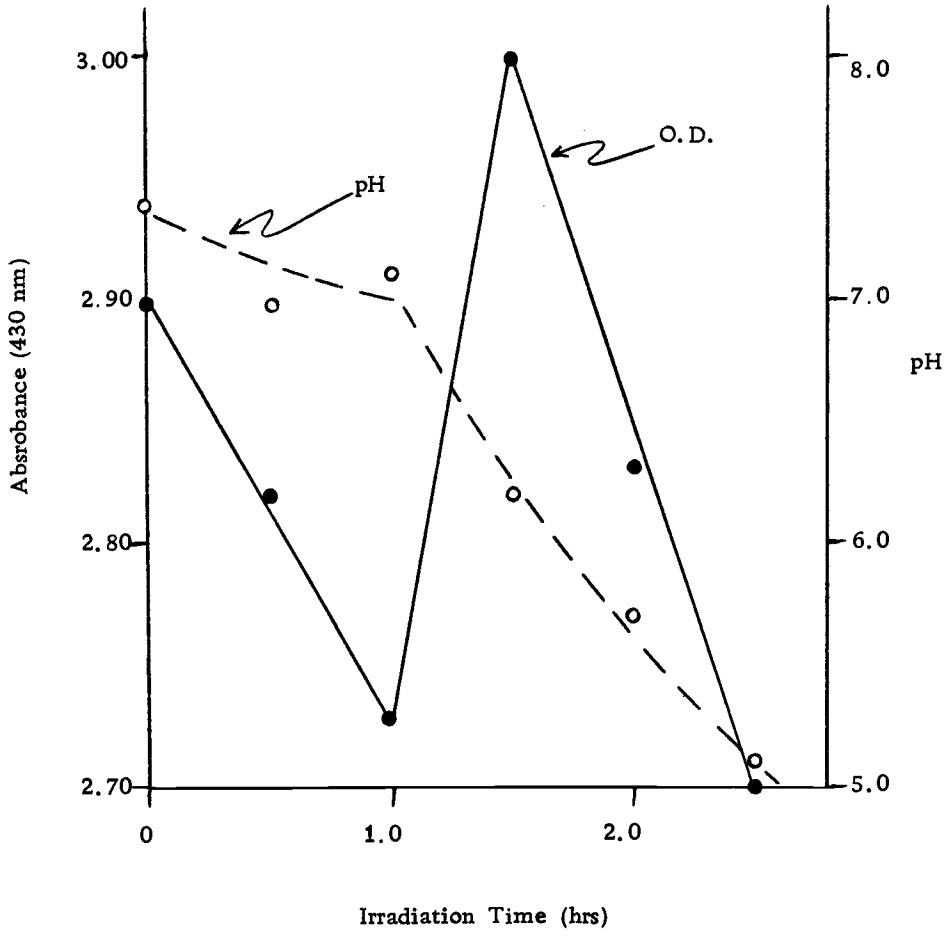


Figure 12. Short irradiation period effects on ligninsulfonate

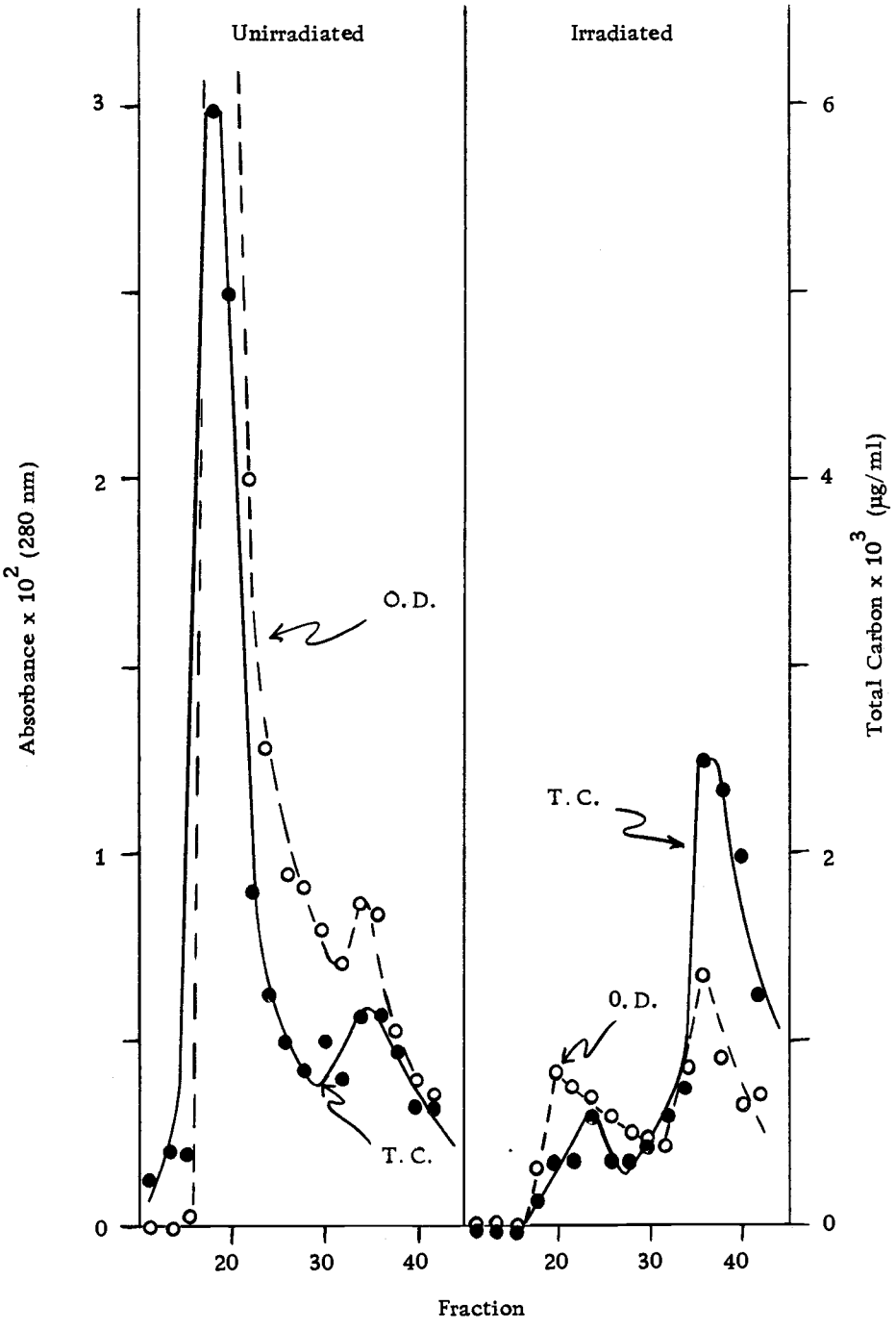


Figure 13. Fractionation of irradiated and unirradiated ligninsulfonate on a Sephadex G-25 column

ligninsulfonate was added to the column, its high phenolic content caused extensive binding to the column. To avoid this problem a 1% solution was run on the column, and the results from the monitored fractions were multiplied by 50 in order to compare them with the irradiated ligninsulfonate profiles.

The unirradiated sample, as seen in Figure 13, was almost entirely excluded from the column. Therefore, nearly all of its molecular weight was in excess of 5000. Also the absorbance at 280 nm, which is a measure of phenolic content, paralleled the total carbon results. On the other hand, the irradiated sample had little, if any, of its components excluded from the column. This indicates that the irradiation process has broken down the ligninsulfonate polymers to molecules of less than 5000 molecular weight. The absorbance at 280 nm does not parallel the total carbon in the irradiated sample. This is most noticeable in the large total carbon peak of low phenolic content occurring in the 30th through 40th ml fractions. As seen in Figure 13 this unique peak is the primary product of the photolysis process.

The portion of the molecular weight range utilized for growth was determined by the use of duplicate samples, one of which was inoculated. After a week of growth and the removal of cells from the inoculated sample, both fractions were prepared for fractionation as previously described. From the results shown in Figure 14, it

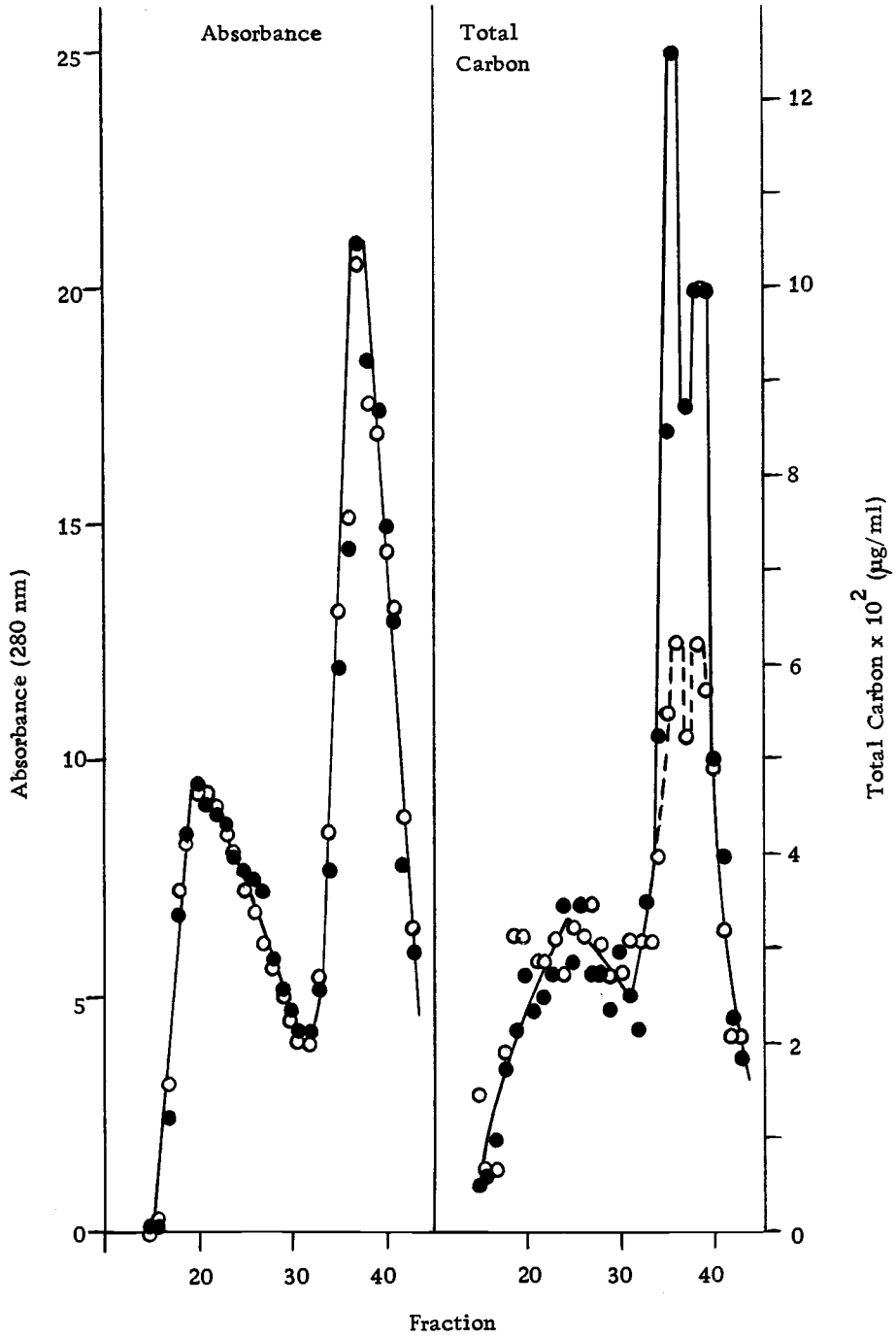


Figure 14. Utilization of irradiated ligninsulfonate (Sephadex G-25)

● = Uninoculated ○ = Inoculated

can be clearly seen that there is no significant change in phenolic content after growth. However, there is a considerable decrease in total carbon in the 30th through 40th ml fraction range. Once again, this is the unique material formed during the photo-oxidation process. It is apparent from this experiment that primarily low molecular weight non-phenolic carbon is utilized during growth.

A determination of the growth capabilities of P. putida on the irradiated ligninsulfonate substrate was achieved next by separately inoculating each fraction. Figure 15 shows that only the fractions betwen the 34th and 41st ml supported good growth. This corresponds exactly to the fractions showing a decrease in total carbon in Figure 14. The first 33 ml eluted from the column did not support appreciable growth. The growth on fractions 34 through 37 exhibited a definite lag period followed by rapid growth which is characteristic of the induction of key degradative enzyme systems. The first growth peak occurred over the fraction range where the phenolic content and total carbon are maximized. Therefore, enough phenolic material is found here to have a marked inductive effect upon growth. A second growth peak is observed at the point where the TC/OD maximum occurs. The TC/OD value is a measure of the non-phenolic carbon content. The growth on these fractions (38 through 41) shows no lag, but still has the characteristic inductive response of the previous fractions. Thus, it appears that the lower molecular

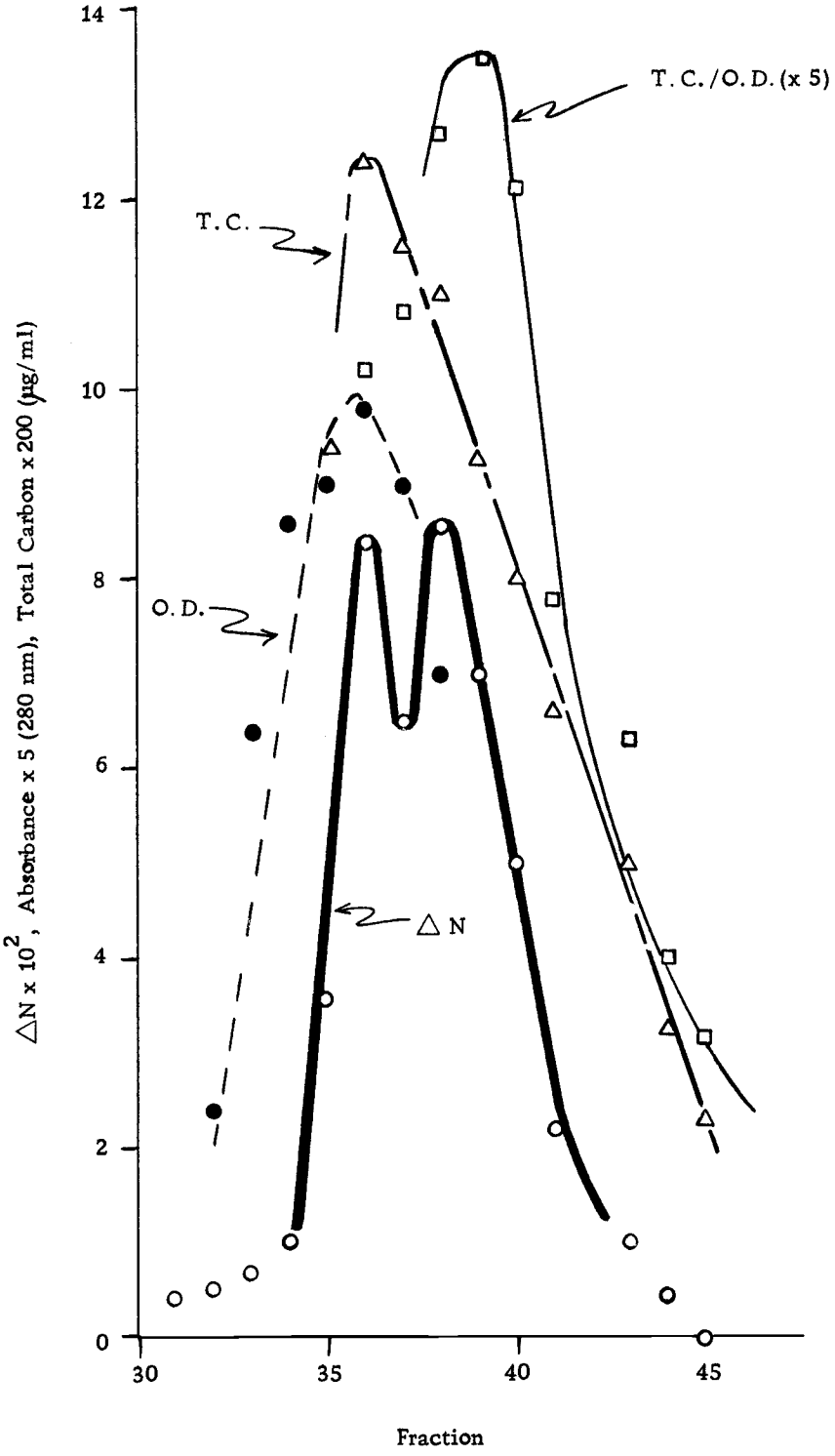


Figure 15. Growth responses on irradiated ligninsulfonate (Sephadex G-25)

weight non-phenolic material present in these fractions can be metabolized constitutively by P. putida. However, there is apparently adequate phenolic material present in these fractions to permit induction. The remaining fractions supporting marginal growth (42 through 45) do not cause a lag or induction of rapid growth. These fractions contain still smaller molecular weight material with too little phenolic content to induce rapid growth.

Throughout this study there has been an apparent interaction between the various molecular weight ranges. In order to test this, fractions supporting similar types of growth were combined and diluted to approximately 100 μg/ml concentrations. The fractions were collected as follows:

Fraction	Volume
a	15 → 28 ml
b	29 → 32 ml
c	33 → 37 ml
d	38 → 41 ml
e	42 → 45 ml
f	46 → 60 ml

A 5 ml aliquot from each fraction was tested for its ability to support growth, and 2 1/2 ml were added to 2 1/2 ml of all other fractions and tested for growth. The results obtained from the interaction of the various fractions are shown in Table 1. Under these experimental

Table 1. Effect on growth of interactions between molecular weight ranges of irradiated ligninsulfonate

Fraction	△ N (growth)		Effect	Total Carbon After Growth		Effect
	observed	predicted	$(\frac{\text{observed}}{\text{predicted}})$	observed	predicted	$(\frac{\text{observed}}{\text{predicted}})$
a	0			160		
a+b	2	(0+2=2)	none	120	(80+58=138)	lower[a]
a+c	12	(0+8=8)	slight +	105	(80+24=104)	none
a+d	12	(0+14=14)	none	185	(80+41=121)	+++
a+e	8	(0+7=7)	none	190	(80+48=128)	+++
a+f	0	(0+0=0)	none	115	(80+44=124)	none
b	4			115		
b+c	8	(2+8=10)	none	100	(58+24=82)	+
b+d	18	(2+14=16)	none	100	(58+41=99)	none
b+e	10	(2+7=9)	none	100	(58+48=106	none
b+f	4	(2+0=2)	none	96	(58+44=102)	none
c	15			47		
c+d	20	(8+14=22)	none	47	(24+41=65)	--
c+e	14	(8+7=15)	none	76	(24+48=72)	none
c+f	7	(8+0=8)	none	55	(24+44=68)	-
d	27			82		
d+e	20	(14+7=21)	none	70	(41+48=89)	--
d+f	10	(14+0=14)	slight -	47	(41+44=85)	---
e	14			95		
e+f	8	(7+0=7)	none	87	(48+44=92)	none
f	0			88		

[a]Probably error due to extremely low amount of growth (△ N=2)

conditions there was no effect from the fraction interactions on the amount of growth. However, the tendency for the larger molecular weight material which does not support growth to limit the carbon utilization of the lower molecular weight material which does support growth, was evident. The original concentrations of these fractions prior to dilution were as follows:

Fraction #	µg/ml
a	940
b	1130
c	5150
d	3660
e	1430
f	480

Therefore the above mentioned effect of the early fraction (a) on the best growth supporting fractions (d and e) would actually be diluted out in the original substrate. Even though the early fraction does show some inhibitory properties, as far as carbon utilization is concerned, it does not affect the amount of growth. Thus, the inhibitory properties of irradiated ligninsulfonate must be the result of an overall interaction between the varied molecular weight ranges.

It is evident from Table 1 that the addition of the best growth supporting fractions (c and d) to later fractions actually increases the carbon utilization. This is probably due to the ability of these

fractions to induce enzymes that metabolize material in the later fractions which is at a concentration too low to allow induction.

There appears to be a considerable amount of non-growth related carbon utilization and inhibition occurring during the growth of P. putida on irradiated ligninsulfonate.

The determination of the concentration dependence on growth of fractions c and d was studied next (Figure 16). Fraction c contains the 33rd through 37th ml fractions and has been shown to contain the material supporting the 1st peak of growth (Figure 15). Fraction d contains the 38th through 41st ml fractions, and this material supports the second peak of growth. Figure 16 shows the effect of adding increasingly larger amounts of 515 μg/ml volumes of fraction c and 590 μg/ml volumes of fraction d to growth tubes containing a total volume of 7 ml. It can be seen that both fractions support linearly increasing growth with increasing substrate concentration. The greater rate of increase in fraction d was probably due to its slightly greater concentration. It is apparent, over these concentration ranges, that carbon was the limiting factor for growth. Furthermore, both fractions c and d produce a diauxic growth response and the induction times for the second burst of growth is characteristic of the particular fraction studied. Figure 17 shows this by depicting the effects of concentration on induction times for these fractions. It can be seen that the induction times of fraction c remain substantially constant while those of fraction d decrease linearly with time. This substantiates the previous data

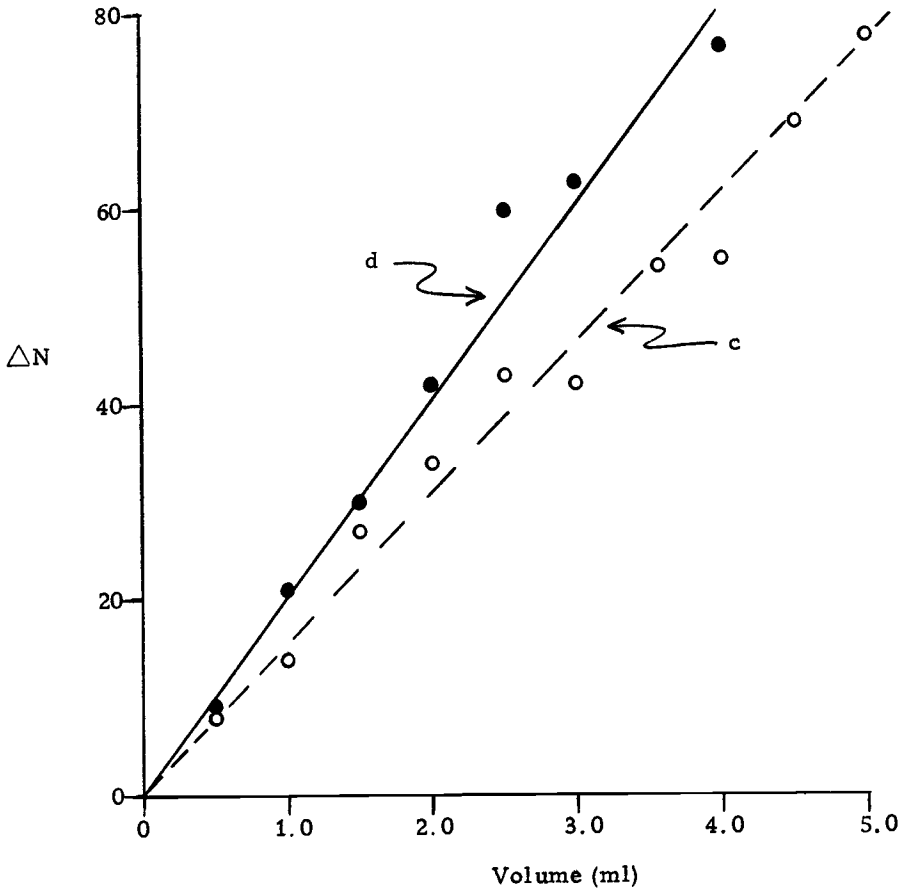


Figure 16. Concentration dependence of fractions c and d as monitored by growth response

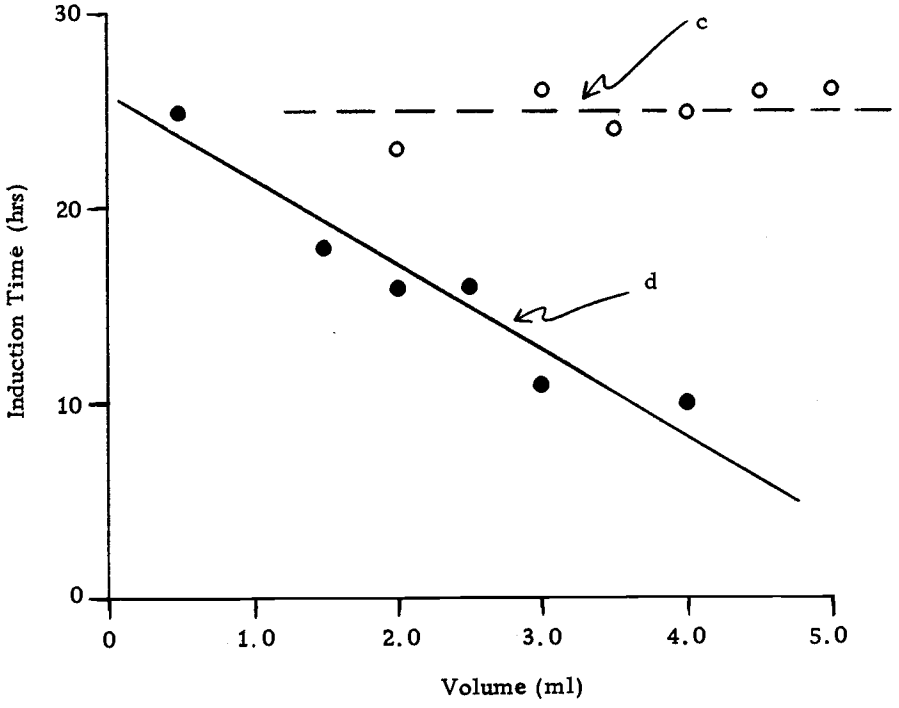


Figure 17. Concentration dependence of fractions c and d as monitored by induction times

indicating that fraction c contains a larger amount of phenolic compounds capable of inducing enzymes than does fraction d. Therefore, even with low amounts of fraction c, there is a maximum induction potential available. With fraction d, on the other hand, the maximum potential is never achieved since the increasing concentrations tend to decrease the induction time.

To determine which compounds are utilized for growth, catechol and vanillin were passed through the Sephadex G-25 column. They came off in the 35th through the 45th ml fractions. This presents strong evidence that the utilizable components in irradiated lignin-sulfonate are in the molecular weight range of these phenolic monomers.

Gas-liquid chromatographic (GLC) analysis of fractions from the Sephadex G-25 column demonstrated that there are six major peaks occurring in the 38th through 41st ml fraction (Figure 18). When inoculated and uninoculated duplicate samples were compared, these six peaks were seen to disappear after growth.

A comparison of the retention times and peak heights of the 38th through 41st ml fraction from these duplicate samples is shown in Table 2. The results from this table indicate that there are seven regions on the chromatograph where utilization has occurred. When model phenolic compounds were derivatized in the same manner as the irradiated ligninsulfonate, a number of peaks were observed in

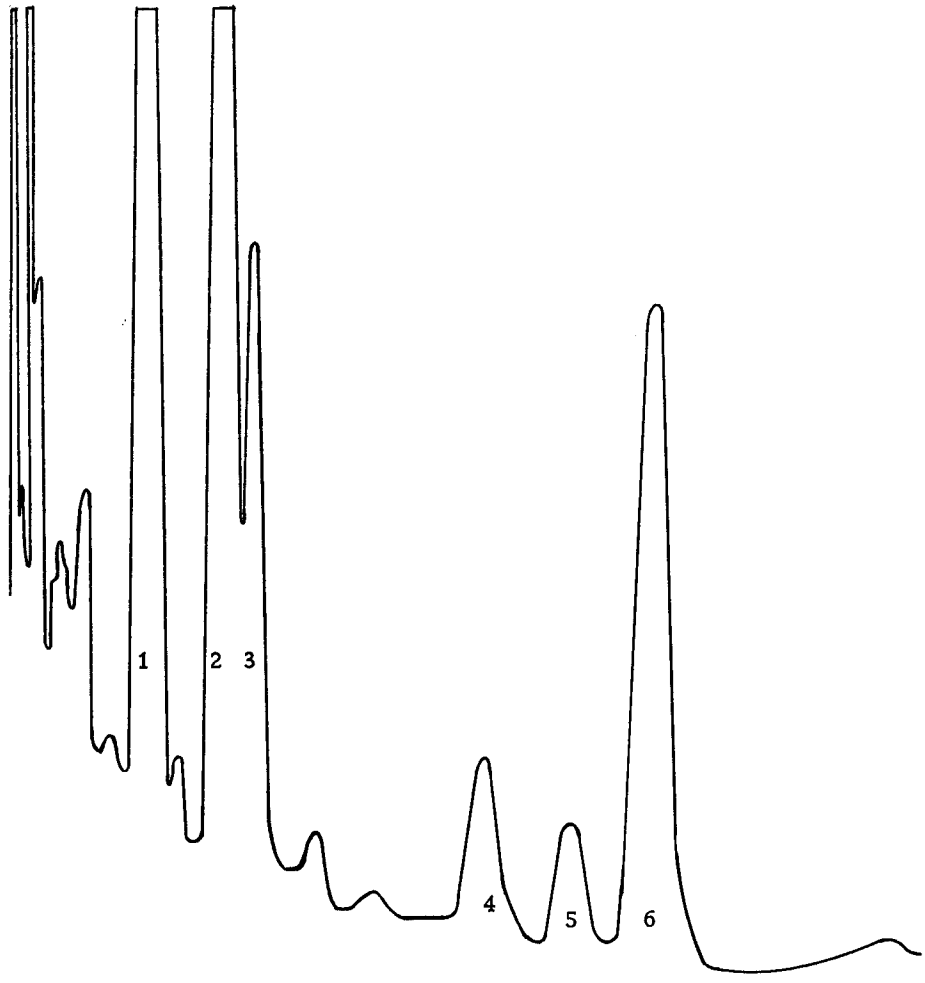


Figure 18. GLC chromatograph of the 39-43 ml fraction from a Sephadex G-25 column (alditol-acetate derivatives)

Table 2. Comparison of retention times and peak heights of inoculated[a] and uninoculated irradiated ligninsulfonate by gas-liquid chromatography.[b]

		Uninoculated		Inoculated	
		Retention time (min.)	Peak height (mm)	Retention time (min.)	Peak height (mm)
attenuation 2					
	I[c]	1	105		
		1 1/2	168	1 1/2	70
		2	18	2	30
		2 1/4	13	2 1/2	18
				3	10
attenuation 1	II	3	175		
		3 1/4	25	3 1/2	33
		4	13		
	III	4 1/2	225	4 1/2	30
		6	13	6 1/2	18
	IV	7	13		
		9	10		
	V	12 1/2	25	12 1/2	5
		13 1/2	5		
	VI	58	38	60	5
	VII	70	18		

[a] Based on the 38th through 41st ml fractions from Sephadex G-25 column.

[b] Alditol acetate derivatives.

[c] Utilization regions.

the chromatograph. Apparently, the procedure utilized to form these derivatives was too harsh for phenolics or each monomer formed a number of derivatives. This procedure (alditol-acetate) is one generally reserved for carbohydrate identification. A closer observation of these multi-peak chromatographs showed that many of the model compounds produced peaks corresponding to the seven regions of utilization. The number of peaks corresponding to the seven utilization regions for the model compounds tested is shown below:

vanillin	7
p-hydroxybenzoic acid	7
benzaldehyde	7
p-hydroxycinnamate	6
vanillic acid	6
protocatechuate	5
phenol	5
ferulic acid	5
caffeic acid	4
benzoic acid	4
catechol	3

A comparison of the retention times of the 38th through 41st ml fraction of irradiated ligninsulfonate with the three model compounds most closely duplicating the seven utilization areas is shown in Figure 19.

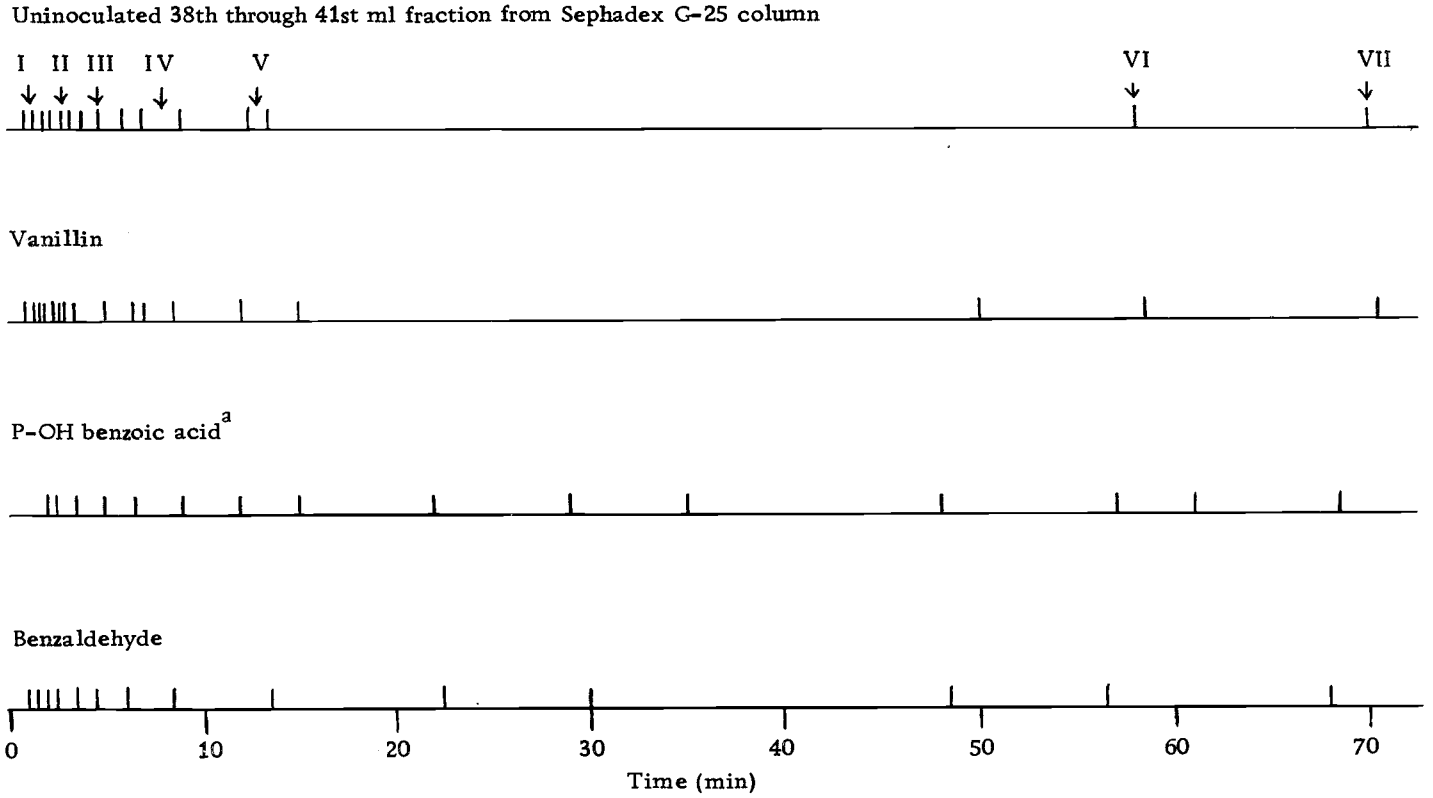


Figure 19. GLC retention times for a growth supporting fraction from a Sephadex G-25 column and for model phenolic compounds (alditol-acetate derivatives)

[a]Solvent front extended to near 2 minutes

Thin layer chromatographic (TLC) comparisons of the inoculated and uninoculated irradiated ligninsulfonate cultures are shown in Figure 20. Ether extracts of duplicate growth and non-growth samples were chromatographed in three solvent systems. The plates were then sprayed with tetrazotized benzidine which forms characteristic colors with phenolic compounds. The red colored spots which disappeared after growth are shown by this dye to be phenolic in nature. The Rf values for these disappearing spots fit the known Rf values for vanillin with these solvent systems (5). Duplicate inoculated and uninoculated irradiated ligninsulfonate samples which were analyzed by GLC were also analyzed by TLC. The fractions showing unique GLC peaks were the only ones which demonstrated an ether-extractable phenolic content.

Phenolic compounds appear to be utilized during growth on irradiated ligninsulfonate, even though a phenolic decrease is not accompanied by a decrease in 280 nm absorbance (Figure 14). These utilizable phenolics are probably responsible for many of the inducible and repressible characteristics of the substrate.

Throughout this study it had been assumed that carbon was the growth-limiting factor. In order to rule out cell density as a limiting factor, the post-growth substrate was reinoculated and not found to support growth. A contol tube that had previously been shaken under growth conditions and then centrifuged was also inoculated for the

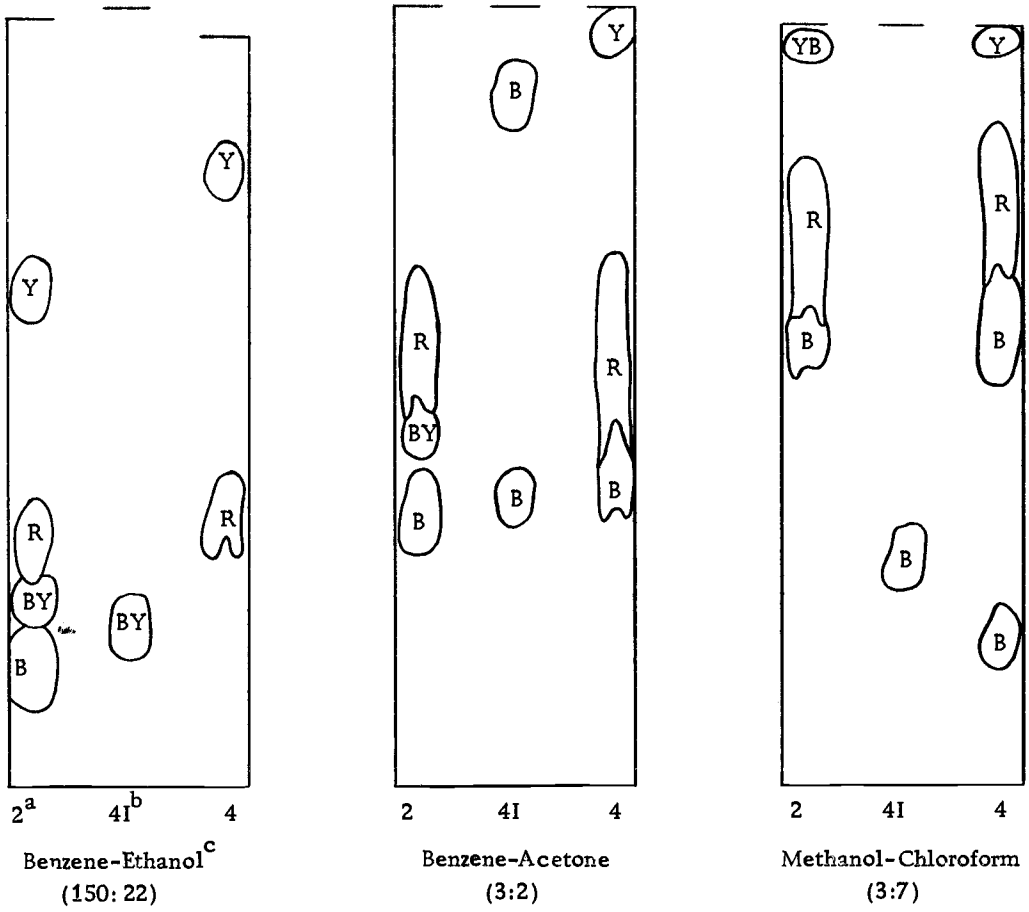


Figure 20. Characterization of TLC plates with tetrazotized benzidine spray.
Y = yellow, R = red, B = brown

[a] Irradiated ligninsulfonate batch number

[b] Inoculated sample of batch #4

[c] Solvent systems - Ether extracts spotted

first time. The control tube supported growth as expected, but exhibited no lag time.

In order to determine the cause of this phenomenon, the following experiment was performed. Three sets of six tubes containing 50%, 70%, and 100% irradiated ligninsulfonate (plus mineral salts) were prepared and three of each set were inoculated. Eighteen additional tubes were prepared in the same manner except the substrate plus mineral salts solutions were centrifuged before inoculation. Finally, a third group of 18 tubes was prepared in which the substrate was centrifuged prior go the addition of mineral salts and subsequent inoculation. A growth curve on one tube for each type of substrate preparation was run normally, one inoculated tube was rerun, and one uninoculated tube was rerun. The results indicated that no carbon was centrifuged out of the medium at any stage of the experiment. Also the amount of carbon utilized was independent of the substrate preparation procedure, and similar lag times were observed after inoculation. In addition, no growth occurred with reinoculation of a previously inoculated tube. Finally, all of the tubes inoculated after four days of shaking showed no lag period. Therefore, it appeared that an interaction between the irradiated ligninsulfonate and the mineral salts occurs during shaking, and this interaction eliminated the normal lag period.

The inoculum for these experiments was prepared by washing

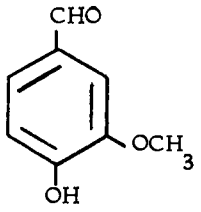
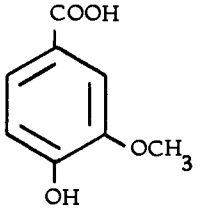
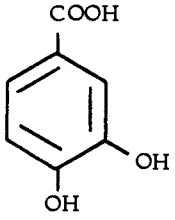
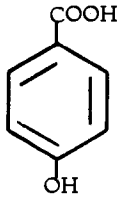
cells from 48-hour-old agar plates containing irradiated lignin-sulfonate as a sole carbon source. These cells were in the stationary growth phase and their metabolic pools were assumed to be depleted. If one irradiated ligninsulfonate broth tube was inoculated from another identical tube containing cells in the log phase of growth, a lag period was not observed in the second growth tube. In this case the metabolic pools of the inoculum could be assumed to be full, eliminating the normal lag time.

Physiological Characterization of *P. putida* (29-43-1) with Respect to the Irradiated Ligninsulfonate Substrate

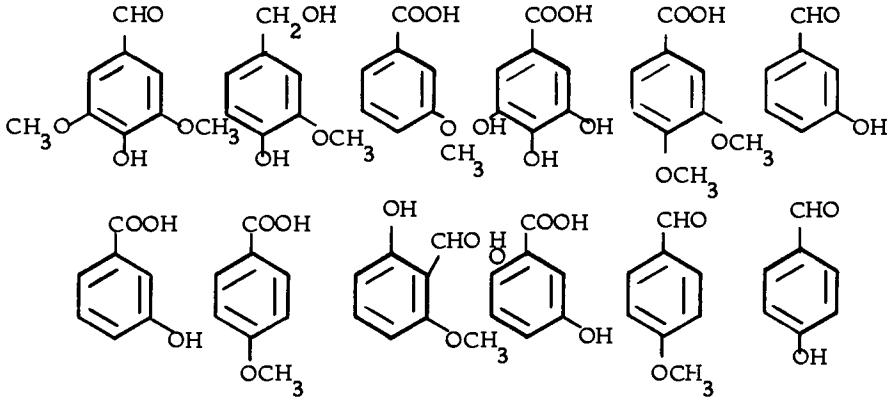
Studies with irradiated ligninsulfonate have established the importance of phenolic compound effects on *P. putida* (29-43-1) growth characteristics. In order to evaluate the ability of this organism to utilize phenolic compounds, the spectrum of degradable lignin-related phenolic monomers was determined. The monomers tested fell into three main groups based on known metabolic pathways in *P. putida*.

Table 3 shows that protocatechuate group compounds require a carboxyl group and a hydroxyl group in the para position. Therefore vanillin metabolism requires an aldehyde group oxidation to a carboxyl group. This extra step results in a longer induction time. The duration of the induction time indicates the reaction sequence

Table 3. Spectrum of utilization of protocatechuic acid group of compounds by P. putida (29-43-1) [a]

Compound	Formula	Induction times (hrs)	Comments
Vanillin		19	1. p-OH group 2. oxidation of aldehyde group required
Vanillic acid		9	1. p-OH group
Protocatechuic acid		3	1. p-OH group
p-OH benzoate		24	1. p-OH group 2. hydroxylation required

Related non-growth supporting monomers:



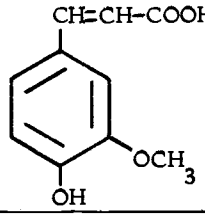
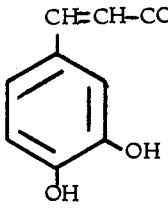
[a] Organism pre-grown on irradiated ligninsulfonate plates

leading to ring cleavage. Using this technique, vanillin metabolism to vanillic acid to protocatechuatic acid is quite clear. The longer induction time with p-OH benzoate indicates a requirement for hydroxylation in protocatechuatic acid formation. The inability of p-OH benzaldehyde to be metabolized indicates that aldehyde group oxidation in vanillin is specific and not the result of a generalized reaction. Finally, a definite steric hindrance is observed when a fourth substituent is added adjacent to the para hydroxyl group.

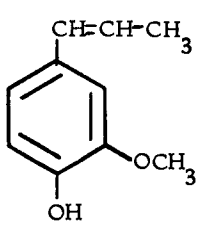
Table 4 indicates similar relationships in the caffeic acid group. Evidently, the side chain with the conjugate electronegative carboxyl group has minimal effects on metabolism. Induction times indicate that enzymes metabolizing the protocatechuate group of compounds may be involved in metabolizing these compounds.

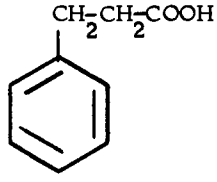
Table 5 depicts the compounds metabolized through two distinct catechol pathways. The lag times are longer with the first three compounds (benzaldehyde, benzoic acid, catechol) since their metabolism is product-induced by the catechol ring-cleavage product. Phenol metabolism, on the other hand, is substrate induced, and the relatively long lag time observed with this compound may be due to other factors, such as phenol interactions with proteins. By noting the non-metabolizable monomers in this group, the effect of methoxyl groups on the catechol molecule and the requirement for ortho hydroxyl groups can be seen. Finally, this organism has the ability

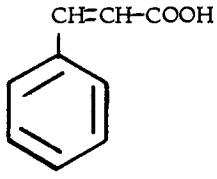
Table 4. Spectrum of utilization of caffeic acid group of compounds by P. putida (29-43-1) [a]

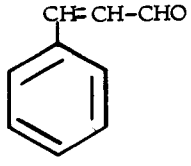
Compound	Formula	Induction time (hrs)	Comments
Ferulic acid		9	1. p-OH group
Caffeic acid		8	1. p-OH group
p-OH cinnamate		15/44 [b]	1. p-OH group 2. hydroxylation required

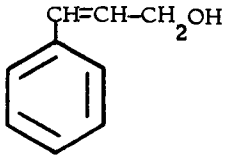
Related non-growth supporting monomers:







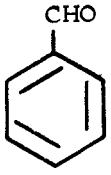
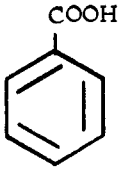
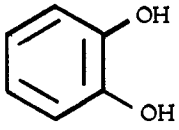
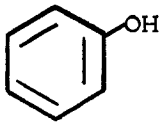




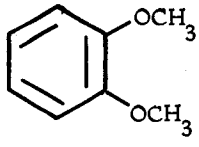
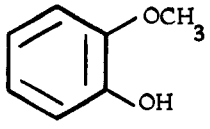
[a] Organism grown on irradiated ligninsulfonate plates

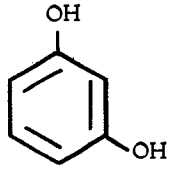
[b] diauxie

Table 5. Spectrum of utilization of catechol group of compounds by P. putida (29-43-1) [a]

Compound	Formula	Induction time (hrs)	Comments
Benzaldehyde		50	1. oxidation of aldehyde group required
Benzoid acid		17	
Catechol		18	
Phenol		33	1. hydroxylation required

Related non-growth supporting monomers:



[a] Organism pre-grown on irradiated ligninsulfonate plates

to oxidize benzaldehyde to benzoic acid which can then be converted to catechol. Induction time sililarity of benzoic acid and catechol indicate that the conversion of benzoic acid to catechol must proceed rapidly.

To evaluate the metabolic route by which these phenolics are degraded, simultaneous adaptation studies were carried out. These studies were based on the premise that cells adapted to attack the primary substrate should be adapted simultaneously to attack all the intermediates formed during the oxidation of that substrate (69). This approach assumes, of course, that all reaction products induce the enzymes which allow catabolism. P. putida appears to be pre-induced on all of the metabolizable phenolics and also on irradiated ligninsulfonate. Organisms from each of these cultures were used to inoculate the test substrates. Compounds in the protocatechuate and caffeic groups are particularly well suited for this type of analysis, since each intermediate induces the enzyme degrading it. Compounds in the catechol group, except for phenol, are not well suited for this type of study since induction of catechol cleavage is product induced.

The amount of growth (ΔN), induction times (lag), and growth rates (slope) for the combinations of inducer and substrate are shown in Tables 6 and 7. The phenylpropanoid compounds of the caffeic acid group appear to be metabolized by the protocatechuate group

Table 6. Simultaneous adaptation studies with phenolic monomers.

Inducers:	Protocatechuic acid △ N lag[b] slope			Vanillic acid △ N lag slope			Vanillin △ N lag slope			p-OH benzoate △N lag slope			Ferulic acid △N lag slope			Caffeic acid △N lag slope		
Substrates:																		
Protocatechuic acid	200	3	40	210	3	52	208	0	52	200	6	33	215	2	43	205	3	68
Vanillic acid	198	6	33	194	5	65	205	3	41	165	14	28	200	3	40	198	4	28
Vanillin	230	23	38	220	11	44	238	3	24	262	17	13	205	4	23	205	5	23
p-OH benzoate	175	23	13	190	20	10	178	19	9	210	9	14	222	17	11	195	22	16
Catechol	245	26	27	200	28	29	202	33	25	200	46	18	212	28	30	215	27	22
Benzoate	195	20	28	210	20	23	220	21	44	195	9	28	180	20	20	225	22	32
Benzaldehyde	158	53	11	165	60	13	55	47	11	182	40	10	220	52	8	100	46	20
Phenol	580	33	39	540	38	54	565	39	51	565	40	51	568	44	63	568	36	44
Ferulic acid	150	6	30	172	6	57	194	3	28	165	15	33	195	3	22	250	6	25
Caffeic acid	193	5	39	238	3	60	240	3	48	238	11	44	250	3	62	240	4	40
p-OH Cinnamate	168	32	23	165	29	8	175	34	21	300	28	24	140	35	9	225	28	20
ILS[a]	25	35	xx	32	35	xx	32	37	xx	25	37	xx	30	25	xx	25	36	xx

[a] Irradiated ligninsulfonate

[b] hours

Table 7. Simultaneous adaptation studies with phenolic monomers.

Inducers:	p-OH Cinnamate			Benzaldehyde			Benzoate			Catechol			Phenol			ILS		
	△N	lag[b]	slope	△N	lag	slope	△N	lag	slope	△N	lag	slope	△N	lag	slope	△N	lag	slope
Substrates:																		
Protocatechuic acid	200	3	33	195	8	28	195	8	65	182	8	36	200	8	40	215	4	36
Vanillic acid	200	4	29	195	12	24	178	10	45	175	13	35	200	14	40	180	8	26
Vanillin	218	9	36	245	22	41	xx	11	15	200	16	33	222	28	16	218	15	27
p-OH benzoate	225	14	16	265	10	16	330	6	22	300	8	33	380	22	48	195	27	16
Catechol	165	35	18	140	51	35	200	13	15	182	35	23	188	25	14	175	48	35
Benzoate	170	13	19	225	13	25	210	9	27	222	10	32	370	19	68	200	23	40
Benzaldehyde	175	34	18	192	22	27	218	65	16	185	70	16	215	28	22	172	52	8
Phenol	575	24	64	542	21	68	535	42	49	595	48	60	555	13	46	570	37	52
Ferulic acid	175	6	25	200	15	33	xx	12	30	255	16	36	230	18	46	250	13	25
Caffeic acid	310	4	31	185	15	33	220	10	28	232	10	39	180	13	36	415	10	59
p-OH Cinnamate	180	25	18	185	32	23	325	16	15	xx	16	7	388	42	44	135	30	8
ILS[a]	26	43	xx	30	35	xx	29	35	xx	24	39	xx	28	45	xx	33	0	xx

[a] Irradiated ligninsulfonate

[b] hours

enzymes. This is indicated by caffeic acid attack with the enzyme catabolizing protocatechuic acid, and ferulic acid attack by the enzyme converting vanillic acid to protocatechuic acid. Therefore, the reaction sequence would be as follows:

$$\text{vanillin} \rightarrow \underset{\text{(ferulic acid)}}{\text{vanillic acid}} \rightarrow \underset{\text{(caffeic acid)}}{\text{protocatechuate}} \rightarrow$$

Para-OH benzoate is reported in the literature to be metabolized through protocatechuate (28, 70). However, only slight induction of protocatechuate degrading enzymes and no induction of caffeic acid degrading enzymes were noted with P. putida induced with this substrate. On the other hand, a relationship between benzoate and p-OH benzoate was indicated, since the organisms grown on either compound strongly induced enzymes for both. The literature indicates that these compounds are metabolized through entirely separate branches of the mandelate pathway (28, 70). Hence, this observation indicates that P. putida (29-43-1) is unique in this respect.

Para-OH cinnamate, the phenylpropanoid equivalent of p-OH benzoate, is metabolized through protocatechuate. However, cells grown on p-OH cinnamate also have induced enzymes for the metabolism of benzoate, phenol, vanillic acid, vanillin, and ferulic acid. Possibly, this multiple induction effect may be due to the large metabolic pools created during slow growth observed with p-OH cinnamate.

Catechol grown cells were induced for benzoate, p-OH benzoate, and p-OH cinnamate degradation. Products of the catechol pathway have been shown to induce protocatechuate pathway enzymes (54), but this does not explain why enzymes for the metabolism of these particular compounds were induced.

The most important aspect of this study was the finding that irradiated ligninsulfonate induced enzymes degrading protocatechuate and vanillic acid. However, this inductive response was not noted with ferulic acid or caffeic acid.

The significance of metabolic pool effects noted in certain portions of the simultaneous adaptation studies was next considered. To evaluate these effects, a fully induced culture, grown on an irradiated ligninsulfonate plate, was used to inoculate the 11 phenolic compounds supporting growth. At the same time a nutrient agar plate was streaked. Induced cells transferred to a nutrient agar plate incurred metabolic pool size alterations. This was a result of metabolic shifts occurring after transfer from a limited to a "luxurious" substrate. Results of successive nutrient agar transfers and subsequent inoculation into phenolic compounds are presented in Table 8. Metabolic pool effects appear to be nonexistent in cells grown on compounds which have substrate-induced ring cleavage capabilities. These compounds belong to the protocatechuate and caffeic acid groups. However, the substrate induced

Table 8. Metabolite pool alteration studies with P. putida (29-43-1)

Compound	Induction times (hrs.)				
	Induced[a]	NA-1[b]	NA-2	NA-3	NA-4
Protocatechuic acid	4	4	4	4	4
Vanillic acid	9	9	9	9	9
Vanillin	22	22	22	22	22
p-OH benzoate	30	34	31	31	34
Ferulic acid	10	10	7	10	10
Caffeic acid	6	6	6	6	9
p-OH cinnamate	16/45[c]	xx	16/35	20/39	19/45
Catechol	24	59	24	none	65
Benzoate	19	36	11	11	11
Benzaldehyde	58	50	25	23	25
Phenol	35	31	30	21	18

[a] Cells grown on irradiated ligninsulfonate plate

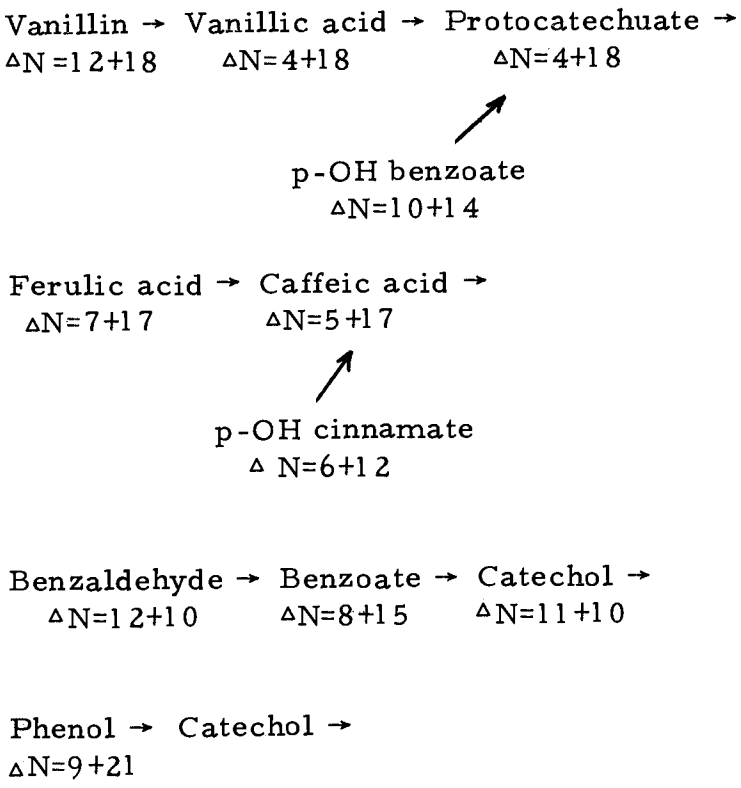
[b] Number of transfers on nutrient agar

[c] Diauxie

ring cleavage observed in phenol metabolism is an exception, in that decreasing induction time with transfers on nutrient agar plates was observed. Product induced ring cleavage, as found in the catechol group metabolism, does show a marked metabolic pool effect due to the large observed variation in induction times.

Metabolic pool alterations appear to markedly influence phenolic compound degradation where product-induced enzymes function in key metabolic steps. These effects were not generally noted when key enzymes were substrate-induced by phenolic compounds.

More information concerning the metabolic pool effects was obtained from experiments in which P. putida (29-43-1) was pre-induced by growth on model phenolic compounds, followed by inoculation into irradiated ligninsulfonate media a few hours after the log period of growth. A diauxic growth response was consistently noted when growth in the inoculated ligninsulfonate culture was monitored. The pathways postulated to occur in the degradation of these model compounds, along with the two periods of diauxic growth are shown below:



The first growth period occurred without a lag, and may be due to nutrient carry over from the model compound inoculum. The second growth phase occurred after a 20 to 25 hour lag period and can be attributed to irradiated ligninsulfonate metabolism.

Such metabolic pool effects can be predicted from previously noted reaction rates and known control mechanisms of P. putida. The large initial growth phase observed with vanillin, p-OH benzoate, benzaldehyde, catechol, and possibly phenol and benzoate induced cells was probably due to the slower metabolism of these compounds when compared to the other model compounds. This slower metabolism could result in certain pools being full at the time the cells

were used for irradiated ligninsulfonate inoculation, and hence, a greater amount of residual growth would be expected to occur.

The lesser second phase of growth seen with p-OH benzoate and p-OH cinnamate induced cells may be an indirect effect of the hydroxylation step required to metabolize these compounds. These induced hydroxylating enzymes may not be required for irradiated ligninsulfonate utilization, thus decreasing the organism's efficiency and resulting growth.

The decreased second phase of growth with benzaldehyde and catechol induced cells reflect tighter control mechanisms resulting from product-induced phenolic ring cleavage. Decreased growth with cells grown on these phenolic compounds, may cause the organism to respond less efficiently when transferred to the irradiated ligninsulfonate medium.

The larger secondary growth observed with phenol-induced cells may indicate the enzymes operative here were substrate-induced. Therefore, phenol degradation does not appear to be negatively controlled and could not subsequently interfere with growth on irradiated ligninsulfonate.

Finally, growth on benzoate preinduced cells presented further evidence for a relationship between benzoate and p-OH benzoate metabolism. The first growth phase was minimal when compared to that observed with benzaldehyde- and catechol-induced cells.

If benzoate was metabolized solely through the catechol pathway, the diauxie curve should resemble that of benzaldehyde and catechol. However, the second growth phase with benzoate-induced cells is much greater than with benzaldehyde- or catechol-induced cells, and resembles growth obtained with p-OH benzoate-induced cells. The fact that benzaldehyde is metabolized through benzoate but does not show these effects indicated that intermediate benzoate levels were not sufficient to induce the p-OH benzoate degrading enzymes.

The extended lag times during irradiated ligninsulfonate utilization seen with model compound-induced cells and absent in irradiated ligninsulfonate-induced cells, may indicate a requirement for the filling of metabolic pools. As noted before, when irradiated ligninsulfonate-induced cells were allowed to remain in the stationary growth phase, a lag time was observed after subsequent irradiated lignin-sulfonate inoculation. This could be due to metabolic pool depletion during the stationary phase of growth. However, when cells growing on an irradiated ligninsulfonate medium were used to inoculate another irradiated ligninsulfonate medium immediately after log growth, no such lag was observed. Thus, all metabolic pools required for irradiated ligninsulfonate utilization appeared to be full.

Irradiated ligninsulfonate effects on phenolic model compound utilization were next determined by adding equal amounts of each

model compound and irradiated ligninsulfonate to culture tubes. The results of the subsequent growth can be seen in Table 9. In general it can be seen that the model compounds were preferentially utilized over the irradiated ligninsulfonate. Also, irradiated ligninsulfonate generally had a marked inhibitory effect on utilization of compounds metabolized through catechol. However, no such effect was usually seen on model compounds metabolized through protocatechuate. The inhibitory effects were seen as increased lag times, decreased growth, and decreased model compound utilization.

Exceptions to the general effects described above were observed. Benzaldehyde and benzoic acid grown cells showed no significant increase in lag time, and benzoate grown cells had little change in total growth. Also, phenol-grown cells showed a marked lag time increase and decreased growth in spite of the phenol degrading enzymes being substrate induced. Another exception was the decreased total growth observed on ferulic acid plus irradiated ligninsulfonate. An identical decrease in growth was observed when cells, preinduced on irradiated ligninsulfonate, were transferred on nutrient agar or glucose plates before inoculating ferulic acid media. Both of these observations may be related to the premature filling of metabolic pools which exert feedback control. These pools could have been filled by the change in metabolism occurring during nutrient agar growth or by irradiated ligninsulfonate

Table 9. Effect of the addition of irradiated ligninsulfonate (ILS) on the utilization of phenolic model compounds.

Compound	Model compound carbon remaining after growth (µg/ml) -ILS	+ILS[a]	Lag change with ILS (hrs.)	Growth change without ILS (△ N)	Growth change with ILS (△N)
Vanillin	29	33	+5	138	+8
Vanillic acid	21	26	0	100	0
Protocatechuic acid	15	16	+2	108	+3
p-OH benzoate	26	17	-12[b]	135	-30
Ferulic acid	20	29	0	220	-125
Caffeic acid	32	29	+2	60[c]	+20
p-OH cinnamate	20	16	-6, -17[d]	60[c]	+5
Benzaldehyde	10	32	0	120	-26
Benzoate	28	97	+6	130	+6
Catechol	15	29	+23	120	+10
Phenol	18	30	+40	290	-158

[a] Total carbon due to ILS subtracted out

[b] less slope

[c] Low growth probably due to insolubility of substrate

[d] Diauxie

addition to the ferulic acid medium.

The irradiated ligninsulfonate effect on phenolic compound utilization appeared to be related to whether the phenolic compound was metabolized through catechol or protocatechuate. Therefore, increasing amounts of irradiated ligninsulfonate were added to constant amounts of catechol and protocatechuate. Then, increasing amounts of catechol and protocatechuate were added to a constant amount of irradiated ligninsulfonate. The results from these experiments are presented in Tables 10 and 11.

As seen in Table 10, increasing the irradiated ligninsulfonate concentration had essentially no effect on protocatechuic acid utilization. In Table 11, growth responses with increasing protocatechuic acid concentration appear independent of the constant irradiated ligninsulfonate present. Thus, metabolism of compounds through protocatechuate, under these conditions, appeared to be independent of irradiated ligninsulfonate.

With catechol, increased lag times were observed when low concentrations of irradiated ligninsulfonate were added to the culture. Even at a relatively low concentration, irradiated ligninsulfonate completely inhibited all growth on catechol. When growth did occur on catechol, with or without added irradiated ligninsulfonate, the rate and amount was always the same.

Tables 10 and 11 show that catechol and irradiated ligninsulfonate

Table 10. Effect of increasing amounts of irradiated lignin sulfonate (ILS) on P. putida (29-43-1) growth with constant amounts of catechol and protocatechuic acid.

% ILS	Total Carbon change during growth (µg/ ml)	% Utilization	Lag Time (hrs.)	Growth (△N)
Constant Protocatechuic acid (30%)[a]				
0	140 → 0	100	8	185
10	200 → 23	89	9	195
20	200 → 50	75	9	180
30	220 → 63	71	14	180
40	235 → 83	65	17	180
Constant Catechol (30%)[a]				
0	160 → 13	92	22	160
10	195 → 45	77	62	160
20	235 → 83	65	88	150
30	250 → 225	10	xx	none
40	295 → 295	0	xx	none

[a] % V/V of volume in growth tube. The concentrations of catechol and protocatechuate are 0.10% W/V. The concentrations of ILS is 0.05% W/V.

Table 11. Effect of increasing amounts of catechol and protocatechuic acid on P. putida (29-43-1) growth with constant amounts of irradiated ligninsulfonate (ILS).

%[a]	Total Carbon change during growth (µg/ml)	% Utilization	Lag Time (hrs.)	Growth (△N)
Increasing Protocatechuic acid:[b]				
0	85 → 58	32	26	8
10	110 → 80	27	13	55
20	180 → 68	62	11	135
30	200 → 68	66	11	178
40	220 → 65	71	11	200
Increasing Catechol:[2]				
0	85 → 58	32	26	8
10	125 → 63	50	39	93
20	185 → 85	54	55	138
30	270 → 260	4	xx	none
40	325 → 300	8	xx	none

[a] % of volume in growth tube. The concentrations of catechol and protocatechuate are 0.10% W/V.

[b] 30% V/V of volume in growth tubes is ILS. The concentration of ILS is 0.05% W/V.

exhibit similar inhibitory characteristics. Thus, catechol-like components in irradiated ligninsulfonate may be significant in explaining the inhibitory properties of this material. When catechol was used as the sole source of carbon, substrate inhibition, similar to that observed with irradiated ligninsulfonate, occurred (Table 12). However, Table 12 also shows that protocatechuic acid, over these same concentrations, exhibits no inhibitory effects.

To determine whether or not catechol had an inhibitory effect on protocatechuic acid utilization, various combinations of these substrates were tested for growth responses. Table 13 shows that catechol and protocatechuic acid were utilized to about the same extent and produced about the same amount of growth. However, the lag time is directly proportional to the catechol concentration. This lag time dependence on catechol concentration and absence of diauxic growth suggest that feedback control exerted by common small molecular weight degradation products may be operative here. Carbon utilization and observed growth remained relatively constant, and therefore, independent of the relative amounts of the two compounds.

Catechol effects on protocatechuic acid utilization differed from the effects exhibited by irradiated ligninsulfonate. Catechol was utilized simultaneously with protocatechuic acid, while irradiated ligninsulfonate was not. Irradiated ligninsulfonate did not influence

Table 12. Effect of increasing concentrations of catechol or protocatechuic acid on the growth of P. putida (29-43-1).

%[a]	Total Carbon change during growth (µg/ml)	% Utilization	Lag Time (hrs.)	Growth (△ N)
Catechol:				
10	31 → 8	74	16	83
20	74 → 7	91	18	162
30	120 → 10	92	23	222
40	175 → 31	82	65	184
50	none	none	xx	none
Protocatechuic acid:				
10	20 → 5	75	8	30
20	39 → 4	90	5	60
30	53 → 2	96	8	110
40	77 → 5	94	8	150
50	99 → 5	95	8	190
60	90 → 5	94	8	250
70	120 → 5	96	8	320

[a]% V/V of volume in growth tube. The concentrations of catechol and protocatechuate are 0.1% W/V.

Table 13. Effect of catechol[a] concentration on utilization of protocatechuic acid.[a]

% Protocatechuic acid[b]	% Catechol	Total Carbon change during growth (μg/ml)	% Utilization	Lag Time (hrs.)	Growth (△N)
70	0	60 → 5	92	6	320
60	10	52 → 5	90	9	330
50	20	99 → 9	91	12	320
40	30	53 → 9	83	15	300
30	40	100 → 5	95	17	250
20	50	60 → 7	88	20	240
10	60	60 → 7	88	25	205
0	70	77 → 10	87	25	240

[a] 0.05% W/V concentration

[b] % V/V of volume in growth tubes

the lag times when added to protocatechuic acid at low concentrations, while catechol did. These differences could be attributed to a very low concentration of catechol-like components in irradiated ligninsulfonate. Possibly the level of these components was sufficient to effect the utilization of catechol or ligninsulfonate, but not protocatechuic acid.

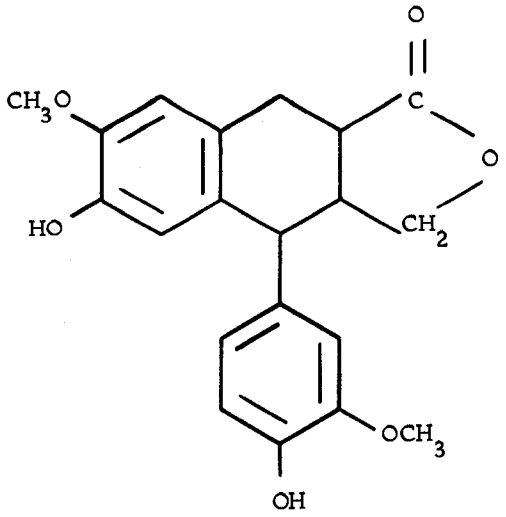
Co-oxidation of catechol or protocatechuate does not appear to enhance irradiated ligninsulfonate utilization. These substrates appear to be metabolized in preference to the irradiated ligninsulfonate. To further investigate the possibility of co-oxidation enhancement of growth on irradiated ligninsulfonate, equal concentrations of glucose, nutrient broth, cas amino acids, or yeast extract were added to irradiated ligninsulfonate cultures. These cultures were compared with control cultures not containing irradiated ligninsulfonate. In all cases, irradiated ligninsulfonate caused longer lag times. When the decrease in total carbon after growth was monitored, it appeared that the co-oxidants were utilized exclusively. This conclusion was arrived at by comparing the decrease in total carbon of the co-oxidant plus irradiated ligninsulfonate cultures with separate co-oxidant and irradiated ligninsulfonate cultures. Total carbon decreases after growth on the combined cultures were similar to those found in the co-oxidant culture without irradiated ligninsulfonate. This indicates that irradiated ligninsulfonate was not appreciably

utilized when grown with co-oxidants. Also substantiating this conclusion was the observation that growth on the co-oxidants, with or without added irradiated ligninsulfonate, appeared similar.

An Alpha-conidendrin Model System for Ligninsulfonate Photolysis

Many problems inherent in ligninsulfonate photolysis are due to characteristics of the ligninsulfonate molecules. Their diverse molecular weight ranges result in a differential susceptibility to the photolytic process. Also, extreme drops in pH follow sulfonate group cleavage. And finally, the effect of the uncleaved sulfonate groups in irradiated ligninsulfonate is difficult to assess.

The alpha-conidendrin molecule is a lignan commonly found in wood. It is a phenylpropanoid dimer linked at the side chains as shown below:



Alpha-condendrin

When an alpha-condendrin solution was photolytically treated in the same manner as a ligninsulfonate solution, an immediate polymerization occurred. The resulting large molecular weight molecules were then broken down to a substrate resembling irradiated ligninsulfonate, but without the interfering sulfonate groups.

Alpha-conidendrin irradiation with ultra-violet light, in the presence of oxygen, causes an immediate polymerization to occur. Further irradiation, breaks down this polymeric material as shown in Figure 21. The pH drops immediately, levels off near neutrality, and eventually begins to rise again.

It was not possible to enrich for an organism that could efficiently utilize alpha-condendrin as a sole source of carbon and energy. However, by irradiating alpha-conidendrin, it was possible to enrich for a number of organisms that could successfully utilize this substrate. Pseudomonas putida (29-43-1) could also utilize this material. The growth responses of P. putida (29-43-1) to alpha-conidendrin irradiated for various lengths of time are shown in Figure 22.

Figure 22 shows that the amount of growth (ΔN) on this substrate was directly proportional to irradiation until carbon became limiting. Growth induction was clearer here than with similar experiments utilizing irradiated ligninsulfonate. The reason for this was that the diauxic type of irradiated ligninsulfonate growth made it difficult to measure the slopes on the growth curves. In the case of irradiated

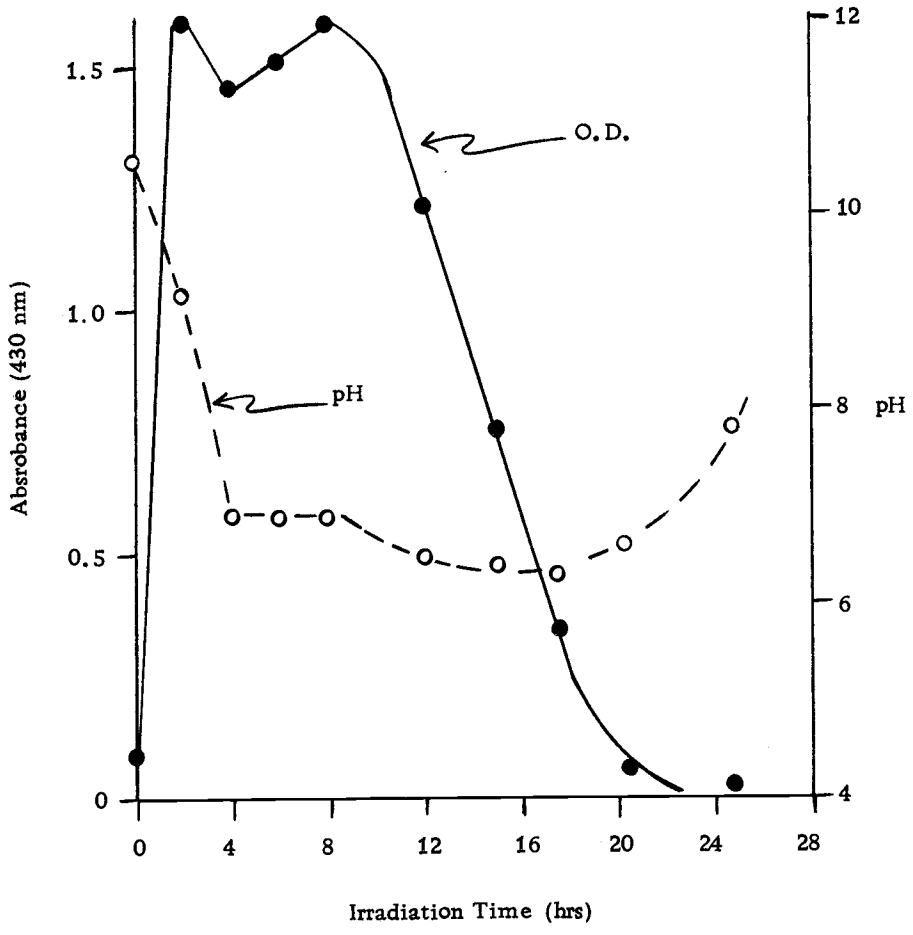


Figure 21. Irradiation of alpha-conidendrin as monitored by pH and absorbance of 430 nm

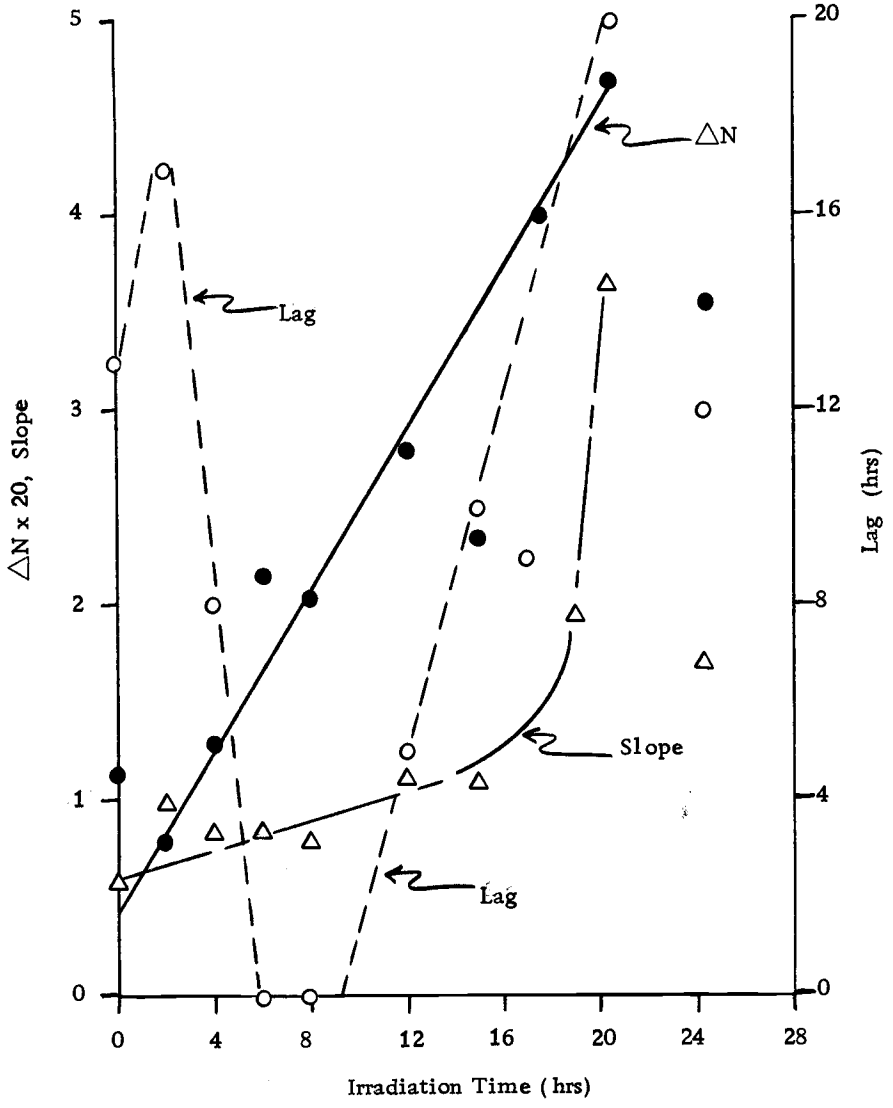


Figure 22. Effect of alpha-conidendrin irradiation time on growth of P. putida (29-43-1)

alpha-conidendrin, no diauxic growth occurred and it was easy to measure the slopes.

With the alpha-conidendrin system, when rapid degradation began at 8 hours of irradiation (Figure 21), the lag period immediately increased (Figure 22). Throughout the shorter photolysis periods slight growth rate (slope) increases occurred; however, a marked increase in these rates occurred between 16 and 20 hours of irradiation. This indicated that the monomeric phenolic content of the irradiated material increased throughout the irradiation process until it reached the level necessary to induce enzymes required for degradation. No major increase in growth (ΔN) occurred at this point, since endogenous enzyme synthesis, needed to metabolize these compounds, occurred at low rates before induction.

The alpha-conidendrin model system provided a means by which the substrate concentration dependence of the growth system could be interpreted. The concentration dependence of the irradiated ligninsulfonate system is shown in Figure 10 while the irradiated alpha-conidendrin system is described in Figure 23. In both cases, growth (ΔN) and carbon utilization increased linearly with substrate concentration. However, the percent utilization remained constant in the alpha-conidendrin system while it continually increased with substrate concentration in the ligninsulfonate system. The continual increased utilization occurring in the ligninsulfonate system may

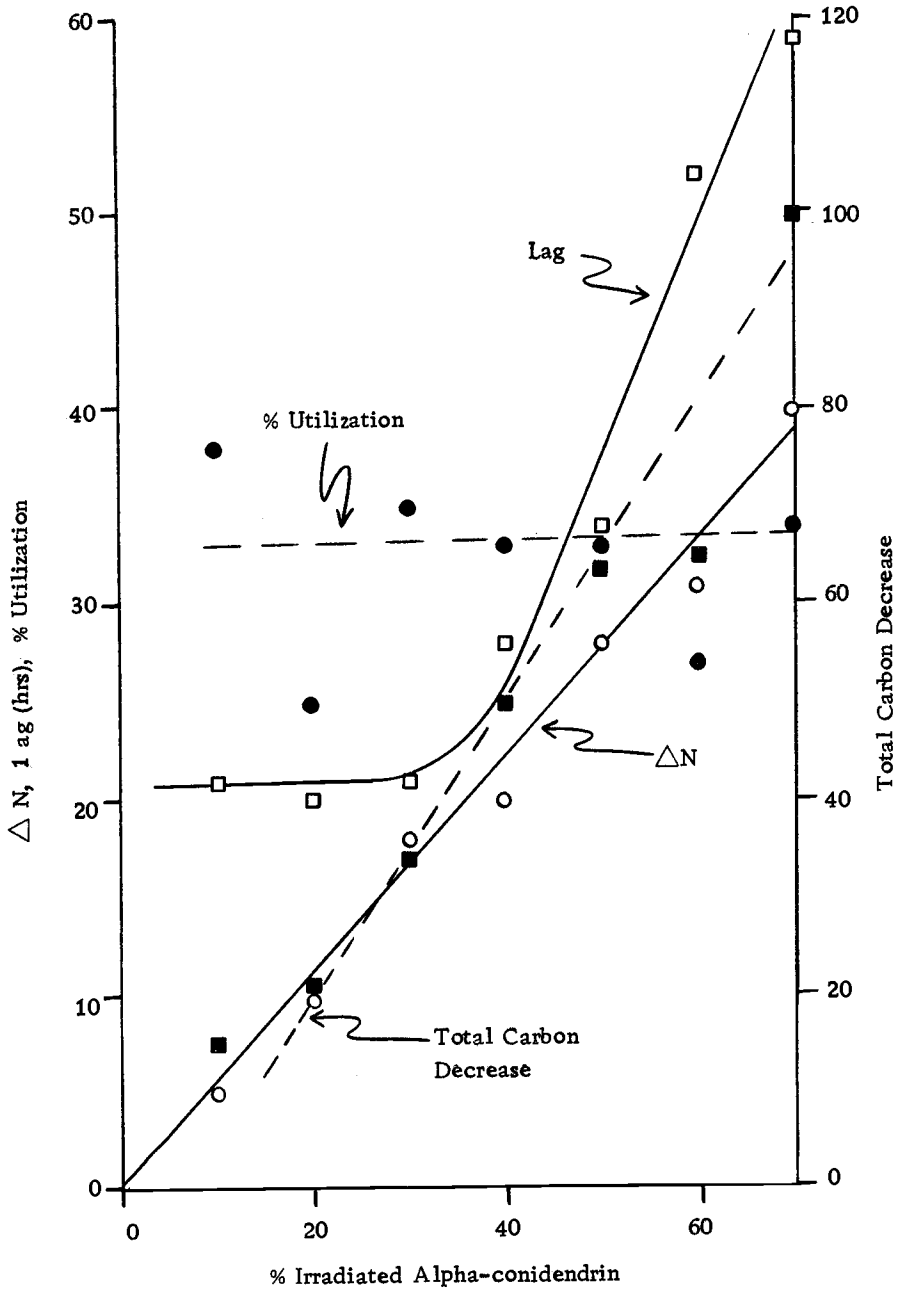


Figure 23. Effect of substrate concentration on growth of P. putida (29-43-1) (irradiated alpha-conidendrin)

have resulted from overcoming the inhibitory effects inherent in this system. This inhibition may well be due to the sulfonate groups present. Another difference inherent in the alpha-conidendrin system was that lag times remained constant until a certain substrate concentration was reached. Additional increases in substrate concentration caused linear lag time increases. This type of response was characteristic of enzyme induction in the degradation of model phenolic compounds of the catechol group. Lag time increases at low substrate concentrations in the ligninsulfonate system were probably due to inhibitory effects which were overcome by the induction of enzymes at higher substrate concentrations. Since inhibition and induction responses both cause increased lag times, a continual increase in lag time with increasing substrate concentration, could result.

The Sephadex G-25 fractionation of irradiated alpha-conidendrin, shown in Figure 24, indicated that the molecular weight range of this material was remarkably similar to that noted for irradiated ligninsulfonate (Figures 13, 14, and 15). It was noted that before irradiation, the alpha-conidendrin molecule was eluted from the column between the 25th and 30th ml fractions. The irradiation process caused polymerization, and this polymer was subsequently broken down to fragments smaller than the original alpha-conidendrin molecule. Fractionation of both irradiated ligninsulfonate and irradiated alpha-conidendrin showed low levels of high molecular

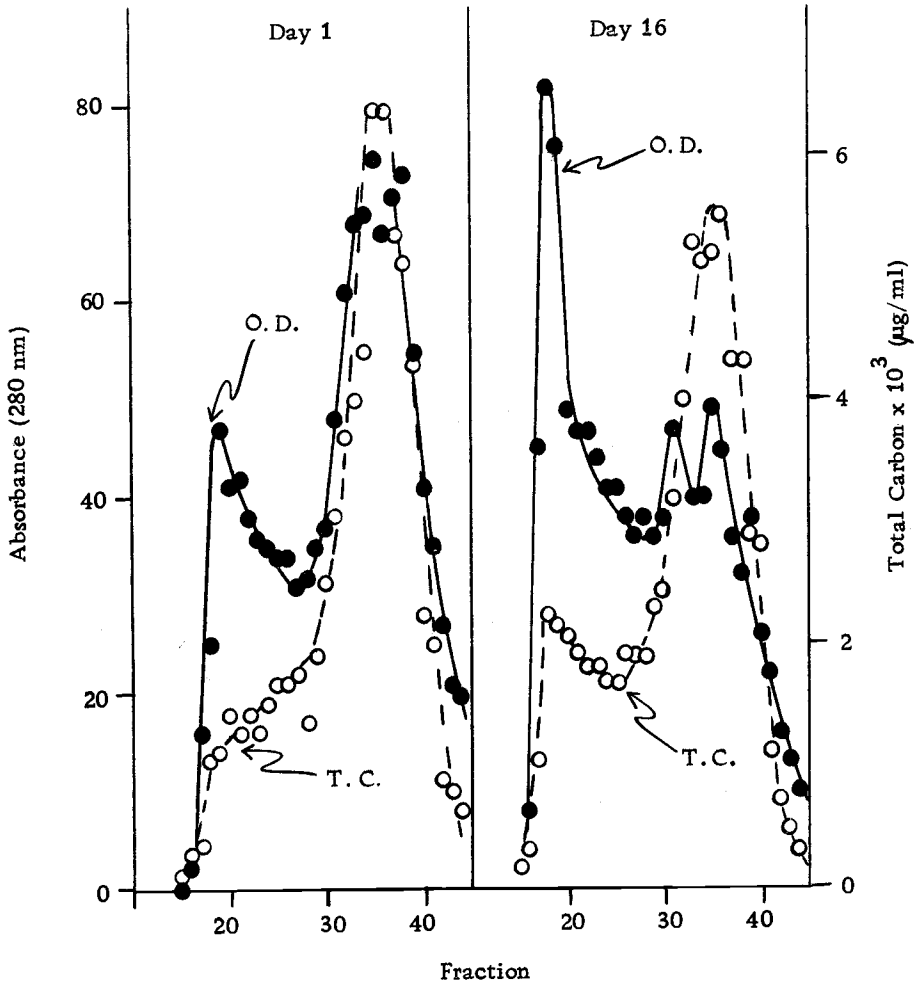


Figure 24. Instability of irradiated alpha-conidendrin

weight, high phenolic content material excluded from the column. Both systems also yielded a large amount of low molecular weight, low phenolic content material occurring over fractions 30 through 40. However, Figure 24 showed that irradiated alpha-conidendrin products were not stable, since after 16 days, a great deal of the low molecular weight phenolic material polymerized sufficiently to be excluded from the column (molecular weight in excess of 5000).

Growth of P. putida (20-43-1) on the 16-day-old irradiated alpha-conidendrin was limited to the O.D. peak occurring at the 35th ml fraction. The new O.D. peak of higher molecular weight material occurring at fraction 31 did not support growth. The fractionation profile of irradiated ligninsulfonate in Figure 15 also showed that absorbance and total carbon peaked near fraction 35, and the growth of P. putida (29-43-1) was limited to these fractions.

An experiment designed to show that carbon limited growth, identical to the one described previously for irradiated ligninsulfonate, was also performed. The alpha-conidendrin system produced essentially the same results as seen with the ligninsulfonate system. For example, carbon was shown to limit growth and interactions between the substrate and mineral salts occurred.

The photolytic treatment of alpha-conidendrin proved to be a valuable tool for the study of irradiated ligninsulfonate. A comparison of the irradiated substrates helped establish the inhibitory nature

of the sulfonate groups on photo-oxidized ligninsulfonate, and also, demonstrated that these groups did not interact with mineral salts to affect growth.

SUMMARY

A commercial sodium ligninsulfonate (Maraspere CB) solution was photolytically modified in the presence of oxygen with a mercury-vapor ultraviolet lamp. The photolytic process was followed by growth responses of a Pseudomonas putida (29-43-1) soil isolate, and by changes in absorbance at 430 nm, pH, and total carbon. A stable and reproducible substrate, producing a maximum growth response, was achieved by irradiating a 0.1% W/V irradiated ligninsulfonate solution at an initial pH of 7.6 (unaltered) and a 2 ml/min. oxygen flow-rate.

Gel fractionation studies (Sephadex G-25) provided evidence that the large molecular weight, high phenolic content ligninsulfonate molecules were photolytically degraded to low phenolic content molecules having molecular weights in the range of phenolic monomers. The non-phenolic portion of this newly formed material was almost exclusively utilized for growth. However, phenolic material resembling vanillin was seen to disappear when followed by gas-liquid chromatography (GLC) and thin-layer chromatography (TLC). Also, the physiological characterization of P. putida (29-43-1) revealed that irradiated ligninsulfonate produced essentially the same growth responses as compounds metabolized through catechol. Therefore, it appeared that irradiated ligninsulfonate provided substantial

non-phenolic utilizable carbon for the organism, but the phenolic material present was sufficient for enzyme induction.

In addition to the inductive characteristics of irradiated lignin-sulfonate, a marked substrate inhibition occurred. This type of self-inhibition was also observed when compounds metabolized through catechol were used as carbon sources. Furthermore, residual large molecular weight polymers were shown to have an inhibitory effect on growth.

The inhibitory effect of sulfonate groups in irradiated lignin-sulfonate was delineated by a comparison of this system with an alpha-conidendrin (a diphenylpropanoid) model irradiation system. The only significant difference in these systems was the absence of sulfonate groups in the latter. This permitted the study of sulfonate group affects indirectly by a comparison of the two systems.

The utilization of up to 50% of the irradiated ligninsulfonate substrate by a common soil bacterium suggested that this photolytic treatment could significantly improve biodegradation. This type of treatment may find applications with other recalcitrant polymers as well.

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